PLATELET AND VASCULAR STUDIES IN MYELOPROLIFERATIVE DISORDERS

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DECLARATION

In accordance with the postgraduate degree regulations of the University of Edinburgh, I declare that this thesis was composed solely by myself, that the work described in this thesis is my own and that it has not been submitted for any other degree.

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ABSTRACT

The myeloproliferative disorders, polycythaemia vera (PV) and primary thrombocythaemia (PT) are relatively indolent diseases but are complicated by thrombosis and bone marrow fibrosis which cause significant morbidity and may reduce life-expectancy especially in younger patients. Our understanding of the pathogenesis of thrombosis and fibrosis in PV and PT and in primary myelofibrosis (PMF) remains incomplete although platelet and vascular abnormalities have been clearly implicated. The recent recognition of the procoagulant activities of cell derived microparticles has led to their study in other prothrombotic disorders, where they are observed to be elevated and to have a pathogenic role.

We have studied a cohort of patients with PV, PT and PMF and described their clinical and laboratory features in comparison to other published observations. The demographic, haematological and molecular characteristics of our cohort were similar to other retrospective analyses, but the occurrence of thrombo-haemorrhagic complications was lower. The presence of vascular abnormalities in these patients was investigated using both established markers and an assay was devised to measure platelet, endothelial, leucocytes and red cell microparticles in platelet poor plasma using flow cytometry techniques. This assay was optimised for pre-analytical variables, the most important of which was found to be sample centrifugation. In keeping with previous studies, increased platelet activation was observed in PV and PT patients compared to healthy controls using both established markers and as evidenced by increased numbers of platelet microparticles. There was no evidence of endothelial disturbance using the soluble endothelial marker E-selectin but we did observe elevated endothelial microparticles in patients compared to controls. Microparticles may therefore be useful as a marker of vascular abnormalities in these disorders and in view of their prothrombotic properties may be an additional pathogenic mechanism in the prothrombotic state and a potential therapeutic target.

In relation to bone marrow fibrosis, plasma levels of platelet α-granule contents, including the pro-fibrotic cytokine transforming growth factor β (TGFβ), were studied. We observed elevated levels of TGFβ in patients compared to controls, with the highest levels in patients with PMF and in those with PT or PV who had more marked fibrosis. Further, levels of TGFβ were strongly associated with another α-granule protein beta-thromboglobulin, suggesting that platelet α-granules may be an important source for this pro-fibrotic cytokine.

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ABBREVIATIONS

AL	Acute leukaemia
BCSH	British Committee for Standards in Haematology
BP	Blood pressure
BTG	beta-thromboglobulin
CAD	Coronary artery disease
CI	Confidence interval
CML	Chronic myeloid leukaemia
CRF	Chronic renal failure
CTAD	Citrate Theophylline Adenosine Dipyridamole
CV	Coefficient of variation
CVA	Cerebrovascular accident
CVD	Cardiovascular disease
CVRF	Cardiovascular risk factors
DM	Diabetes Mellitus
DVT	Deep vein thrombosis
ECLAP	European Collaboration on Low-Dose Aspirin in Polycythaemia vera
ECM	Extracellular matrix
EEC	Endogenous erythroid colonies
ELISA	Enzyme linked immunoassay
EMP	Endothelial microparticles
Epo	Erythropoietin
(s)E-sel	(soluble) E-selectin (CD62E)
ESL	E-selectin ligand
ESRF	End-stage renal failure
FITC	Fluorescein Isothiocyanate
FVII	Factor VII
GP	Glycoprotein

HUVEC	Human umbilical vein endothelial cells
(s) ICAM	(soluble) Intercellular adhesion molecule
IFN	Interferon
IHD	Ischaemic heart disease
IL	Interleukin
LMP	Leucocyte microparticles
mAb	Monoclonal antibodies
MF	Myelofibrosis
MPD	Myeloproliferative disorder
MPD NOS	MPD not otherwise specified
MPL	Thrombopoietin receptor
mRNA	Messenger ribonucleic acid
NFkB	Nuclear factor kappa beta
OR	Odds ratio
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PE	Phycoerythrin
PECAM	Platelet endothelial cell adhesion molecule (CD31)
PF 1+2	Prothrombin fragments 1+2
PF4	Platelet factor 4
PLA	Platelet leucocyte aggregates
PMF	Primary myelofibrosis
PMP	Platelet microparticles
PNH	Paroxysmal nocturnal haemoglobinuria
PPP	Platelet poor plasma
PPS	Phosphatidylserine
PRP	Platelet rich plasma
(s)P-sel	(soluble) P-selectin (CD62P)
PSGL	P-selectin glycoprotein ligand

PV	Polycythaemia vera
PVD	Peripheral vascular disease
PVSG	Polycythaemia vera Study Group
PT	Primary thrombocythaemia
RCM	Red cell mass
RMP	Red cell microparticles
RR	Relative risk
sd	Standard deviation
SEM	Standard error of the mean
STAT	Signal transducers and activators of transcription
TAT	Thrombin-antithrombin
TF	Tissue factor
TGFβ	Transforming growth factor beta
TIA	Transient ischaemic attack
(s)TM	(soluble) Thrombomodulin
TMD	Transient myeloproliferative disorder
TNF	Tumour necrosis factor
Tpo	Thrombopoietin receptor gene
TTP	Thrombotic thrombocytopenic purpura
VCAM	Vascular cell adhesion molecule
VTE	Venous thromboembolism
VWF	Von Willebrand Factor
WHO	World Health Organisation

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Myeloproliferative disorders – Clinical Aspects

1.1.1 Epidemiology

The myeloproliferative disorders (MPD) include polycythaemia vera (PV), primary thrombocythaemia (PT) and primary myelofibrosis (PMF) and are largely adult diseases with an increasing incidence with age. The majority of cases are diagnosed after the fifth decade. PV more commonly affects men in comparison to the female predominance of PT. Depending on the population, annual incidences 0.2 to 28 per 100,000/year are reported for PV (McMullin *et al*, 2005) and 1-2.5/100,000/year for PT (Jensen *et al*, 2000b;Mesa *et al*, 1999). The Leukaemia Research Fund registry, which covers approximately 20% of the UK, reported a combined incidence of PV, PT and PMF of 2.27 per 100,000 population per year between 1984 and 1993 (McNally *et al*, 1997). The incidence of both appears to be rising (Jensen *et al*, 2000b) but this is likely to be due to the identification of asymptomatic cases from abnormal peripheral blood counts performed for other indications.

1.1.2 Pathophysiology and molecular aspects

Despite their phenotypic differences PV, PT and PMF share a number of common clinical and pathological features. Chronic myeloid leukaemia (CML) was previously included in this group of diseases but since the identification of its molecular origin, specifically the BCR-ABL translocation, it is considered to be a separate entity. Clinically they are all characterised by the overproliferation of mature cells of one or more haematopoetic cell lines (erythrocytes in PV, megakaryocytes and platelets in PT) and they can all be complicated by thrombosis, haemorrhage, progression to marrow fibrosis and transformation to acute leukaemia.

The common clonal stem cell origin of these disorders was originally identified by examination of X-linked polymorphic markers (Fialkow *et al*, 1981) and later by patterns of X-chromosome inactivation (El Kassar *et al*, 1997). However, using this technique, in up to 50% of PT patients a clonal population cannot be identified (El

Kassar *et al*, 1997;Harrison *et al*, 1999;Shih *et al*, 2002), although this may be a consequence of the limited sensitivity of the assay to detect low levels of clonal haematopoiesis.

Cytokine and growth factor hypersensitivity is another shared feature. *In vitro* studies in PV demonstrate erythroid colony formation in the absence of exogenous erythropoietin (Epo) (Liu *et al*, 2003) and in PT, thrombopoietin (Tpo) hypersensitivity is observed (Axelrad *et al*, 2000). In PV, this translates *in vivo* to erythroid proliferation despite low Epo levels consequent on the suppression of renal Epo synthesis by a negative feedback mechanism. In PT the picture is of inappropriately high Tpo levels despite thrombocytosis. In the normal situation a rise in the platelet count results in increased Tpo binding to its receptor (c-mpl) on the platelet surface. This reduces the amount of free Tpo available to stimulate megakaryocyte maturation and proliferation. In PT it is thought that abnormalities of the receptor reduce its binding to Tpo, resulting in high circulating levels despite the high platelet count (Schafer, 2006).

Clearly the shared stem cell origin of these disorders cannot explain their phenotypic diversity. Recently a variety of mutated tyrosine kinases (or related molecules) which result in abnormal signal transduction have been identified. The first discovery was in CML where the chromosome translocation (t9:22) produces a fusion kinase gene (BCR-ABL). This dysregulated tyrosine kinase is responsible for myeloid overproliferation in CML and specific inhibitors of the molecule are now the cornerstone of treatment (Deininger *et al*, 2000). Similarly a dysregulated platelet derived growth factor receptor has been revealed in a proportion of patients with idiopathic hypereosinophilic syndrome, a rarer MPD (Cools *et al*, 2003). These findings prompted the suggestion that similar mechanisms may be at work in the other MPD. Subsequently, a number of groups simultaneously published results showing the presence of an acquired point mutation of the JAK2 gene (V617F) in a proportion of PV, PT and PMF patients (Baxter *et al*, 2005;James *et al*, 2005;Kralovics *et al*, 2005;Levine *et al*, 2006).

The JAK2 protein is a member of the Janus kinase family of tyrosine kinases that are associated with haematopoetic cytokine and growth factor receptors. Its role in intracellular signalling and the effect of the V617F mutation in MPD is comprehensively reviewed by Khwaja (Khwaja, 2006). Following specific growth factor receptor-ligand binding, the JAK molecules are activated leading to phosphorylation of the cytoplasmic domains of the receptors. This promotes downstream signalling in the STAT (signal transducers and activators of transcription) pathway and the activation or suppression of genes involved in the growth factor response. The V617F mutation affects the negative regulatory pseudokinase domain (JH2) of the protein. It is a gain of function mutation that expresses a constitutively activated JAK2 tyrosine kinase. The mutated JAK2 can therefore bind to the Epo or Tpo receptors and activate downstream signalling pathways in the absence of growth factors. *In vitro* studies have shown that expression of JAK2 V617F can induce Epo hypersensitivity in cultured cell lines (James *et al*, 2005) and in murine bone marrow transplantation models produces a PV like phenotype (Wernig *et al*, 2006). In JAK2 V617F positive PV patients, erythroid progenitors heterozygous or homozygous for the mutation show increased sensitivity to Epo compared to normal progenitors from the same patients (Dupont *et al*, 2007).

The presence of the JAK2 V617F mutation has been demonstrated in over 80% of patients with PV and 25-60% of PT and PMF patients (Baxter *et al*, 2005;James *et al*, 2005;Jones *et al*, 2005;Kralovics *et al*, 2005;Levine *et al*, 2005). This important discovery raises further questions: How does one mutation result in three phenotypically distinct disorders? What is the molecular mechanism in the cases negative for this mutation? These issues are currently the subject of investigation but likely explanations include differences in mutant allele load and the presence of alternative or additional mutations.

Homozygosity for the mutation is thought to occur by mitotic recombination and in contrast to 25-30% homozygosity in PV patients, almost all (>97%) JAK2 V617F positive PT patients are heterozygous for the mutation (Baxter *et al*, 2005;James *et*

al, 2005;Kralovics *et al*, 2005;Levine *et al*, 2005). In a study of 962 patients with PV or PT, the presence of homozygosity conferred phenotypic differences at diagnosis with a higher haematocrit, higher leucocyte count and lower platelet count (Vannucchi *et al*, 2007b). Splenomegaly was more frequent in homozygous patients. In PT patients, homozygosity was independently associated with the occurrence of cardiovascular events.

Other mutations affecting exon 12 of the JAK2 gene have been described in a number of JAK2 V617F negative PV patients. These mutations did not appear to confer a distinct phenotype, with clinical and bone marrow features similar to those of JAK2 V617F positive patients. Gain of function mutations of the thrombopoietin receptor gene (MPL) have also been identified in patients with MPD (Pardanani *et al*, 2006;Pikman *et al*, 2006). The mutations (MPL W515L and MPL W515K) result in the constitutive activation of the thrombopoietin receptor and JAK-STAT pathway and consequently growth factor independence. In a cohort of 1182 patients, MPL mutations were identified in around 5% and 1% of PMF and PT patients respectively but were not found in PV (Pardanani *et al*, 2006). In keeping with this finding, expression of the MPL mutation in a mouse model produces a clinical picture characterised by marked thrombocytosis (Pikman *et al*, 2006) compared to the PV phenotype induced by the JAK2 V617F mutation (Wernig *et al*, 2006). In some cases the MPL and JAK2 V617F mutations were present concurrently, suggesting that they may have complementary actions.

1.1.3 PV and PT – clinical and laboratory features and diagnosis

PV is characterised by an erythroid excess in the absence of a secondary cause. This is usually reflected by a raised haematocrit and haemoglobin but these changes may be masked in the presence of iron deficiency, which not uncommonly develops. Direct measurement of red cell mass (RCM) may be used to exclude an apparent erythrocytosis where the haemoglobin is elevated but RCM is normal. Diagnosis of PV requires the exclusion of secondary causes of erythrocytosis driven by elevated

Epo levels (McMullin *et al*, 2005). These include hypoxic conditions or abnormal haemoglobins which cause a physiological Epo response or pathological conditions of excess Epo production, such as renal tumours. Clonal cytogenetic abnormalities, identified in 10-20% of patients or the JAK2 V671F mutation (or similar) can confirm the primary nature of the erythrocytosis. Other myeloproliferative features which support the diagnosis of PV include leucocytosis and/or thrombocytosis, splenomegaly, typical bone marrow features of trilineage hyperplasia and evidence of cytokine independence. Up to 60% of patients present with thrombotic or less commonly haemorrhagic complications (Elliott & Tefferi, 2005). Patients may also report pruritus, constitutional upset or symptoms of hyperviscosity including headache and dizziness.

PT is characterised by a persistent thrombocytosis in the absence of a reactive cause such as chronic inflammatory conditions or iron deficiency states. Positive features supporting the diagnosis of PT include splenomegaly and clonal genetic abnormalities including JAK2 V617F but this is present in only around 50% of cases. PT may also be confused with other haematological conditions including myelodysplastic disease, PV or pre-fibrotic hypercellular PMF. Some studies have reported that PT patients with the JAK2 V617F mutation have features more like those of PV compared to those without the mutation. At diagnosis they have higher haemoglobin, neutrophil counts and increased bone marrow erythropoietic and granulopoietic activity, as well as lower ferritin and Epo levels (Campbell *et al*, 2005;Wolanskyj *et al*, 2005). Like PV, up to 40% of patients present with thrombo-haemorrhagic events (Elliott & Tefferi, 2005).

The diagnosis of these diseases has evolved as their pathogenesis has become better understood. This has resulted in the development of different but overlapping diagnostic criteria for PV including those of the Polycythaemia Vera Study Group (PVSG), British Committee for Standards in haematology (BCSH) and the World Health Organisation (WHO) (McMullin *et al*, 2005;McMullin *et al*, 2007;Michiels *et al*, 2006;Murphy *et al*, 1986;Tefferi *et al*, 2007) (Appendix 1). All PV criteria require that a true erythrocytosis is established and that secondary causes are

excluded. The early PVSG criteria also included presence of other myeloid features including splenomegaly as support for the diagnosis of PV. These criteria were retained in the BCSH 2005 guidelines with the addition of tests of clonality and growth factor independence. The WHO criteria allow the use of elevated haemoglobin values alone (in comparison to RCM or haematocrit) in defining erythrocytosis and recognise clonality and growth factor independence but also include typical bone marrow features as supporting evidence for PV. The identification of the JAK2 V617F mutation in almost all PV patients has had the greatest influence on diagnosis. Consequently, the BCSH and WHO diagnostic criteria have been revised to include it as a positive criterion in combination with evidence of erythrocytosis.

The diagnosis of PT in patients with a persistent thrombocytosis above 600 $x10^9$/L is mainly one of exclusion of both reactive causes and other MPD (Michiels *et al*, 2006;Murphy *et al*, 1986;Tefferi & Vardiman, 2007). Identification of the JAK2 V617F mutation (or MPL mutations) confirm the primary nature of the thrombocytosis but it is still necessary to exclude other haematological conditions by careful assessment for erythrocytosis or bone marrow features of dysplasia or myelofibrosis. As before and until further responsible mutations are identified, the diagnosis of the 50-60% of PT cases which are JAK2 V617F negative remains one of exclusion.

Assessment of fibrosis is an important part of the histological assessment of the bone marrow and a number of difficulties are recognised. Fibrosis reflects an increase in the deposition of reticulin or collagen in the extracellular tissue and may be localised or diffuse. In the normal bone marrow, no reticulin, individual fine fibres or small focal networks may be present. Increased reticulin with diffuse fibre networks and coarse fibres is frequently observed in abnormal marrow and may be reactive to haematological or non-haematological malignant disease. Reticulin fibrosis is a feature common to the MPD but is rarely marked in uncomplicated PT or PV, in contrast to the fibrotic stage of PMF where the presence of coarse reticulin fibres and collagen is diagnostic (Bain *et al*, 2001)

The patchy nature of marrow fibrosis and differences in the processing of biopsies may influence the quantification of fibrosis (Buesche *et al*, 2006). Further problems are posed by differences in the histopathological diagnostic criteria used and a variety of fibrosis grading systems have been employed in different regions at different times (Thiele *et al*, 2005;Bauermeister, 1971). A European consensus based on a number of published systems and the commonly used Bauermeister criteria are shown in Table 1.1.

There are also differences between the diagnostic criteria for the myeloproliferative disorders in the differentiation of PT from early pre-fibrotic MF. Using the PVSG criteria, differentiation of PT from PMF is defined by the absence of collagen fibrosis or affecting less than 1/3 of the biopsy with no splenomegaly or leucoerythroblastic film features (Murphy *et al*, 1986). In contrast, the WHO criteria place greater emphasis on the positive use of trephine histology to distinguish "true PT" from the early stages of myelofibrosis. They define pre-fibrotic MF by the presence of atypical megakaryocyte features (clustering, maturation defects or bare nuclei) , in contrast to PT where the megakaryocytes are expected to be large and mature (Tefferi & Vardiman, 2007). However in a recent study the trephines of 370 PT patients enrolled in the PT-1 trial were reviewed by haematopathology experts who were unable to distinguish the two diagnoses according to the WHO classification (Wilkins *et al*, 2008). This reflects the ongoing controversy as to whether there is a spectrum of disease in PT patients from those without marrow fibrosis through sub-clinical fibrosis to those who will ultimately develop clinical MF; or alternatively that pre-fibrotic MF is a distinct entity commonly misdiagnosed as PT which can be distinguished according to histological features at presentation and which has a significantly higher likelihood of progression to clinical MF. The interpretation of case-control and cohort studies that estimate the risk of progression to myelofibrosis may therefore be confounded by such sampling and procedural variables.

Bauermeister (1971)	Grade 0	Normal	No reticulin fibres
	Grade 1	Normal	Occasional fine fibres and foci of fine fibre networks
	Grade 2	Normal	Fine fibre network throughout. No coarse fibres.
	Grade 3	Reticulin fibrosis	Diffuse fine fibre network with scattered coarse fibres.
	Grade 4	Myelofibrosis	Diffuse often coarse fibre network with collagen fibres.
European Consensus (2005)	MF-0	Normal	Scattered linear reticulin with no intersections
	MF-1		Loose network of reticulin with many intersections,
	MF-2		Diffuse/dense increase in reticulin with extensive intersections, occasional focal bundles of collagen
	MF-3		Diffuse/dense increase in reticulin with extensive intersections with coarse bundles of collagen, often associated with significant osteosclerosis

Table 1.1 Fibrosis grading systems

Bauermeister fibrosis grading system and the European Consensus on grading bone marrow fibrosis (Bauermeister, 1971;Thiele *et al*, 2005)

1.1.4 PV and PT - Complications and Prognosis

Haemorrhagic or thrombotic events at presentation are reported in 30-55% of PT and PV patients (Harrison *et al*, 2005;Jensen *et al*, 2000b;Marchioli *et al*, 2005;Passamonti *et al*, 2004;Wolanskyj *et al*, 2006) The natural history of PV and PT is of death and disability from vascular and haematological complications but with treatment, prognosis can be improved towards that of age matched controls. Complications fall into two main categories: vascular, including haemorrhagic episodes or arterial and venous thrombosis and haematological, including progression to acute leukaemia (AL) or myelofibrosis (MF). Studies of PT and PV populations published over the last 20 years give us some idea of the incidence of these complications at presentation and follow-up, overall survival and the associated risk factors. However the retrospective nature of many of these studies and differences in diagnostic criteria, patient population, length of follow-up and reporting methods mean that the results are not directly comparable and often appear conflicting.

1.1.4.1 Thrombosis

Thrombosis is the most common complication in the MPD. At the time of presentation thrombosis is reported in up to 55% of patients depending on the demographics of the study cohort (Cortelazzo *et al*, 1990;Harrison *et al*, 2005;Marchioli *et al*, 2005;Tefferi *et al*, 2001;Wolanskyj *et al*, 2006). In 114 PT patients who were over the age of 60 and/or had had a previous thrombosis, those who did not receive cytoreductive therapy had an annual incidence of first thrombosis of 24% (Cortelazzo *et al*, 1990). In contrast, in patients treated with hydroxycarbamide the rate was 4%, comparable to that reported in other treated PT groups (Harrison *et al*, 2005) and to the 5% annual incidence reported in treated PV patients (Marchioli *et al*, 2005). In younger patient cohorts lower incidences of 0.8-2.8% per year have been reported (Alvarez-Larran *et al*, 2007).

At both presentation and follow-up the rates of arterial thrombosis are higher than those of venous thrombosis. The most frequent arterial events are cerebrovascular, followed by cardiac then peripheral thrombosis (Harrison *et al*, 2005;Jensen *et al*, 2000b;Marchioli *et al*, 2005). As in the general population, deep vein thrombosis is commoner than pulmonary embolism or intra-abdominal thrombosis (Harrison *et al*, 2005;Marchioli *et al*, 2005) although the MPD are the single commonest causal factor in intra-abdominal thromboses, present in around 30-50% of cases (Denninger *et al*, 2000).

1.1.4.2 Haemorrhage

Haemorrhage is a less common complication in the MPD and is reported in around 6-12% of cases at presentation with an annual incidence during follow-up of 2-3% (Harrison *et al*, 2005;Jensen *et al*, 2000b;Marchioli *et al*, 2005;Wolanskyj *et al*, 2006). In the study by Wolanskyj and colleagues, which had longer follow-up than the others, the 10 year cumulative probability of haemorrhage was 15.1% and at 20 years 18.1%. Minor skin and mucous membrane bleeding is reported most commonly but there can be major bleeding episodes, the majority of which are severe gastrointestinal bleeding (Elliott & Tefferi, 2005;Harrison *et al*, 2005). The risk of haemorrhagic episodes in PV patients has been associated with older age, longer duration of disease and previous haemorrhage (Marchioli *et al*, 2005).

In PT patients, a platelet count of over 1000-1500 x 10^9/L at presentation is thought to be associated with an increased risk of haemorrhage and has been attributed to the finding of acquired von Willebrand disease in such patients (Budde *et al*, 1984), with an inverse relationship between the platelet count and plasma levels of large von Willebrand factor (VWF) multimers (Budde & van Genderen, 1997). It is proposed that the increased platelet mass binds more large VWF multimers which are then effectively removed from the circulation, producing a haemorrhagic tendency.

1.1.4.3 Haematological transformation

Incidences of 5-24% for the development of MF in PT have been reported with the incidence increasing with age and time from diagnosis (Cervantes *et al*, 2002;Murphy *et al*, 1986). Estimation of the incidence of transformation to MF and AL is therefore likely to be influenced by the demographics of the study cohort and the length of patient follow-up.

In studies with shorter follow-up of less than 10 years, AL and MF occurred in less than 3% of patients (Alvarez-Larran *et al*, 2007;Harrison *et al*, 2005;Marchioli *et al*, 2005;Tefferi *et al*, 2001). Similarly in a retrospective study of 831 PV and PT patients with a median follow-up of 9.6 years, the calculated 15 year cumulative risks of AL or MF were 5% and 1.5% for PV and PT patients respectively (Passamonti *et al*, 2004). However in a retrospective analysis of 322 PT patients with a median follow-up of 13.6 years, cumulative probabilities of 1.4% and 3.8% at 10 years rising to 8.1% and 19.9% at 20 years for AL and MF respectively, were reported (Wolanskyj *et al*, 2006).

With regard to patient age, in 195 patients diagnosed with PT by PVSG criteria and followed up for a median of 7.2 years (1.9-24) the actuarial probability of MF was 2.7% at 5 years, 8.3% at 10 years and 15.3% at 15 years (Cervantes *et al*, 2002). In contrast, in 126 patients diagnosed with PT before age 40, using the same criteria, the actuarial probability of freedom from MF at 10 years was calculated to be 97% (Alvarez-Larran *et al*, 2007).

A number of studies have compared the diagnosis of PT by PVSG criteria or the 2001 WHO classification which places greater emphasis on bone marrow histology and differentiation from MF (Thiele & Kvasnicka, 2003;Thiele *et al*, 2002;Florena *et al*, 2004). These suggest that up to three-quarters of patients diagnosed with PT by PVSG criteria would be diagnosed with early or pre-fibrotic MF by WHO criteria. In two of these studies, they also examined sequential bone marrow biopsies performed at follow-up examinations in some of the patients (Thiele *et al*, 2002;Thiele &

Kvasnicka, 2003). These suggested that the development of reticulin fibrosis on repeat marrow examination or overt MF was very uncommon in patients with "true PT" diagnosed by WHO criteria but was much more frequent in patients with early or pre-fibrotic MF. However these studies had a limited follow-up of 38±30 months and 39±31 months respectively. A similar retrospective analysis of 322 patients diagnosed with PT by the 2001 WHO criteria had a median follow-up of 13.6 years and at least a decade in around three-quarters of the patients (Wolanskyj *et al*, 2006). In contrast, they observed a cumulative risk of transformation to MF of 3.8% at 10 years, 19.9% at 20 years and 28.9% at 30 years in "true" PT, similar to the results from earlier studies.

In PV, marrow reticulin is reported to be increased in 8-15% of patients at the time of diagnosis (Spivak, 2002). Long term follow-up (12-25 years) of 204 patients enrolled in the first PVSG trials showed the risk of MF to increase with time from diagnosis with an overall risk of 50% at more than 15 years (Najean *et al*, 1994). In this study, in patients treated with radiophosphorus MF was not observed before the 10^{th} year but was reported in 20% of patients at 15 years, and 50% at 20 years. In comparison, phlebotomy alone was associated with earlier progression to MF. More recently the ECLAP study (European Collaboration on Low-dose Aspirin in PV), which prospectively assessed the longer term outcomes of PV as determined by current clinical practice, the rate of haematological transformation (to overt MF or AL) was 1.3/100 persons/year (Marchioli *et al*, 2005). Longer disease duration predicted the risk of MF with a relative risk (RR) of 5.74 (95% confidence interval (CI) 1.15- 21.77) at 6 years and 15.24 (4.22-55.06) at 10 years.

In patients with post-PV/PT MF, the clinical features are similar to those of PMF. There is infiltration of the marrow by fibrotic tissue resulting in pancytopenia and leucoerythroblastic features in the peripheral blood film. Consequently patients develop symptoms of anaemia, which is often transfusion dependent, are at increased risk of infection and experience haemorrhagic complications of thrombocytopenia. Splenomegaly, as a result of extramedullary haemopoiesis, contributes to the pancytopenia and there may be local pain from stretching of the capsule and splenic

infarct. Constitutional symptoms including fever, sweats and weight loss may also be present. The International Working Group for myelofibrosis research and treatment recently published proposed criteria for the diagnosis of post-PV and post-PT myelofibrosis. These require documentation of a previous diagnosis of PV or PT by the WHO rather than the PVSG criteria and bone marrow fibrosis of Grade 3-4 according to the Bauermeister classification (Bauermeister, 1971) in addition to other clinical and laboratory features (Barosi *et al*, 2007). Treatment is mainly supportive although judicial use of chemotherapy may be instituted for symptomatic splenomegaly, constitutional symptoms or thrombocytosis. Splenectomy may also be considered but is associated with significant peri- and post-operative morbidity and mortality. A small number of young patients with high-risk disease may be suitable for allogeneic stem cell transplantation (Cervantes, 2005).

1.1.4.4 Survival and risk stratification

In untreated PV and PT survival is reduced due to vascular complications (Chievitz & Thiede, 1962). Most studies consider treated populations and in PT patients survival has been reported to approach that of age-matched populations although the relatively small patient numbers and short follow-up periods in some of these studies limit their interpretation (Rozman *et al*, 1991;van Genderen *et al*, 1997). In a larger study of 187 PT patients the findings were similar for older patients but for those diagnosed before the age of 55 years the relative risk of death was four times greater than for healthy age-matched controls (Bazzan *et al*, 1999). Two studies with longer follow-up (median 9.3 years and 13.6 years) have suggested a median survival for PT patients of 18-22 years (Passamonti *et al*, 2004;Wolanskyj *et al*, 2006). Survival was similar to that of an age-matched population in the first 10 years but worsened thereafter, RR 2.21 (95% CI 1.74-2.76) at 20 years and RR 3.37 (1.84 – 5.56) at 30 years (Wolanskyj *et al*, 2006). PV patients had reduced survival compared to either PT patients or an age matched population, of 65% at 15 years compared to 73% (p=0.01) (Passamonti *et al*, 2004).

A variety of risk factors for thrombotic complications and overall survival have been identified. Those most strongly and consistently associated with thrombotic risk are older age (>60 years) and prior history of thrombosis (Gruppo Italiano, 1995;Landolfi *et al*, 2007;Marchioli *et al*, 2005;Passamonti *et al*, 2004;Wolanskyj *et al*, 2006). More recently an elevated white cell count at diagnosis has been shown to be independently associated with thrombosis in PT (Carobbio *et al*, 2007;Wolanskyj *et al*, 2005) and PV (Landolfi *et al*, 2007), where it was also associated with leukaemic transformation and reduced survival in a retrospective analysis (Gangat *et al*, 2007).

The role of traditional cardiovascular risk factors is less clear but some studies have found associations with tobacco use (Alvarez-Larran *et al*, 2007;Cortelazzo *et al*, 1990;Landolfi *et al*, 2007;Wolanskyj *et al*, 2006), diabetes mellitus (DM) (Landolfi *et al*, 2007;Wolanskyj *et al*, 2006) and cardiac failure (Landolfi *et al*, 2007). There is also limited data from small retrospective studies on the influence of concurrent thrombophilias on thrombotic risk. In PT patients an increased incidence of anti-β_2 glycoprotein I IgM antiphospholipid antibodies, reduced protein C and S levels and an association between carriage of the Factor V Leiden mutation and incidence of venous thrombosis have all been reported (Harrison *et al*, 2002;Jensen *et al*, 2002;Ruggeri *et al*, 2002).

Based on these risk factors, a number of models for risk stratification have been proposed in an attempt to identify those patients at highest risk of thrombosis and so to apply appropriate management strategies. At the most simplistic level, high risk patients aged over 60 years and/or with a prior history of thrombosis and low risk patients who meet neither of these criteria are easily identified. The identification of an intermediate risk group of patients by inclusion of cardiovascular risk factors and additional age criteria is less clear. Extreme thrombocytosis above 1000-1500 x 10^9/L is also considered to confer an increased thrombo-haemorrhagic risk, although substantive evidence for this is lacking. Prospective data from the PT-1 trial which includes such a group will be useful in determining its discriminative strength with regard to their thrombotic risk and treatment needs. A stratification model which

includes the presence of leucocytosis at diagnosis in addition to age and thrombotic history has been shown to be predictive of survival using retrospective data (Gangat *et al*, 2006). An example of a risk stratification model is outlined in Table 1.2.

In patients with PMF the median survival of 4 years from diagnosis is significantly shorter than the 14 years for age and sex-matched controls. However low risk patients can be identified (by the presence of normal haemoglobin and leucocyte counts and the absence of peripheral blasts or constitutional symptoms) whose median survival may be more than twice this (Cervantes, 2005). The major causes of death are infection, haemorrhage, cardiac failure and leukaemic transformation (Reilly, 1997). In patients developing overt post-PT or post-PV MF life expectancy is likely to be similarly affected. The recent studies re-evaluating the diagnosis of PT patients using the WHO criteria suggest that some of the shortened survival reported in these patients may be accounted for by misclassified early or pre-fibrotic MF (Thiele *et al*, 2002;Thiele & Kvasnicka, 2003).

	Low risk	Intermediate risk		High risk
Age (years)	< 40 y **and**	<40 y **and**	40-60 y **and**	> 60 y **or**
Platelet count x10^9/L	<1000 **and**	<1000 **and**	<1000 **and**	>1000 **or**
Prior thrombosis or haemorrhage	NO **and**	NO **and**	NO **and**	YES **or**
Cardiovascular co-morbidities*	NO **and**	NO **and**	NO **and**	YES
Inherited thrombophilia or cardiovascular risk factors	NO	YES**	YES/NO	-

Table 1.2 Risk stratification for thrombosis

Example of risk stratification for thrombosis in patients with PV or PT adapted from previously published models (Barbui *et al*, 2004;Harrison, 2005b;McMullin *et al*, 2005). *Cardiovascular co-morbidities include hypertension, diabetes and congestive cardiac failure. **The inclusion of an intermediate risk group, based on the presence of inherited thrombophilia or cardiovascular risk factors, in otherwise low risk patients, is not agreed.

1.1.5 Treatment

The goal of treatment in PV and PT is to reduce morbidity and mortality by reducing the incidence of thrombo-haemorrhagic complications without increasing the risk of fibrotic or leukaemic transformation. In both conditions antiplatelet agents are key in the reduction of thrombotic risk. In PV, venesection is generally first line therapy to normalise the haematocrit but cytoreductives may be required if venesection is not tolerated or if control of thrombocytosis is required. Cytoreductives may also have additional benefits in the reduction of thrombotic risk over and above their role in normalisation of peripheral blood counts. In PT, normalisation of the platelet count can be achieved using a number of different cytotoxic and non-cytotoxic agents.

In order to achieve a balance between the benefits of these therapies and concerns regarding their potential leukaemogenic effects, individual patients are stratified according to their thrombotic risk (Table 1.2). Thus, exposure to agents whose long term risks are uncertain is avoided in low risk patients but is considered acceptable, given the established benefits of such treatment, in patients at higher thrombotic risk. Despite the lack of clear evidence for the contribution of traditional cardiovascular risk factors to thrombotic risk, there is consensus of opinion that for individual patients, cessation of smoking and the appropriate management of hypertension, cholesterol and overweight is likely to be beneficial.

Aspirin

Aspirin has well established benefits in both the primary and secondary prevention of cardiovascular disease in haematologically normal persons. Results from the ECLAP study have supported the benefit of low-dose aspirin in PV (Landolfi *et al*, 2004). In this study, 518 PV patients with no clear indication or contra-indication for antiplatelet therapy were randomised to either low-dose aspirin or placebo. Aspirin use reduced the combined primary endpoint of non-fatal myocardial infarction, stroke, major venous thromboembolism or death from a cardiovascular cause (RR

0.40; 95%CI 0.18-0.91) although overall mortality and cardiovascular mortality were not significantly reduced. There was no significant increase in major haemorrhage in the aspirin group. Aspirin is therefore recommended for all PV patients in the absence of a contraindication.

Despite the lack of similarly robust evidence for its benefit, aspirin is also widely used in PT. A retrospective analysis of low dose aspirin use 68 PT patients, reported a reduced incidence of thrombosis in patients treated with aspirin, alone or with cytoreduction, compared to those receiving no treatment (van Genderen *et al*, 1997). All patients were low risk for haemorrhage (platelet counts below 1000 x 10^9/L and no history of haemorrhage) and there was an increase in the incidence of minor bleeding episodes only. A number of factors provide some rationale for the use of anti-platelet therapy in PT, including evidence of platelet activation (Jensen *et al*, 2000a;Musolino *et al*, 2000;Robertson *et al*, 2007;Villmow *et al*, 2002) and increased platelet thromboxane A_2 (Landolfi *et al*, 1992). Aspirin is also an effective treatment to reverse the microcirculatory symptoms of PT such as erythromelalgia (van Genderen *et al*, 1996) and is indicated in haematologically normal patients for the prevention of cerebral ischaemia, the commonest vascular complication in PT patients. It is generally avoided in patients with platelet counts of over 1000 x10^9/L due to the potential haemorrhagic risks associated with this level of thrombocytosis (van Genderen *et al*, 1997).

Venesection

Phlebotomy to maintain the haematocrit within the normal range remains the cornerstone of therapy in PV. The incidence of thrombosis was shown to increase progressively for haematocrit above 0.44 (Pearson & Wetherley-Mein, 1978) and cerebral blood flow improves by 73% with a haematocrit below 0.45 (Harrison, 2005b). On the basis of these studies a target haematocrit of less than 0.45 is generally accepted. Results from the ECLAP study showed that the majority of patients were tightly controlled with a haematocrit of between 0.44-0.47 throughout

the study and that differences in haematocrit within the range of 0.40-0.55 were not predictive of thrombosis (Di Nisio *et al*, 2007).

Cytoreduction

In patients with either PV or PT and who are at high risk of thrombosis (see Table 1.2), cytoreduction is indicated to maintain the platelet count below $400x10^9$/L (Barbui *et al*, 2004;Harrison, 2005b). However this target platelet count is based on expert recommendation rather than a clear evidence base. In the ECLAP study of PV patients, where this recommendation was used, platelet counts of up to 600 x 10^9/L were seen and compared to those below $400x10^9$/L were not predictive for thrombosis (Hazard ratio 0.96, 95% CI 0.66-1.38) (Di Nisio *et al*, 2007). In PT, cytoreduction is not recommended in low risk patients but the question of benefit in intermediate risk patients is currently being addressed by the intermediate arm of the PT-1 study. Cytoreductive therapy may also be required in PV patients intolerant of venesection at a level adequate to maintain a haematocrit in the target range (McMullin *et al*, 2005). An isolated marked thrombocytosis (above 1000-$1500x10^9$/l) in otherwise low risk patients is generally considered to confer increased risk of both thrombosis and haemorrhage and may therefore be an indication for cytoreduction although evidence in support of this is lacking.

The choice of cytoreductive is dictated by efficacy, safety and tolerability and these factors are partially dependent on the individual.

Hydroxycarbamide (formerly hydroxyurea)

In PT patients with a high risk of thrombosis, hydroxycarbamide is effective in controlling platelet counts and reducing the incidence of thrombosis (Cortelazzo et al, 1990;Harrison et al, 2005). In a comparison between hydroxycarbamide and no cytoreduction in 114 high risk patients, thrombosis developed in 3.6% or treated

patients compared to 24% of the untreated group at a median follow-up of 24 months (Cortelazzo et al, 1990).

Whether the rate of leukaemic transformation seen in patients treated with hydroxycarbamide is any higher than that of the underlying disease is a matter of debate. There is no increased risk of leukaemia in young patients treated with hydroxycarbamide for sickle cell disease however this population does not have an underlying stem cell disorder (de Montalembert & Davies, 2001). The initial results from the PT-1 study, with a median follow-up of 39 months, did not show an increased risk of leukaemic transformation with hydroxycarbamide compared to anagrelide, a non-cytotoxic drug (Harrison, 2005a); the long-term follow-up data will be even more important in providing some answers to this issue. Hydroxycarbamide is also associated with an increased risk of dermatological side effects including mouth ulcers, leg ulcers and skin malignancies (Guillot *et al*, 2004).

At present hydroxycarbamide is considered to have the lowest leukaemic risk of the cytotoxic agents and is generally the first line choice in high-risk patients over the age of 40 years. In younger patients and pregnant women, non-cytotoxic agents such as interferon and anagrelide may be preferred. Second line therapy is also indicated in patients who are intolerant of hydroxycarbamide due to side effects or if the dose required for adequate control of the platelet count results in unacceptable anaemia or neutropenia. In this situation alternative or dual therapy with anagrelide may be considered. In older patients, particularly where compliance is problematic, alternative cytotoxics, including alkylating agents such as radiophosphorus or busulphan, that may have more leukaemogenic potential may be appropriate (Barbui *et al*, 2004;McMullin *et al*, 2005).

Anagrelide

Anagrelide selectively blocks megakaryocyte differentiation and proliferation and thus reduces the platelet count. It is therefore an attractive option for cytoreduction

as, from its pharmacological effects it would not be expected to increase the risk of acute leukaemia. It has been widely used for some time in the United States and its safety and efficacy was retrospectively analysed in 934 PT and 208 PV patients, of whom 189 had not received any other cytoreductive therapy (Fruchtman *et al*, 2005). Control of the platelet count to less than 600 $x10^9$/L (or a reduction of more than 50% from baseline) was achieved in around two-thirds of PT patients. The rate of leukaemic transformation was 2-3%, comparable to that reported in previous studies. With regard to tolerability, 40% of patients reported drug related adverse events, most commonly mild to moderate gastrointestinal symptoms, headache or dizziness, oedema and cardiac symptoms such as palpitations.

This type of retrospective analysis is helpful but until recently there had been a lack of randomised controlled trials of anagrelide with prolonged follow-up. In the high risk arm of the PT-1 study 809 patients with PT and a high risk for thrombosis were randomised to treatment with aspirin and either anagrelide or hydroxycarbamide (Harrison *et al*, 2005). Control of the platelet count was similar but there were statistically significant differences in the rates of vascular events between the groups. The anagrelide group had more than double the rate of arterial thrombosis (Odds Ratio (OR) 2.16; 95% CI 1.27-3.69) with significantly more transient ischaemic attacks (OR 5.72; 2.08-15.73). In comparison the rate of venous thrombosis was lower (OR 0.27; 0.06-0.71) especially for deep vein thrombosis.

That these discrepancies were seen despite comparable platelet counts, suggests that hydroxycarbamide may have additional anti-thrombotic effects. For example the white cell count was lower in patients receiving hydroxycarbamide and neutrophil activation has been demonstrated in PT in association with increased markers of coagulation activation (Falanga *et al*, 2000). Further analysis revealed a differential effect of the JAK2 V617F mutation on the therapeutic response. Patients positive for the mutation were more sensitive to the platelet lowering effect of hydroxycarbamide and those on anagrelide experienced significantly more arterial thromboses compared to those on hydroxycarbamide (15 v 9 events, $p = 0.03$) (Campbell *et al*, 2005).

There was also more serious haemorrhage in the anagrelide group (Odds ratio (OR) 2.61; 95% CI 1.27-5.33), particularly gastrointestinal. This may be explained by the anti-platelet aggregation effects of anagrelide in combination with aspirin. Unexpectedly, the results suggested an increased rate of transformation to myelofibrosis in the anagrelide group but the lack of baseline bone marrow examinations means that the pre-existence of more pre-fibrotic changes in the anagrelide group cannot be excluded. The side effect profile was consistent with the results from retrospective studies and more patients on anagrelide withdrew from the study due to these or because of serious adverse events.

Interferon alpha

A further non-cytotoxic option in both PV and PT, is interferon-alpha (IFN-α) which controls counts in up to 90% of patients (Langer *et al*, 2005;Lengfelder *et al*, 1996). In some patients, the JAK2 V617F allele load is also reduced (Langer *et al*, 2005;Samuelsson *et al*, 2006). Unfortunately its side effect profile means that its use tends to be limited by tolerability and 25-30% of patients stop the treatment mainly due to flu-like symptoms and depression (Langer *et al*, 2005;Lengfelder *et al*, 1996).

1.2 Pathophysiology of thrombosis in myeloproliferative disorders

The elevated haematocrit and thrombocytosis are contributory factors to the thrombotic risk in PV and PT but clinical evidence suggests that they are not wholly responsible and a number of other abnormalities of the vascular system have been identified.

1.2.1 Haematocrit and thrombocytosis

The effects of haematocrit and thrombocytosis on thrombotic risk have been reviewed by Elliot and Tefferi (2005). Haematocrit is the major determinant of whole blood viscosity *in vitro* but *in vivo* other factors are also involved. Higher haematocrit levels significantly reduce cerebral blood flow due both to the increased viscosity and to the negative regulatory effect of the higher arterial oxygen concentrations consequent on the raised haematocrit. The axial migration of red cells in normal flow conditions also means that the increased haematocrit narrows the plasma/platelet zone and displaces it further towards the vessel wall. This has multiple effects, increasing the shear forces to which the platelets are exposed and increasing platelet-platelet and platelet-endothelial interactions.

The effect of the absolute platelet count on thrombotic tendency is unclear and no significant correlation of degree of thrombocytosis and thrombosis has been observed. In high risk PT patients, reducing the platelet count may reduce the incidence of thrombosis but this may be due to more general myelosuppressive treatment effects rather than the reduced platelet count itself. In keeping with this, in the high risk arm of the PT-1 trial, significantly more arterial events were observed in the anagrelide treated group compared to those on hydroxycarbamide despite equivalent control of the platelet counts (Harrison *et al*, 2005).

1.2.2 Platelet activation

Evidence of increased platelet activation has been widely observed in MPD patients with increases in platelet expression of the activation markers P-selectin (P-sel)(CD62) and thrombospondin, levels of soluble P-sel and platelet α-granule contents and a greater proportion of platelet microparticles (Arellano-Rodrigo *et al*, 2006;Bellucci *et al*, 1993;Falanga *et al*, 2000;Falanga *et al*, 2005;Jensen *et al*, 2000a;Musolino *et al*, 2000;Robertson *et al*, 2007). P-sel levels have also been variably correlated with thrombotic history (Arellano-Rodrigo *et al*, 2006;Falanga *et al*, 2005;Jensen *et al*, 2000a;Musolino *et al*, 2000;Robertson *et al*, 2007;Villmow *et al*, 2002). The cause of this platelet activation is unknown but leucocyte induced activation, abnormalities of platelet receptors and increased platelet thromboxane A_2 have been suggested. More recently, an association between the presence of the JAK2 V617F mutation and markers of platelet activation has been reported (Robertson *et al*, 2007).

Clinical observations also provide support for the role of platelet activation in the thrombotic manifestations of MPD. Microvascular erythromelalgic symptoms are highly sensitive to the effects of aspirin and markers of platelet activation were observed to fall in line with symptom resolution following aspirin treatment (van Genderen *et al*, 1996). Evidence from the ECLAP study in PV patients also showed that aspirin was beneficial in reducing the risk of cardiovascular events (Landolfi *et al*, 2004).

1.2.3 Leucocytes

Markers of leucocyte activation such as membrane CD11b and leucocyte alkaline phosphatase (LAP) are elevated in MPD patients alongside increased plasma levels of neutrophil enzymes (Falanga *et al*, 2000;Villmow *et al*, 2002). Increased platelet-leucocyte interactions (PLA) are also observed, similar to other vascular conditions, such as myocardial ischaemia and stroke, and correlate with markers of both

leucocyte and platelet activation (Falanga *et al*, 2005;Jensen *et al*, 2001;Villmow *et al*, 2002). Following the *in vitro* stimulation of whole blood with a neutrophil agonist, an increase in neutrophil activation markers and PLA was observed, an effect that was attenuated in PT patients who were treated with aspirin (Falanga *et al*, 2005). Leucocyte activation and degranulation releases enzymes including myeloperoxidase and elastase and *in vitro* these cause endothelial cell detachment and release of VWF and the anticoagulant protein thrombomodulin (TM). They may also affect the balance of the coagulation system by the inactivation of anticoagulant proteins (Esmon, 2005). In keeping with these observations, two recent large clinical studies of PV and PT patients have shown that elevated leucocyte counts are predictive for thrombosis (Landolfi *et al*, 2007;Gangat *et al*, 2007). An effect on leucocytes by myelosuppressive therapy may also explain its beneficial effects over and above those of non-myelosuppressive cytoreduction in reducing thrombotic risk.

1.2.4 Endothelial dysfunction and prothrombotic state

Evidence also exists for endothelial abnormalities in MPD but reports are inconsistent. Soluble TM, VWF antigen and E-selectin have been studied as *in vivo* markers of endothelial disturbance (Bellucci *et al*, 1993;Falanga *et al*, 2000;Landolfi *et al*, 2007;Musolino *et al*, 2000;Robertson *et al*, 2007). Elevated serum levels were observed in some studies and variably correlated with the thrombotic history but in others were no different from controls. Impaired flow mediated vasodilatation, a feature of many vascular conditions, was also observed in PV patients compared to controls matched for cardiovascular disease. Interestingly, this effect was not correlated with either the haematocrit or platelet count (Neunteufl *et al*, 2001).

Evidence for an underlying state of increased thrombin activity in MPD patients comes from the observation of increased levels of prothrombin fragments, fibrin degradation products and thrombin-antithrombin complexes (Bellucci *et al*, 1993;Falanga *et al*, 2000;Robertson *et al*, 2007). However, similar to measures of endothelial dysfunction, these findings are not consistently observed. Rather than

implying a primary role for endothelial dysfunction or increased thrombin activation it may be that these observations simply reflect the influence of other prothrombotic factors on the vasculature including age and co-morbidities.

1.2.5 Molecular mechanisms

Studies of the effects of clonality on thrombotic risk using X chromosome inactivation patterns suggest that a proportion of PT patients have polyclonal disease and may have a reduced thrombotic risk (Harrison *et al*, 1999;Shih *et al*, 2002). However in the latter of these studies, patients with monoclonal disease were significantly older, a recognised risk factor for thrombosis. In a more recent publication no association of clonality with thrombosis or with markers of platelet or coagulation activation were observed but patients with the JAK2 V617F mutation had increased levels of P-sel (Robertson *et al*, 2007). In PT patients this mutation is associated with higher leucocyte counts (Campbell *et al*, 2005;Carobbio *et al*, 2007;Kittur *et al*, 2007;Wolanskyj *et al*, 2005) and in PV a gene dosage effect on leucocyte count and activation is observed (Vannucchi *et al*, 2007a). Since P-sel levels and leucocyte count have been correlated with thrombosis, we might predict an association between JAK2 V167F mutation status and thrombosis. However such a correlation has been observed in only two retrospective studies of over 100 patients and only with venous (not arterial) thrombosis (Campbell *et al*, 2005;Kittur *et al*, 2007) whilst other similarly sized studies have not reported such an association (Carobbio *et al*, 2007;Wolanskyj *et al*, 2005).

1.3 Pathophysiology of myelofibrosis

Marrow studies in PMF demonstrate a marked increase in neovascularisation of the stromal tissue together with an increased deposition of extracellular matrix (ECM) components - collagens, fibronectin, laminin and vitronectin. This is a cytokine-mediated reactive phenomenon and in particular, growth factors including transforming growth factor β (TGFβ) and platelet derived growth factor (PDGF) have been implicated. Bone marrow and platelets are a major source of these factors (Fava *et al*, 1990). Imbalances in the levels of tissue metalloproteinases, which break down the ECM and their inhibitors, are also observed. It is likely that similar mechanisms are involved in the development of fibrosis in PV and PT. The recently identified JAK2 tyrosine kinase and MPL mutations seen in a proportion of PMF, PT and PV may also have a causative role in the development of fibrosis.

1.3.1 Platelets, megakaryocytes and cytokines

Several observations suggest a key role for megakaryocytes and platelets in the pathogenesis of marrow fibrosis. Megakaryocyte hyperplasia is a prominent feature in PMF and the presence of dysplastic forms may be an important distinguishing feature from PT (Tefferi & Vardiman, 2007). In addition, the marrow stromal reaction is often noted to be maximal in areas of megakaryocyte clustering. CD34 positive peripheral blood stem cells isolated from patients with PMF and cultured with Tpo generate greater numbers of megakaryocytes than normal CD34 positive cells and show reduced rates of apoptosis (Ciurea *et al*, 2007). Marrow fibrosis is also a prominent feature of both acute megakaryoblastic leukaemia and transient myeloproliferative disorder (TMD) which is characterised by an increase in circulating megakaryoblasts (Hattori *et al*, 2001).

TGFβ and PDGF are the cytokines most strongly implicated in the pathogenesis of myelofibrosis. Megakaryocytes are a major site for their synthesis and both are stored in platelet α-granules (Fava *et al*, 1990). The functions of PDGF and TGFβ

are reviewed by Miyazono (1989) and Reilly (1992). PDGF stimulates the growth and cell division of fibroblasts. TGFβ has a number of actions; it increases the expression of genes coding for collagen, fibronectin and other stromal elements; it has strong angiogenic properties; and it shifts the balance of tissue metalloproteinases and their inhibitors towards increasing the ECM.

The roles of TGFβ and PDGF are partly inferred from studies of other haematological disorders where fibrosis is a prominent feature. TMD, an acute leukaemia-like disorder seen in children with Down syndrome, is characterised by organ fibrosis and circulating megakaryoblasts. The blasts and liver tissue from TMD patients with hepatic fibrosis showed increased expression of the PDGF genes and TGFβ gene expression was increased in blast cells from both TMD and acute megakaryocytic leukaemia (Hattori *et al*, 2001). Similarly in PMF bone marrow, increased expression of the genes for PDGF and its receptor was observed in advanced PMF compared to the pre-fibrotic state (Bock *et al*, 2005). In hairy cell leukaemia, where fibrosis is a prominent feature, TGFβ was present at higher concentrations in the bone marrow, serum and plasma of 13 patients compared to healthy controls (Shehata *et al*, 2004). In this study, *ex-vivo* experiments also demonstrated that TGFβ enhanced the production and deposition of reticulin and collagen fibres by bone marrow fibroblasts. Elevated serum levels of TGFβ have also been observed in PMF patients compared to controls (Rameshwar *et al*, 1998).

The role of TGFβ is also supported by the results of *in vitro* experiments. Studies have used a mouse model of MF where Tpo is over-expressed by infection with a Tpo encoding retrovirus (Tpo^{high} mice). This results in the development of a myeloproliferative syndrome with severe marrow and splenic fibrosis in wild type mice but in contrast no reticulin deposition was seen in TGF-β null mice (Chagraoui *et al*, 2002). Similarly in a rat model, supra-pharmacological doses of recombinant human megakaryocyte growth and development factor induced predominantly reticulin fibrosis in the bone marrow associated with an increase in bone marrow and plasma TGFβ levels (Yanagida *et al*, 1997). As TGFβ levels normalised, reversal of the fibrotic changes in the bone marrow was observed.

Although a role for TGFβ and PDGF in fibrosis has been established, the mechanisms of their increased production and release are less clearly defined. Decreased platelet PDGF activity but increased megakaryocyte PDGF mRNA expression (Katoh *et al*, 1988;Katoh *et al*, 1990;Lev *et al*, 2002) and increased plasma and urinary PDGF concentrations (Gersuk *et al*, 1989;Lev *et al*, 2002) have been reported in patients with MPD. Consistent with this, intraplatelet levels of β-thromboglobulin (BTG) and platelet factor 4 (PF4), which are also stored in platelet α-granules, are reported to be decreased in MPD (Katoh *et al*, 1988), while plasma and urine levels are elevated (Sacchi *et al*, 1986). This would support a mechanism whereby defects of α-granule storage or release could result in elevated levels of PDGF and TGFβ in the plasma and/or bone marrow.

One potential confounding factor in these studies is that poor technique may cause *in vitro* platelet activation and release of α-granule contents. BTG and PF4 are present in platelets in similar amounts however *in vivo* there is an increased ratio of BTG to PF4. Rapid clearance of PF4 through binding to endothelial cells compared to a slower renal clearance of BTG is thought to account for this difference (Dawes *et al*, 1978). It has been proposed that these properties can be exploited to detect artefactual *in vitro* platelet activation when there is a comparable elevation of both BTG and PF4, in contrast to a normal sample with low levels of both or *in vivo* platelet α-granule release with elevated BTG to PF4 ratio (Kaplan & Owen, 1981).

Electron microscopy studies of megakaryocytes in TPO^{high} mice, have shown extensive emperipoiesis of neutrophils, associated with intracytoplasmic rupture of α-granules (Schmitt *et al*, 2000). Increased megakaryocyte P-sel expression was also observed suggesting this may mediate sequestration of neutrophils via the P-selectin glycoprotein ligand (PSGL), leading to α-granule rupture and growth factor release. Similar findings were observed in bone marrow trephines from 12 patients with PMF.

Intracellular signalling involving NFkB (nuclear factor kappa beta) has also been implicated in the abnormal release of TGFβ. Spontaneous NFkB activation is

reported in monocytes in PMF and results in increased interleukin-1 (IL-1) and consequently TGFβ production by an autocrine mechanism (Rameshwar *et al*, 2000). NFkB activation is also reported in megakaryocytes and circulating CD34 positive cells in PMF (Komura *et al*, 2005). In TPO^{high} mouse models of MF, increased plasma levels of IL-1α are observed (Wagner-Ballon *et al*, 2006) and treatment with the NFkB inhibitor bortezomib was shown to reduce NFkB activation and plasma IL-1α. Bone marrow and spleen fibrosis and myeloproliferation was also reduced via a reduction in marrow TGFβ (Wagner-Ballon *et al*, 2007).

1.3.2 Molecular mechanisms

The fibrotic effects of the JAK2 V617F mutation, detected in around 50% of PMF, can be deduced from mouse models transplanted with BM expressing either the wild type or mutated JAK2 protein. Expression of JAK2 V617F but not the wild type resulted in clinico-pathological features similar to PV in humans including erythrocytosis, leucocytosis, megakaryocyte hyperplasia and bone marrow reticulin fibrosis (Wernig *et al*, 2006). Megakaryocyte hyperplasia and dysplasia was seen even in the absence of thrombocytosis, suggesting that quantitative or qualitative megakaryocyte changes may be more important in the pathogenesis of fibrosis than the thrombocytosis per se. Strain specific differences were seen in the mice, in particular in levels of reticulin fibrosis, suggesting the influence of other undefined factors.

Several groups have now shown that compared to PMF, more patients with post-PV MF are homozygous for the JAK2 V617F mutation (Passamonti *et al*, 2006;Popat *et al*, 2006;Tefferi *et al*, 2005). Gene dosage of JAK2 V617F was also measured in megakaryocytes from 68 patients with JAK2 V617F positive PT, PV, PMF and pre-fibrotic MF (Hussein *et al*, 2007). Significantly lower levels of mutated JAK2 alleles were found in the megakaryocytes from patients with PT or prefibrotic MF compared to patients with PV or PMF with manifest fibrosis. The difference between pre-fibrotic and fibrotic MF might suggest a causative role for gene dosage in fibrotic

progression but sequential follow-up showed no direct correlation between development of homozygosity for JAK2 V617F and the progression of fibrosis.

The gain-of-function mutations of the Tpo receptor MPL reported in a small number of patients with JAK2 V617F negative PMF have also been studied in murine models. Similar to the JAK2 V617F mutation, expression of MPL W515L but not wild-type resulted in a myeloproliferative disorder characterized by marked thrombocytosis, splenomegaly due to extramedullary haematopoiesis and increased reticulin fibrosis (Pikman *et al*, 2006). Both the JAK2 V617F and MPL mutations cause deregulated MPL signalling which is mimicked by the Tpo^{high} mouse models in which fibrosis is a prominent feature. However the discrepancy between the frequent presence of these mutations in PMF, PV and PT, but a significant degree of fibrosis in only a proportion, implies that other factors must also be involved.

1.4 Microparticles, vascular disease and thrombosis

Microparticles are submicron plasma particles which are formed from platelets, endothelial cells, leucocytes and erythrocytes, by the exocytic budding of cell membranes. During their formation, the symmetry of the plasma membrane lipid bilayer is altered, resulting in the exposure of a phospholipid rich surface. Microparticles also bear antigens expressed on the surface of the cells from which they originate. It is this phospholipid rich surface and the expression of functional molecules such as tissue factor or selectins that mediate the biological actions of microparticles in the initiation and propagation of thrombus and in cellular interactions. Elevated levels of microparticles are found in a number of conditions associated with vascular dysfunction, thrombosis and inflammation.

1.4.1 Biological aspects of microparticles

1.4.1.1 Formation of microparticles

Microparticles are constitutively released from the surface of cells but their formation can be up-regulated by cell activation or apoptosis. Other small vesicles which are also released from cells include exosomes, pre-formed intracellularly and released by fusion with the cell membrane, and apoptotic bodies which are formed late in the process of cell death. Membrane microparticles differ from these vesicles in both their mechanism of formation and their functional properties. After cell activation or apoptosis is triggered, there is a rise in cytosolic calcium concentration, leading to kinase activation and phosphatase inhibition which induce cytoskeletal changes. Consequently there is alteration of the normal lipid bilayer of the cell membrane, with "flip-flopping" of the internal negatively charged phospholipids to the external surface. Membrane blebbing and microparticle formation then results (VanWijk *et al*, 2003). Microparticle membrane composition therefore reflects the membranous elements of the cell of origin. Their surfaces are rich in negatively charged phospholipids, chiefly phosphatidylserine (PPS), and they bear surface

antigens of their parent cell including functional molecules such as tissue factor (TF) or selectins.

A number of cell activation and apoptosis triggers which induce microparticle formation have been identified including chemical stimuli such as cytokines, thrombin and endotoxin, or physical stimuli such as shear stress or hypoxia (VanWijk *et al*, 2003). In platelet microparticle (PMP) formation it has been shown that platelet glycoprotein (GP) receptors and adhesion molecules can also be involved. For example, the GPIb receptor mediates adhesion to VWF and, under hydrodynamic flow, stretching of the platelet membrane occurs followed by separation of areas of tethered membrane and the production of microparticles (Reininger *et al*, 2006). Platelet P-sel levels correlate with PMP levels in mice and *in vitro,* P-sel immunoglobulin can induce microparticle formation in human blood; this effect is abolished by blocking antibodies to the counter-receptor PSGL (Hrachovinova *et al*, 2003).

It is likely that the nature of the trigger influences both the number of the microparticles released and their physical and functional properties. For example, *in vitro*, endothelial cells can be induced to express different profiles of surface antigens, such as adhesion molecules or tissue factor, by different stimuli. Many of these same stimuli can also induce microparticle release. It might therefore be expected that the induced endothelial cell changes will be reflected in the composition and antigenic profile of the microparticles which will in turn determine their pathophysiological effects. This was demonstrated by culturing endothelial cells from brain, kidney and coronary arteries and exposing them to apoptotic or activating stimuli (Jimenez *et al*, 2003b). The endothelial microparticles (EMP) released following apoptotic stimuli had higher levels of surface Annexin V binding to PPS and of constitutive endothelial cell markers such as CD31 (Platelet Endothelial Cell Adhesion Molecule, PECAM). In contrast, EMP induced by activation with tumour necrosis factor α (TNFα) expressed higher levels of inducible antigens, such as E-selectin (E-sel, CD62E), which were also increased on the parent endothelial cells. Additionally, microvascular endothelial cells released significantly

more microparticles overall compared to the macrovascular coronary artery endothelium. The formation of phenotypically heterogeneous microparticles is represented in Figure 1a.

1.4.1.2 Microparticles – coagulation activity

Microparticles are likely to support coagulation and thrombus formation in a number of different ways. Under certain conditions they can also exhibit anticoagulant properties dependent on their origin and the stimulus to release. Accordingly, microparticles may contribute to the complex regulation of the balance between an anti- or prothrombotic vasculature. A schematic summary of the postulated role of microparticles in the development of localised thrombus is shown in Figure 1c.

In addition to coagulation factors and calcium ions, cell membrane phospholipids are necessary for the optimal activity of the coagulation system. Negatively charged phospholipids, mainly PPS, have a catalytic role promoting the formation of the intrinsic tenase and the prothrombinase enzyme complexes on the membrane surface. This phospholipid surface has traditionally been thought to be provided by activated platelets (previously known as platelet factor 3) but microparticles can also support this process with their PPS rich surfaces. Microparticles expressing TF can also be identified in some circumstances, thus providing a suitable environment both to initiate and to propagate coagulation (Figure 1b).

Microparticles have been shown to support coagulation by both FVII/TF dependent and independent pathways. Using a thrombin generation assay to study the procoagulant potential of microparticles, Pereira and colleagues reported that platelet free plasma from patients with antiphospholipid syndrome had an increased endogenous thrombin potential compared to healthy controls. This effect was dependent on the presence of PMP and correlated with microparticle numbers (Pereira *et al*, 2006). Combes and colleagues found that TNF stimulation of cultured human umbilical vein endothelial cells (HUVEC) resulted in an increase in the

release of EMP expressing surface TF (Combes *et al*, 1999). The addition of increasing concentrations of these EMP to a coagulation assay shortened the plasma clotting time compared to EMP from unstimulated HUVEC. The effect was not seen in FVII deficient plasma, showing the procoagulant activity of the EMP to be FVII/TF dependent in this situation. In contrast, EMP induced *in vitro* by cisplatin treatment were found to be highly procoagulant and thrombin generation was not reduced by blocking antibodies to TF or FVII (Lechner *et al*, 2007). Similarly microparticles have been identified in healthy volunteers which supported low grade thrombin generation, independent of TF (Berckmans *et al*, 2001).

Microparticles can also contribute to the development of platelet and fibrin rich thrombus at sites of vascular injury, through the recruitment of cells and concentration of tissue factor (Figure 1c). In mouse models fluorescently labelled microparticles were observed to accumulate in areas of developing thrombus (Hrachovinova *et al*, 2003). Activated endothelium at sites of vascular injury and activated platelets within developing thrombus both express P-sel which may bind to monocyte microparticles expressing TF via the P-sel receptor PSGL-1. In mice lacking P-sel/PSGL-1 or in the presence of blocking antibodies, platelet thrombi with minimal tissue factor and fibrin are formed (Falati *et al*, 2003). Similarly PMP from activated platelets can mediate leukocyte-leukocyte interactions *in vitro* via binding of P-sel to PSGL (Forlow *et al*, 2000). Such interactions could lead to increased numbers of leucocytes and leucocyte microparticles (LMP) on surfaces expressing P-sel thus promoting the concentration of TF and localised thrombin generation within developing thrombus.

1.4.1.3 Microparticles – effects on endothelium

Endothelial dysfunction is a common feature of many vascular disorders including atherosclerosis, diabetes, antiphospholipid syndrome, thrombotic thrombocytopenic purpura and sickle cell disease, where it is likely to have an important pathogenic role. Elevated levels of microparticles have been reported in all of these disorders

(see later). Correlation between EMP levels and other serum markers of endothelial dysfunction including TM and endothelial adhesion molecules has also been reported (Koga *et al*, 2005;Nomura *et al*, 2005;Ogura *et al*, 2004). Soluble intercellular adhesion molecule (sICAM), which has been found to be related to endothelial dysfunction and coronary artery disease (CAD), was measured concurrently with EMP in patients with DM (Koga *et al*, 2005). Levels of both EMP and sICAM were elevated compared to non-diabetic controls and were greater in DM patients with CAD than those without CAD. EMP were also assessed in patients following stem cell transplantation and rose in parallel with levels of vascular cell adhesion molecule-1 (VCAM-1) and E-sel (Nomura *et al*, 2005).

Comparison of microparticle levels with *in vivo* measures of vascular dysfunction shows similar results. In patients with end-stage renal failure (ESRF) a strong correlation between EMP levels and reduced flow mediated brachial artery dilation and increased indices of arterial stiffening was observed (Amabile *et al*, 2005). *In vitro*, microparticles from patients with ESRF, but not healthy controls or microparticle supernatant, caused impaired endothelial dependent vasorelaxation in rat aorta and reduced nitric oxide release. These effects correlated strongly with EMP levels and could be induced by purified EMP alone. Circulating EMP may therefore contribute to the vascular changes seen in ESRF through inhibition of the endothelial nitric oxide pathway. Similarly, an inverse correlation was found between EMP numbers and endothelium dependent vasodilatation of the coronary arteries in diabetic patients undergoing angiography, where EMP numbers correlated with the presence of coronary artery lesions (Koga *et al*, 2005).

These observations suggest that EMP may not only provide an indication of the local or general state of the endothelium induced by activating or injurious external stimuli but may themselves induce changes in endothelial function.

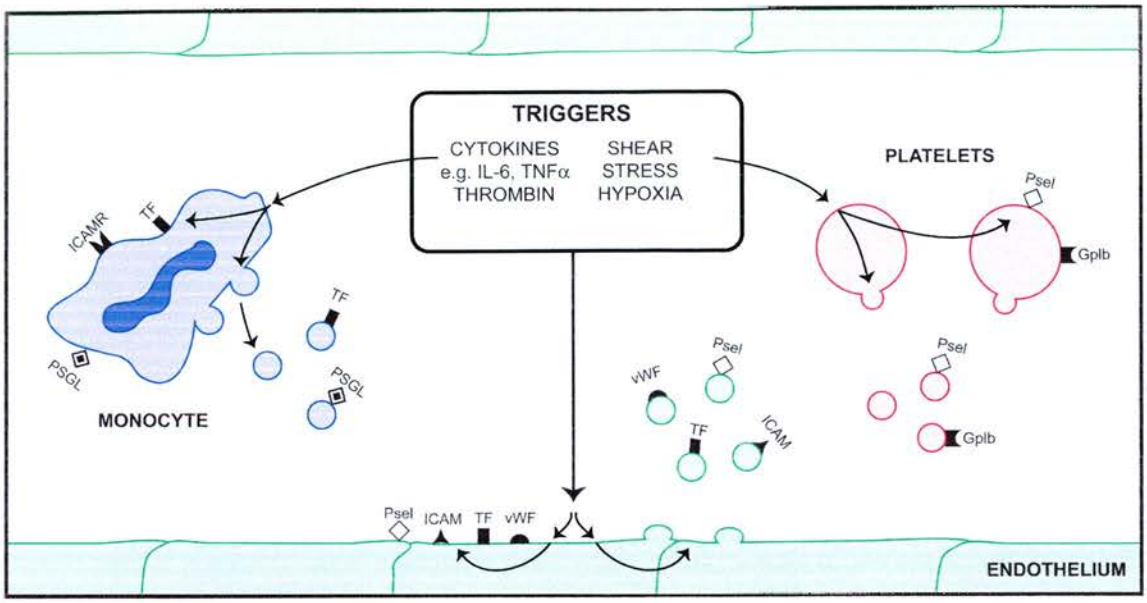


Figure 1a: Formation of microparticles.
GPIb – glycoprotein Ib, ICAM – intercellular adhesion molelule, ICAMR – ICAM receptor, Psel – P-selectin, PSGL – P-selectin glycoprotein ligand, TF – tissue factor, vWF – von Willebrand Factor.

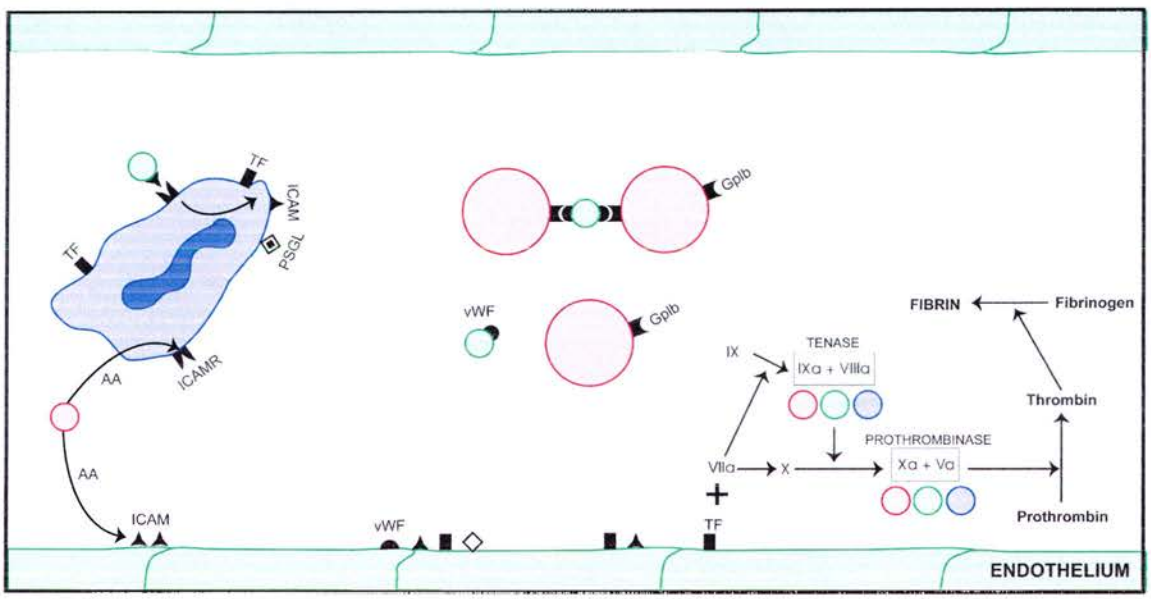


Figure 1b: Functions of microparticles in cellular interactions and coagulation.
AA – Arachidonic acid.

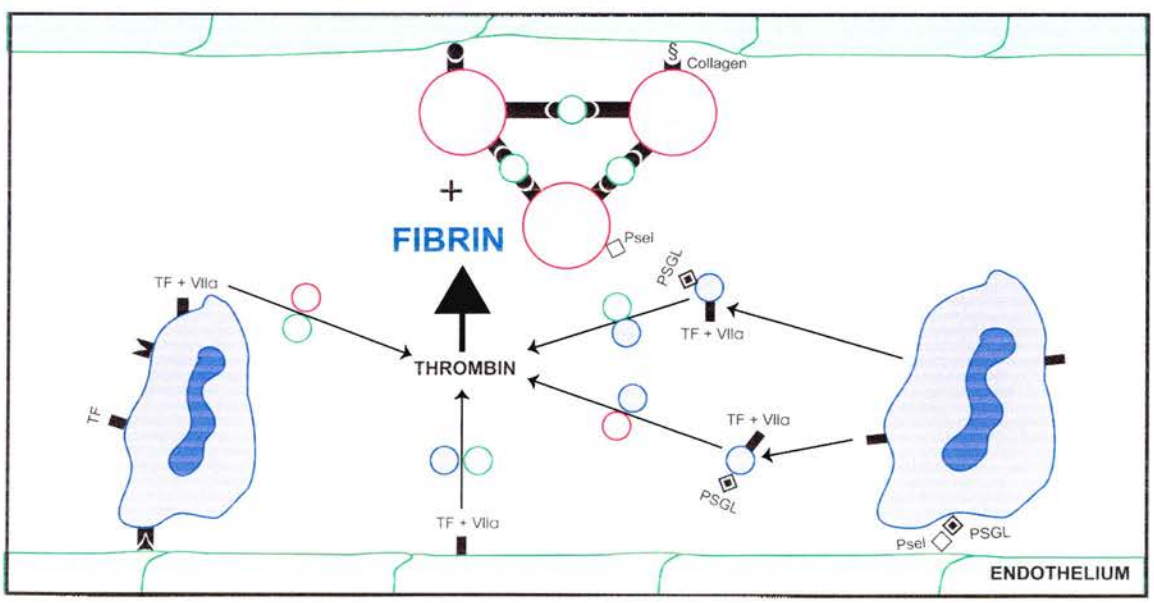


Figure 1c: Postulated role of microparticles in the development of thrombus.

1.4.1.4 Microparticles - cellular interactions

Microparticles bear antigens of their cell of origin and can transfer these surface molecules to other cell types. In doing so they may alter the biological activity of the recipient cells. Additionally, the binding of microparticle surface antigens to their specific counter-receptor may induce intracellular signalling pathways. Some of the known cellular interactions of microparticles are illustrated in Figure 1b.

EMP induced by *in vitro* stimulation of HUVEC with TNFα showed heterogeneous expression of a number of adhesive receptors including PECAM-1, ICAM-1, VCAM-1, E-sel and αvβ3 integrin. After incubation with these EMP, cultured monocytic cells also expressed these endothelial antigens at the cell surface (Sabatier *et al*, 2002b). The results suggested that this phenomenon was likely to involve receptor binding rather than membrane fusion. In addition, the co-incubated monocytes had increased levels of TF mRNA and increased TF dependent procoagulant activity. This effect was significantly inhibited by the addition of blocking antibodies to ICAM-1 and its counter-receptor suggesting it to be at least partly dependent on specific EMP and monocyte adhesion molecule interactions.

A key feature in atherosclerosis is monocyte adhesion to endothelial cells followed by subendothelial migration. Cytokines such as IL-1β and TNF-α can affect this process by inducing the synthesis or up-regulation of leucocyte-endothelial adhesion molecules. The *in vitro* stimulation of both monocytes and endothelial cells by high shear stress induced PMP, resulted in significantly increased production of IL-8, IL-1β and TNF-α and increased the expression of adhesion molecules on both cell types (Nomura *et al*, 2001;Barry *et al*, 1998). PMP have also been shown *in vitro*, to increase platelet aggregation and to induce endothelial cell expression of cyclo-oxygenase-2 and production of prostaglandin I_2 (Barry *et al*, 1997). These effects could be replicated by arachidonic acid isolated from the PMP lipids (Barry *et al*, 1998;Barry *et al*, 1997) which suggests that microparticles may also be able to modulate cell functions by the direct delivery of active substances.

Microparticles may also provide a novel mechanism of cross-talk between the cellular elements of the inflammatory and coagulation systems. Microparticles induced by *in vivo* stimulation of healthy volunteers with a chemotactic peptide were able to induce IL-6 release and TF expression by endothelial cells *in vitro*. This was associated with a TF dependent increase in the procoagulant activity of the endothelial cells, an effect which appeared to be mediated by LMP (Mesri & Altieri, 1999). Similarly, the addition of neutrophils, cell free supernatant or purified microparticles, but not microparticle free supernatant to cultured endothelial cells induced the release of IL-6 and IL-8 (Mesri & Altieri, 1998). Thus leucocytes activated by inflammatory cytokines may release microparticles which can promote procoagulant endothelial changes.

1.4.2 Definition and *in vivo* measurement of microparticles

The definition of microparticles and the methods used in their measurement vary between research groups and these factors are likely to have an important influence on the observed results. A forum addressing this methodological variation suggested that most laboratories defined microparticles by size (less than 1μm) and expression of cell lineage-specific antigens. Isolation and measurement techniques are more variable but most commonly microparticles are measured in platelet poor plasma or microparticle suspensions by either flow cytometry or activity assays (Biro *et al*, 2004;Dignat-George *et al*, 2004;Hugel *et al*, 2004;Jimenez *et al*, 2004;Jy *et al*, 2004;Nomura, 2004;Shet *et al*, 2004) .

Despite the variation in absolute numbers, studies which have assessed the relative proportions of microparticle subtypes in plasma show fairly consistent results. In general PMP are found to be the most abundant with LMP, EMP and erythrocyte (RMP) microparticles accounting for the remainder (Daniel *et al*, 2006;Berckmans *et al*, 2001) although this profile may be altered in some disease states.

1.4.3 Microparticles in vascular disorders

Microparticles have been studied in many conditions which share common pathophysiological features with evidence of platelet and coagulation activation and vascular dysfunction, including cardiovascular disease and DM, venous thrombosis, thrombotic microangiopathies, antiphospholipid syndrome. A limited amount of information is also available regarding the role of microparticles in primary haematological diseases complicated by thrombosis. A summary of the results of these studies is given in Tables 1.3 and 1.4.

1.4.3.1 Cardiovascular disease and venous thrombosis

Endothelial dysfunction, vascular inflammation and a prothrombotic state arise in patients with CAD, the vascular complications of diabetes, hypertension, cerebrovascular disease and venous thrombosis (VTE). As might be expected from the pathophysiologic processes involved, elevated microparticles have been reported in all of these diseases.

In 25 patients with deep vein thrombosis or pulmonary embolism, EMP levels were markedly elevated in patients with VTE compared to controls (Chirinos *et al*, 2005). PMP were not elevated despite higher levels of platelet expression of the activation marker P-sel. Increased leucocyte expression of the activation marker CD11b and EMP-monocyte conjugates in the VTE patients was also seen.

Overall microparticles are elevated in diabetic patients but studies have found differences in the microparticle profile in relation to disease type and the presence of complications. In type I DM the procoagulant potential of microparticles, as measured by a prothrombinase assay, was elevated and correlated with degree of glycaemic control (Sabatier *et al*, 2002a). In contrast, although total numbers of microparticles were elevated in type II DM there was no associated increase in their procoagulant potential. Levels of PMP and monocyte microparticles have been

shown to correlate with the extent of diabetic retinopathy which is associated with microvascular damage (Ogata *et al*, 2006).

In a prospective observational study of 217 diabetic patients referred for investigative angiography, elevated EMP levels were predictive for the presence of coronary artery lesions, OR 3.5 (1.8-6.9). Further, it was a more significant independent risk factor than length of diabetic disease, lipid levels or the presence of hypertension (Koga *et al*, 2005). Interestingly, elevated EMP levels were predictive in identifying a subpopulation of diabetic patients without typical anginal symptoms who had angiographic evidence of CAD.

Procoagulant microparticles and in particular EMP are elevated in patients with acute coronary syndromes compared to either patients with stable anginal symptoms or normal controls (Bernal-Mizrachi *et al*, 2003;Mallat *et al*, 2000). This likely reflects the degree of acute vascular injury and inflammation at the time of measurement. In addition LMP are abundant in atherosclerotic plaques and elevated circulating CD11a positive LMP were reported to be predictive for subclinical atherosclerotic lesions (Chironi *et al*, 2006).

Circulating EMP are also elevated in acute ischaemic stroke (CVA) (Simak *et al*, 2006). It was observed that while PPS positive EMP were elevated in all acute CVA compared to controls, endoglin positive EMP were elevated only in the moderate-severe group. Endoglin expression is associated with endothelial apoptosis and is upregulated on cultured endothelial cells under hypoxic conditions. These findings may therefore reflect more extensive endothelial injury in the more clinically severe group. Notably, the patient samples were collected at an average of 37 hours following hospital admission suggesting an ongoing procoagulant state.

DISEASE	STUDY	TMP	PMP	EMP	LMP	RMP
VTE	Chirinos *et al,* 2005		(ns↑)	↑		
Type I DM	Sabatier *et al,* 2002	↑	↑	↑		
Type II DM	Sabatier *et al,* 2002	↑	=	=		
DM retinopathy	Ogata *et al,* 2006		↑		↑	
DM + CAD	Bernal-Mizrachi *et al,* 2004			↑		
ACS	Bernal-Mizrachi *et al,* 2003		↑	↑		
	Mallat *et al,* 2000	↑	=	↑	=	
Acute CVA	Simak *et al,* 2006		(ns↑)	↑	=	=
CRF	Amabile *et al,* 2005	↑	↑	↑		↑
	Amabile *et al,* 2006		↑	↑	=	
Hypertension	Preston *et al,* 2003		↑	↑		

Table 1.3 Microparticles in cardiovascular disorders

Summary of results of published studies of plasma microparticle levels in patients with cardiovascular disorders compared to healthy controls.

DISEASE	STUDY	TMP	PMP	EMP	LMP	RMP
Acute TTP	Jimenez *et al,* 2003			↑		
SCD	Shet *et al,* 2003	↑	=	=	↑	↑
PNH	Hugel *et al,* 1999		↑			=
	Simak *et al,* 2004		↑/=	↑	=	=
MPD	Villmow *et al,* 2002		↑			

Table 1.4 Microparticles in prothrombotic haematological disorders

Summary of results of published studies of plasma microparticle levels in prothrombotic haematological disorders compared to healthy controls.

Abbreviations: total (TMP), platelet (PMP), endothelial (EMP), leucocyte (LMP) and erythrocyte (RMP) microparticles. Venous thromboembolism (VTE), diabetes mellitus (DM), coronary artery disease (CAD), acute coronary syndrome (ACS), cerebrovascular accident (CVA), chronic renal failure (CRF), thrombotic thrombocytopenic purpura (TTP), sickle cell disease (SCD), paroxysmal nocturnal haemoglobinuria (PNH), myeloproliferative disorders (MPD).↑- statistically significant increase; (ns↑) - non-significant increase; = - no difference; ↓ - statistically significant reduction.

Patients with chronic renal failure (CRF) are also at increased risk of accelerated cardiovascular disease and have evidence of endothelial dysfunction. In keeping with this, elevated levels of EMP have been found in CRF (Amabile *et al*, 2005;Faure *et al*, 2006), however no difference was identified in EMP levels between those with or without a history of vascular disease. Assessment of *in vivo* measures of endothelial dysfunction in ESRF shows a strong correlation with levels of EMP (Amabile *et al*, 2005). The study of microparticles may also provide insight into the causes of endothelial dysfunction; culture of HUVEC with uraemic toxins induced EMP formation supporting a direct effect of uraemia on endothelial function (Faure *et al*, 2006). Hypertension is another important factor in renal disease and the associated shear stress is trigger for microparticle formation. In severe hypertension, in the absence of CRF, both EMP and PMP are elevated and correlate with systolic pressure (Preston *et al*, 2003).

1.4.3.2 Thrombotic thrombocytopenic purpura

Microvascular endothelial injury, triggering the formation of platelet rich thrombi is thought to be of primary importance in the pathogenesis of thrombotic thrombocytopenic purpura (TTP) and related disorders. When plasma from patients with acute TTP was incubated with cultured brain and renal microvascular endothelial cell lines and a 5-6 fold increase in EMP generation compared to control plasma was observed (Jimenez *et al*, 2001). There was also a proportional increase in the procoagulant activity of the EMP as measured by the Russell Viper Venom Time. The phenotype of the EMP generated by TTP plasma was similar to that of EMP induced by culture with activating rather than apoptotic stimuli (Jimenez *et al*, 2003b), with an increased ratio of $CD62E^+$ to $CD31^+$ EMP. In addition, more than 60% of the $CD62E^+$ EMP co-expressed VWF (Jimenez *et al*, 2003a) in the form of ultra large VWF multimers. These EMP strongly induced stable platelet aggregation, in the presence of ristocetin, by a VWF-dependent mechanism.

In relation to the clinical findings, elevated numbers of EMP in the plasma of patients with acute TTP compared to normal controls or those in remission were observed. The EMP phenotype in the patients also reflected that found *in vitro*, with an increased ratio of $CD62E^+$ to $CD31^+$ EMP. The level of co-expression of VWF on the $CD62E^+$ EMP was five times that of the normal controls. These studies suggest a pathophysiological role for microvascular EMP in TTP through the expression of ultra large VWF and the induction and stabilisation of platelet aggregates.

1.4.3.3 Primary haematological diseases

The vaso-occlusive episodes of sickle cell disease were previously thought to be secondary to vessel occlusion by sickled erythrocytes. More recent evidence suggests that other factors are important, in particular, microvascular endothelial activation and endothelial-erythrocyte adhesion. In keeping with the prothrombotic clinical phenotype, *in vivo* markers of coagulation and fibrinolysis are elevated including prothrombin fragments (PF1+2), thrombin-antithrombin complexes (TAT) and D-dimer (Switzer *et al*, 2006).

Erythrocyte microparticle levels are significantly increased in sickle cell patients compared to controls and account for the majority of circulating microparticles. However, endothelial and monocyte microparticles are also increased compared to healthy controls both in the steady state and greater still in vaso-occlusive crises, supporting the theory of endothelial activation (Shet *et al*, 2003). A proportion of these endothelial and monocyte microparticles express TF and shorten the plasma clotting time, an effect partially inhibited by anti-TF antibodies. The total levels of microparticles and the TF positive subset also correlated with D-dimer, TAT and PT 1+2 measurements.

Elevated levels of microparticles have also been reported in paroxysmal nocturnal haemoglobinuria (PNH), another prothrombotic disorder (Simak *et al*, 2004;Hugel *et*

al, 1999). In one study PMP appeared to account for the majority and RMP were infrequent but they did not measure LMP or EMP. The procoagulant potential of the microparticles was confirmed using a prothrombinase assay (Hugel *et al*, 1999). In contrast, Simak found that EMP were elevated in the PNH patients as a whole, but that individual patients had increased numbers of PMP (Simak *et al*, 2004). However they also observed that the majority of microparticles in both patients and normal controls were RMP in contrast to most other studies where PMP predominate, suggesting that methodological differences may have influenced the results.

1.5 Hypothesis, aims and objectives

With current treatment, the life expectancy for patients with PV and PT is significantly improved from that of the natural history of the disease. However, thrombotic and fibrotic complications continue to contribute significant morbidity to patients with these conditions and may reduce survival, in particular for younger patients. Our understanding of the pathophysiology of these complications is incomplete but in both there is evidence for platelet abnormalities and, in thrombosis, other vascular disturbances. Phenotypic differences dependent on the presence of the recently recognised JAK2 V617F tyrosine kinase mutation in a proportion of patients have been described but whether this mutation has any direct role in the complications of these disorders is unclear.

The pathogenesis of thrombosis in these disorders is likely to be multifactorial and in addition to platelet activation, there is evidence for leucocyte activation, endothelial disturbance and a procoagulant state. Similar abnormalities are associated with elevated microparticles from platelets, leucocytes, erythrocytes and endothelial cells in other prothrombotic disorders. To date, only one published study has measured PMP in MPD and they reported an increased proportion of PMP which correlated with markers of platelet activation. In conjunction with the recognised procoagulant properties of microparticles, these observations suggest that investigation of microparticles in myeloproliferative disorders may extend our understanding of the pathogenesis of thrombosis in these diseases.

Platelets and megakaryocytes have been shown to have a central role in bone marrow fibrosis through the effects of pro-fibrotic cytokines in particular TGFβ and PDGF. However the mechanisms by which elevated levels of these cytokines are present remain elusive. Possible mechanisms include specific release possibly as a result of aberrant intracellular signalling or non-specific "leak" consequent on defects in the packaging or storage of the cytokines in intracellular α-granules. Measurement of circulating levels of these profibrotic cytokines along with other factors contained within the platelet α-granules, may provide additional evidence for the latter theory.

Aims and Objectives

- To identify and describe the local MPD population
    - Description of the clinical and laboratory features of the local MPD population and relation to findings from larger studies
    - Description of the management of the local MPD population in comparison to existing guidelines
    - Assessment of clinical and laboratory parameters in relation to the JAK2 V617F mutation
    - Assessment of the effect of the JAK2 V617F mutation on patient diagnosis

- To set up a flow cytometry assay for the detection of plasma microparticles
    - Review of currently published methods of microparticle isolation
    - Optimisation and standardisation of preanalytical variables in sample collection and processing
    - To identify platelet, endothelial, leucocyte and red cell microparticles by size and surface antigen expression
    - To devise a suitable method of microparticle quantitation

- To investigate microparticles and established markers of vascular function in patients with myeloproliferative disorders

    - Measurement of platelet, endothelial, leucocyte and red cell microparticles in patients with MPD compared to controls

    - Relation of microparticle levels to clinical features, haematological and molecular parameters

    - Measurement of established markers of platelet and endothelial function and coagulation activation in patients with MPD compared to controls

    - Relation of markers to clinical features, haematological and molecular parameters and comparison with findings from previous studies

    - Assessment of the correlation of microparticle numbers with established vascular markers in MPD patients and controls

- To investigate circulating levels of profibrotic cytokines in comparison to other platelet α-granules proteins in patients with myeloproliferative disorders

    - Measurement of TGFβ and PDGF in MPD patients compared to controls

    - Correlation of TGFβ and PDGF levels with the platelet α-granule proteins BTG and PF4

    - Correlation of levels of α-granule proteins with clinical, haematological and molecular parameters

    - Correlation of levels of α-granule proteins with the histological degree of fibrosis

CHAPTER 2

CLINICAL AND LABORATORY CHARACTERISTICS

2.1 Introduction

PV and PT are relatively rare diseases and until recently our insight into their pathogenesis has been limited and studies of the incidence of complications and the effect of therapeutic interventions have been small and retrospective. However in the last 5 years, the publication of a number of large clinical trials and observational studies and the identification of a JAK2 tyrosine kinase mutation in a proportion of these patients, have significantly advanced our understanding in this field. These studies and the clinical and laboratory features of PV and PT are discussed fully in Chapter 1 but are summarised here for reference to our own observations in the current study.

Around 30-50% of patients with PV or PT present with a thrombotic, or less commonly, a haemorrhagic episode or experience these as a complication during follow-up. In PT patients, arterial microvascular symptoms such as CVA and erythromelalgia predominate whereas in PV, venous thrombosis and large vessel arterial disease are commoner. The factors most strongly and consistently associated with thrombotic risk are older age (>60 years), prior history of thrombosis and more recently, elevated white cell count at diagnosis (Carobbio *et al*, 2007;Gruppo Italiano, 1995;Landolfi *et al*, 2007;Marchioli *et al*, 2005;Passamonti *et al*, 2004;Wolanskyj *et al*, 2006). The older age of most PV and PT patients means that traditional cardiovascular risk factors are commonly present but their role in thrombotic risk is less clear. In a smaller proportion of patients, haematological progression to acute leukaemia or myelofibrosis occurs with increasing time from diagnosis.

The diagnosis of these diseases has evolved as their pathogenesis has become better understood. In particular the identification of the JAK2 V617F tyrosine kinase mutation has significantly simplified the diagnosis of PV in the majority of cases and has prompted review of the diagnosis in "PV" patients negative for the mutation. In PT patients with the mutation, a phenotype with more similarities to PV is described, with higher haematocrit and leucocyte counts, signs of iron deficiency and lower

platelet counts. Some studies have suggested that it may also be associated with an increased risk of venous thrombosis.

The aim of therapy in PV and PT is to reduce the risk of vascular complications without increasing the risk of leukaemic transformation. Risk stratification is therefore used to help guide management decisions (Table 1.2). Concerns about the potential leukaemogenic risk of the standard cytoreductive hydroxycarbamide mean that observation or non-cytotoxic alternatives may be preferred in those at low thrombotic risk or younger patients. A recent study of aspirin in PV patients has confirmed its predicted benefits in reducing thrombotic risk and this benefit has been extrapolated to PT patients.

We have identified the PV and PT population in Edinburgh and in this chapter we describe the demographics, clinical and laboratory features at diagnosis and the complications experienced by those who participated in the main study. The frequency of the JAK2 V617F mutation in this population has been established and compared to previously published results and we have examined its relation to clinical and laboratory parameters. We have also reviewed the diagnosis of PV patients negative for the JAK2 V617F mutation.

2.2 Materials and Methods

2.2.1 Patient identification and recruitment

Patients with a working diagnosis of PT or PV, based on either the PVSG or BCSH criteria (Appendix 1), were identified from the haematology out-patient services at the Royal Infirmary of Edinburgh (RIE) and the Western General Hospital (WGH) Edinburgh. Letters of invitation, patient information sheets and consent forms were sent to patients one week prior to their clinic appointments (Appendix 2). Patients who wished to participate returned their completed consent form by post or at their clinic visit and thereafter a study visit was arranged to coincide with their next clinical appointment.

2.2.2 Data collection

Clinical history and haematological parameters at diagnosis, molecular data and follow-up information was obtained by review of the patient case records. Cardiovascular disease (CVD) was defined as a history of ischaemic heart disease (IHD) (including angina, acute coronary syndrome or myocardial infarction), cerebrovascular disease (transient ischaemic attack (TIA) or stroke) or peripheral vascular disease (PVD). Cardiovascular risk factors (CVRF) included diabetes mellitus, hypertension, hypercholesterolemia, smoking and cardiac failure. Vascular events included cardiovascular thrombosis, TIA or stroke, deep vein thrombosis (DVT) or pulmonary embolism (PE) or microvascular symptoms including vascular headache and erythromelalgia. Vascular events at presentation included those which occurred up to one year prior to the diagnosis of MPD.

2.2.3 Ethics

Ethical approval for the study was obtained from the Lothian Local Research Ethics Committee.

2.2.4 Statistical analysis

For normally distributed data the mean and standard error of the mean (SEM) are presented and comparisons between groups were made using the student's t-test. For non-normally distributed data, the median and interquartile range (IQR) is presented and the non-parametric Mann-Whitney *U* test was used for between group comparisons. Associations between qualitative variables were investigated with the Fisher's exact test for 2 x 2 tables. A value of $P<0.05$ (2-tailed) was considered statistically significant. Statistical analysis was performed using Minitab 15 software (Minitab Inc, US).

2.3 Results

2.3.1 Edinburgh MPD patient population and study cohort

At the start of the study period 185 patients with a diagnosis of PV (50) or PT (135) documented in their case records were identified over two hospital sites. Just under half of these (42%) participated in the study. Table 2.1 shows the breakdown of the numbers invited to take part, those who consented and those who finally participated in the study.

We compared the basic demographics of the participating (study cohort) and non-participating patients. Of all the patients identified, 72.9% had PT compared to 76% of those who participated in the study. The study cohort were younger than those who did not participate with a mean age (standard error (SEM)) of 64.7 (1.7) v 71.5 (1.5) years ($p=0.003$). This was mainly due to differences in the PT group, 64.3 (2.0) v 73.7 (1.7) years ($p<0.001$), whereas the mean age of the PV patients in the study cohort was similar to those not participating, 66.0 (3.3) v 66.3 (2.8) years ($p=0.945$). In the overall local MPD population there was no significant difference in age between the PT and PV patients, 69.6 (1.3) v 66.2 (2.1) years respectively ($p=0.172$).

2.3.2 Study cohort – Clinical characteristics

Age and time from diagnosis

The study cohort consisted of 78 patients, 59 PT and 19 PV. The age, sex and follow-up period for the patients is detailed in Table 2.2. There was no significant difference in these parameters between the PV and PT patients. In the patients recruited from the RIE the median time from diagnosis was 71.3 (IQR 34.8-129.6) months compared to 30.8 (20.7-43.8) months for the WGH patients ($p<0.001$).

	All (n)	PT(n)	PV (n)
RIE			
Total	**101**	**74**	**27**
Invited to participate	73	51	22
Consented	48	37	11
Withdrew/DNA	5	1	4
In study	**43**	**36**	**7**
WGH			
Total	**84**	**61**	**23**
Invited to participate	58	42	16
Consented	37	24	13
Withdrew/DNA	2	1	1
In study	**35**	**23**	**12**

Table 2.1 Local MPD patient population and study cohort

Breakdown of the numbers of PV and PT patients identified at the Royal Infirmary of Edinburgh (RIE) and the Western General Hospital Edinburgh (WGH) and those participating in the current study. Around two-thirds of those invited to participate gave their consent however some withdrew thereafter or did not attend (DNA).

Vascular co-morbidities and cardiovascular risk factors

Data on the presence of pre-existing CVD (diagnosed prior to the diagnosis of PT or PV) and CVRF (identified at any time before or after the diagnosis of PT or PV) was available from the case records for 76 patients (Table 2.2). There was no documented history of CVD or CVRF in 40 patients (51.9%). In 21 patients there was a documented history of CVRF but no CVD and five of these patients had more than one risk factor. The commonest CVRF in this group was hypertension in 18 patients. In total, three patients had DM and one patient had cardiac failure. Smoking at the time of study entry was reported by only five patients. A documented history of CVD prior to the diagnosis of MPD was present in 15 patients (19%), five of whom had multiple vascular co-morbidities. A similar proportion of PV and PT patients had CVRF but more PT patients had had cardiovascular or venous events prior to diagnosis, although this was not statistically significant.

To establish the prevalence of undiagnosed hypertension we performed a small audit of consecutive patients at the MPD clinic. A single blood pressure (BP) reading was taken in 32 patients; 20 PT, 10 PV and 2 PMF. The mean age of these patients was similar to the study cohort at 65.5 and 64.7 years respectively ($p=0.76$). A documented history of hypertension was present in 34.4% of the audit group compared to 36.8% of the study cohort. Twenty-one of the 32 audit patients had no history of hypertension; 13 had a normal BP (below 140/90), six had borderline systolic hypertension (systolic BP 140-150) and only two had a BP of over 150/90. In the 11 patients with a documented history of hypertension, four had a normal BP, three had borderline readings and four had a BP over 150/90.

	Entire cohort n = 78	PT n = 59	PV n = 19	PT v PV p value
Age at study entry (mean, SEM)	64.7 (1.7)	64.3 (2.0)	66.0 (3.3)	0.663
Age at diagnosis (mean, SEM)	59.5 (1.7)	58.5 (2.1)	62.4 (3.2)	0.319
Male sex, n (%)	32 (41.0)	25 (42.3)	7 (36.8)	1.000
Presentation data	**n = 76**	**n = 57**	**n = 19**	
Cardiovascular risk factors, n (%)				
Any	33 (43)	25 (43.8)	8 (42.1)	1.000
Diabetes mellitus	3 (3.9)	3 (5.3)	0	0.569
Hypertension	27 (35.1)	21 (36.8)	7 (36.8)	1.000
Hyperlipidaemia	5 (6.6)	7 (12.3)	0	0.182
Smoking	5 (6.6)	5 (8.8)	1 (5.3)	1.000
Congestive cardiac failure	1 (1.3)	1 (1.8)	0	1.000
Pre-existing cardiovascular or venous disease, n (%)				
Any	17 (22.4)	16 (28.1)	3 (15.8)	0.372
Ischaemic heart disease	9 (11.8)	8 (14.0)	2 (10.5)	1.000
Cerebrovascular disease	8 (10.5)	7 (12.3)	1 (5.3)	0.671
Peripheral vascular disease	4 (5.3)	5 (8.8)	0	0.320
Venous thrombosis	4 (5.3)	3 (5.3)	1 (5.3)	1.000
Thrombosis at presentation, n (%)				
Any	22 (28.9)	16 (28.1)	6 (31.6)	0.777
Arterial	15 (19.7)	12 (21.0)	3 (15.8)	1.000
Venous	7 (9.2)	4 (7.0)	3 (15.8)	0.351
Haemorrhage at presentation, n (%)	6 (7.9)	3 (5.3)	3 (15.8)	0.151

	Entire cohort n = 78	PT n = 59	PV n = 19	PT v PV p value
Complicating events	**n = 77**	**n = 58**	**n = 19**	
Follow-up from diagnosis (months) (median, IQR)	43.2 (24.9, 97.4)	43.8 (29.5, 103.6)	43.2 (23.3, 51.8)	0.191
Thrombosis, n (%)				
Any	7 (9.1)	6 (10.3)	1 (5.3)	0.674
Arterial	6 (7.8)	6 (10.3)	0	0.327
Venous	2 (2.6)	1 (1.7)	1 (5.3)	0.435
Haemorrhage, n (%)	0	-	-	
Transformation, n (%)				
acute leukaemia	0	-	-	
myelofibrosis	4 (5.2)	4 (6.9)	0	0.567

Table 2.2 Clinical characteristics of patient cohort

Clinical characteristics of PV and PT patients at the time of diagnosis and at study entry. P values for comparison of PV and PT groups are shown.

Clinical presentation

Data on clinical presentation was available for 76 patients. An incidental finding of raised haematocrit or thrombocytosis was the commonest presentation occurring in 61.5% of patients. The remainder of patients had a history of haemorrhagic or thrombotic events at or within the 12 months prior to presentation (Table 2.2).

A history of arterial events was the commonest symptomatic presentation occurring in 14 patients (19.7%). These included four TIA, two CVA, four peripheral arterial thrombosis and four patients with microvascular symptoms (erythromelalgia or headache). There was no prior history of CVD or CVRF in seven of these patients, five had CVRF only and two patients had a history of PVD or IHD. Haemorrhagic symptoms were the least common presentation and included two epistaxis, two minor gastrointestinal bleeding episodes, one intra-articular knee bleed and one rectus sheath haematoma.

Complications arising after the diagnosis of PT or PV

Thrombotic complications were recorded in seven patients (9%) following diagnosis; six arterial (four TIAs and two peripheral thromboses) and two VTE. No haemorrhagic complications were observed (Table 2.2).

In four patients originally diagnosed with PT, progression to clinical MF was recorded after 12, 27, 54 and 84 months follow-up. Leucoerythroblastic film features were present in all four patients and two had documented splenomegaly at the time of diagnosis of post-PT MF. All had bone marrow trephines confirming Grade 3-4 fibrosis. The diagnostic bone marrow trephine reports were available for three of the patients; all reported an increase in reticulin and two reported megakaryocyte atypia at initial presentation. One patient died within 6 months of the diagnosis of post-PT MF; the others survive with subsequent follow-up of 10, 83 and 120 months. No leukaemic transformations were observed.

Risk stratification and management

None of the patients in the study cohort were assessed to be low risk for thrombosis (for risk stratification see Table 1.2). Seven patients (two PV and five PT) were considered at intermediate risk and the remaining 71 patients were in the high risk category. In the PV group 18/19 were undergoing or had previously had venesection and 15 patients were receiving hydroxycarbamide. In the PT group 53/59 were currently treated with hydroxycarbamide and of these, three were receiving combined therapy with anagrelide. Two patients were receiving single therapy with anagrelide or interferon respectively. No cytoreductive therapy was given in three PT patients aged between 40 and 59 years with no history of or risk factors for thrombosis (intermediate risk). One further patient under 40 years, who had presented with a retinal vein thrombosis in association with thrombocytosis, and who was considering pregnancy, was being observed while hydroxycarbamide was withheld. Other treatment given prior to study entry included anagrelide in one patient, interferon in two patients, radiophosphorus in four patients and busulphan in one patient.

15 PV and 48 PT patients (78.9% and 81.4% respectively) were on aspirin at the time of study entry and three PT patients were on an alternative antiplatelet drug. Warfarin was used in three PV patients for VTE (in two cases recurrent) and in five PT patients, two with recurrent VTE, one with a previous arterial thrombosis, one with atrial fibrillation and one with a mechanical heart valve. Combination therapy with warfarin and aspirin was used in only two of these patients. 15 patients were prescribed statin therapy.

2.3.3 Haematological and molecular parameters

The values for the haematological parameters in the PT and PV patients at diagnosis and at study entry are shown in Table 2.3. 17 patients with PT (29.3%) had a platelet count greater than 1000 x 10^9/L at diagnosis.

JAK2 V617F

The JAK2 V617F mutation was present in 17/19 PV patients (89.7%) and 24/54 PT patients (44.4%). A comparison of the haematological parameters at diagnosis in the PT patients according to JAK2 mutation status is shown in Table 2.4. Haemoglobin and haematocrit values were significantly higher at diagnosis in patients with the JAK2 V617F mutation compared to wild type. The mean platelet count was higher in patients with wild type JAK2 but this was not statistically significant.

Haematological parameters at diagnosis			
	PT (n = 59)	PV (n = 19)	PT v PV p value
Haemoglobin (g/l) Mean (SEM)	139.9 (2.1)	185.8 (2.9)	**<0.001**
Haematocrit Mean (SEM)	0.41 (0.01)	0.56 (0.01)	**<0.001**
Leucocyte count ($x10^9$/L) Median (IQR)	9.1 (8.0, 10.5)	10.0 (8.6, 13.0)	0.118
Platelets ($x10^9$/L) Median (IQR)	788 (620, 1102)	447 (319, 660)	**<0.001**
Haematological parameters at study entry			
	PT (n=59)	PV (n=19)	PT v PV p value
Haemoglobin (g/l) Mean (SEM)	133.5 (3.7)	139.6 (2.1)	0.167
Haematocrit Mean (SEM)	0.38 (0.009)	0.42 (0.009)	**0.002**
Leucocyte count x 10^9/L Median (IQR)	5.6 (3.7-11.5)	8.4 (3.4-18.1)	**0.018**
Platelets x 10^9/L Median (IQR)	405 (350,486)	351 (285,456)	0.078

Table 2.3 Haematological parameters in patient cohort

Haematological parameters at the time of diagnosis and study entry in PT and PV patients. Values for the mean and SEM or the median and IQR are shown. P values for statistically significant differences are shown in bold.

	JAK2 wild type (n=29)	JAK2 V617F (n=23)	p value
Haemoglobin (g/L) Mean (SEM)	132.9 (2.7)	149.7 (2.5)	**<0.001**
Haematocrit Mean (SEM)	0.38 (0.02)	0.45 (0.01)	**<0.001**
Mean cell volume (fl) Mean (SEM)	87.9 (1.0)	88.0 (1.7)	0.974
Leucocyte count (x 10^9/L) Median (IQR)	9.3 (8.5, 10.6)	8.5 (7.4, 10.2)	0.213
Platelets (x 10^9/L) Median (IQR)	792 (619, 1222)	769 (618, 946)	0.357

Table 2.4 Haematological parameters in PT patients according to JAK2 mutation

Haematological parameters at the time of diagnosis in PT patients according to the presence of the JAK2 V617F mutation. Values for the mean and SEM and the median and IQR are shown. P values for statistically significant differences are shown in bold.

2.3.4 Association of clinical and laboratory variables with thrombosis

There was no difference in the overall incidence of thrombosis between the PT and PV groups (p=1.0, Fisher's exact test). Arterial thrombosis (at or after presentation) was commoner in PT patients and venous thrombosis was commoner in PV patients but these differences were not statistically significant (Table 2.1).

There was no significant difference in age at study entry between patients who had or had not experienced a thrombosis (mean age (SEM) 62.5 (2.9) v 66.4 (2.0) years respectively, p=0.275) and age over 60 years was not associated with a history of thrombosis (p=0.312). However patients who had presented with a thrombotic event were younger at diagnosis (53.8 (3.1) v 62.4 (2.0) years (p=0.026). Patients who had experienced a complicating thrombosis had a median follow-up of 193.5 months compared to 37.8 months for those without (p<0.001).

A history of CVD was recorded in 16/77 patients at the time of diagnosis of their MPD. There was no association of a history of CVD with arterial, venous or any thrombosis at presentation; a thrombotic event at presentation was recorded in 3/16 patients with a history of CVD v 18/61 without (p=0.534). CVRF were recorded in an additional 21 patients and a history of either CVD or CVRF combined was not associated with thrombosis at presentation, with events in 9/37 patients with CVD or CVRF v 12/40 patients without (p=0.617).

Thrombosis arising after the diagnosis of PV or PT (complicating thrombosis) occurred in seven out of 34 patients with a previous history of either thrombosis or CVD (at or prior to MPD diagnosis) but none of 43 patients with no such history (p=0.002). Five out of the six patients with a complicating arterial thrombosis had a previous history of CVD (p<0.001). The other patient had hypertension and hypercholesterolemia and had originally presented with symptoms of erythromelalgia.

Six patients presented with a VTE, one of whom had a past history of VTE and had a subsequent VTE. One further patient had a VTE following diagnosis and had a

history of previous arterial thrombosis and erythromelalgia. No association between VTE and a previous history of such was detected (p=0.365).

There was no difference in the haematocrit or platelet count at diagnosis between patients with or without thrombosis at presentation (haematocrit, mean (SEM) 0.45 (0.02) v 0.45 (0.01), p=0.94; platelets, median (IQR) 662 (510, 1052) v 681 (574, 987) x 10^9/L, p=0.579) but the leucocyte count was higher in those with thrombosis (median (IQR) 10.4 (8.4, 13.9) v 8.9 (8.1, 10.3) x 10^9/L, p=0.023). Patients with a leucocyte count above the median value of 9.1 x 10^9 /L were more likely to have a recorded thrombotic event at or following diagnosis, 16/36 v 7/37 (p=0.024). This appeared to be mainly due to thrombosis at presentation, 14/36 v 6/37 (p=0.038) rather than as a complicating event (3/36 v 2/37). In the PT patients the presence of the JAK2 mutation was not associated with any thrombosis, with events in 6/23 JAK2 V617F positive patients v 12/30 wild type patients (p=0.384) or with venous thrombosis alone (p=0.620).

2.3.5 Diagnosis of PV and the use of JAK2 V617F mutation status

Molecular status, with regard to JAK2, was known for all 19 PV patients in the study cohort and was negative in two of these. A further five patients outwith the study who had a working diagnosis of PV were shown to be negative for the JAK2 V617F mutation. We therefore reassessed the original diagnoses in those patients negative for the mutation using the PVSG criteria, BCSH guidelines (2005) and the WHO criteria (2001 and 2008) (Appendix 1). Diagnostic data for each of the patients is shown in Table 2.6.

Patient Age (years) and gender	A 73 F	B 78 F	C 62 F	D 57 M	E 56 F	F 65 M	G 79 M
Absolute erythrocytosis							
Haemoglobin(g/L)	18.0	17.1	18.8	18.4	17.2	19.2	19.0
Haematocrit	0.54	0.53	0.56	0.52	0.50	0.55	0.58
Red Cell Mass	124%	0.03l/kg	155%	131%	145%	159%	213%
Plasma Volume	*	Reduced	Normal	Normal	*	*	*
Secondary causes							
Smoker	No	Yes	Ex	Yes	Yes	No	No
Arterial oxygen	98%	11.7kPa	14.5kPa	10.6kPa	98%	94%	98%
Other	*	*	*	*	*	*	*
Myeloid features							
Splenomegaly	No	*	No	No	12cm	13.5cm	No
Cytogenetics	*	*	Normal	*	Normal	Normal	Normal
Bone Marrow	50% cellularity	*	Panmyelosis	Erythroid hyperplasia	No	Erythroid hyperplasia	80% cellularity
Leucocytes(x 10^9/l)	<12	6.4	5.1	12.2	11.8	8.1	7.4
Platelets (x10^9/l)	250	343	278	294	270	182	516
Erythroid independence							
▪ Epo	*	*	*	*	*	Low	Normal
▪ EEC	*	*	*	*	*	*	*
Presentation	Asymptomatic	Asymptomatic	Asymptomatic	DVT	Asymptomatic	*	Dizziness
Management	Aspirin Venesection	Venesection	Clopidogrel Venesection	Warfarin Venesection HC- stopped	Aspirin Venesection	*	Aspirin Venesection HC

Table 2.6 Diagnostic data for JAK2 V617F negative polycythaemia patients

Diagnostic data for patients diagnosed with PV but negative for the JAK2 V617F mutation. * indicates missing data. Cut-off values for diagnosis of an absolute erythrocytosis vary according to the criteria used and are detailed in the text. The upper limit of normal for leucocyte count is 11.0 x10^9/l and for platelets is 450 x 10^9/l. Abbreviations: kilopascals (kPa), serum erythropoietin level (Epo), endogenous erythroid colonies (EEC), deep vein thrombosis (DVT), hydroxycarbamide (HC).

In only one patient (G) by one set of criteria (WHO 2001) was a positive diagnosis of PV reached. A summary of the diagnostic outcome of the use of each of the criteria is shown in Table 2.7. All seven patients met the definition of an absolute erythrocytosis based on the haemoglobin values used in the WHO criteria, however patients A and B did not meet this criteria based on either haematocrit or measured RCM. No patient had complete investigations for alternative causes of erythrocytosis although arterial oxygen measurements and abdominal ultrasound scans excluded hypoxic lung disease or renal disease. Only two patients were investigated for evidence of erythroid independence.

Patient C had typical bone marrow features to support a diagnosis of PV. Patient D had a leucocyte count of 12.2 x 10^9/L supporting a diagnosis of PV by PVSG or WHO criteria, but as a smoker did not meet the higher cut-off in the BCSH criteria. Patient E had borderline splenomegaly on ultrasound scan, in support of a diagnosis of PV. However in these three patients there was insufficient data to confirm or exclude a diagnosis of PV at least by the WHO criteria and in most cases by the PVSG or BCSH criteria

Patient F had a reduced serum Epo and borderline splenomegaly. Investigations for alternative mutations affecting JAK2 exon 12 and for the von Hippel Lindau and erythropoietin receptor gene mutations that cause congenital erythrocytosis, were negative. The diagnosis therefore lies between PV and idiopathic erythrocytosis. Patient G had bone marrow hypercellularity with increased erythroid and megakaryocyte activity and a peripheral thrombocytosis but a normal Epo level. He did not meet the BCSH criteria for PV but did achieve this diagnosis by the WHO 2001 criteria.

Patient	PVSG 1967	BCSH 2005	WHO 2001	WHO 2008
A	Not true erythrocytosis	Not true erythrocytosis	Insufficient	Insufficient
B	Not true erythrocytosis	Not true erythrocytosis	Insufficient	Insufficient
C	Not PV	Not PV	Insufficient	Insufficient
D	Insufficient	Not PV	Insufficient	Insufficient
E	Not PV	Insufficient	Borderline	Insufficient
F	Not PV	Borderline	Borderline	Insufficient
G	Insufficient	Not PV	PV	Insufficient

Table 2.7 Differential diagnostic outcomes for JAK2 V617F negative polycythaemia

The diagnosis of patients labelled as PV but negative for the JAK2 V617F mutation was reviewed using the criteria of the PVSG, BCSH, WHO. This table details the outcome for each patient according to each set of criteria. In many cases there was insufficient data to confirm or refute a diagnosis of PV.

2.4 Discussion

Clinical and laboratory features

Almost half of the PV and PT patients that we identified participated in the study. Their make-up reflected the characteristics of the local patient population with more PT than PV patients. This is in contrast to the findings of the Leukaemia Research Fund UK registry (McNally *et al*, 1997) which recorded a higher incidence of PV. As our data indicate prevalence they may be affected by different life expectancies in the groups. The patients in the study cohort were younger than the overall local PV and PT population largely due to the PT study patients being younger than their counterparts who did not participate. More of these older patients may have been considered unable to give informed consent due to dementing co-morbidities or may have declined to participate due to perceived difficulties in attending the clinic appointments. The mean age of the total patient group (68.7 years) and the study cohort (64.7 years) was similar to that reported in the literature (Gangat *et al*, 2006;Gangat *et al*, 2007;Harrison *et al*, 2005;Marchioli *et al*, 2005). There were more women than men in the study group (3:2), with similar ratios in the PV and PT subgroups. This sex distribution contrasts with the equal ratios or predominance of men reported in PV (Gangat *et al*, 2007;Marchioli *et al*, 2005;McMullin *et al*, 2005). At entry to the study, patients had been followed-up from diagnosis for a median of almost 4 years and a maximum of over 26 years. Follow-up was shorter for patients from the WGH site because management of this patient group had commenced there only three years previously.

An asymptomatic presentation was recorded in over 60% of the patients, in keeping with the trend towards increasing numbers of patients diagnosed following the incidental finding of haematological abnormalities on blood counts performed for unrelated reasons (Jensen *et al*, 2000b). Thrombo-haemorrhagic symptoms at presentation were recorded in just over a third of patients, at the lower end of the range reported in the literature (Elliott & Tefferi, 2005;Harrison *et al*, 2005;Jensen *et al*, 2000b;Marchioli *et al*, 2005;Passamonti *et al*, 2004;Wolanskyj *et al*, 2006).

Consistent with the findings in these other retrospective studies, these episodes were mainly thrombotic with twice as many arterial thromboses (including microvascular symptoms) compared to venous (18.7 v 9.3%) and the commonest symptoms were cerebrovascular in origin.

Around half of the patients had no documented history of CVD or CVRF prior to diagnosis with PV or PT. CVD was recorded in 20% of patients and in a quarter of these there was more than one diagnosis. IHD and cerebrovascular disease were the commonest diagnoses, each in 10% of patients, similar to the 16% prevalence of CAD in a casenote review of 180 of PV and PT patients (Ganti *et al*, 2003). Only 4/78 patients had a diagnosis of PVD, similar to the ECLAP patients (Marchioli *et al*, 2005) but much lower than the 35% recorded in the high risk arm of the PT-1 trial (Harrison *et al*, 2005).

In a further 25% of the patients, the presence of one or more CVRF was recorded. This was most commonly hypertension, present in 35% of the study cohort, similar to the findings in the high risk arm of the PT-1 trial (23%) and the ECLAP study (39%) of PV patients (Harrison *et al*, 2005;Marchioli *et al*, 2005). We attempted evaluate whether undiagnosed hypertension might be a significant issue. In 21 patients, age-matched to our study cohort, who were attending the MPD clinic and who did not have a diagnosis of hypertension, the majority (62%) were normotensive. A further 29% had borderline systolic hypertension and only 2/21 were clearly hypertensive. Although all would require further assessment with repeat BP measurement, this suggests that undiagnosed hypertension is not widespread but may be significant for individual patients. Less than 10% of the patients reported smoking at the time of diagnosis. This seems low compared to the 25% rate reported in the general Scottish population (Corbett J *et al*, 2007) and other study populations (Gangat *et al*, 2007;Harrison *et al*, 2005;Marchioli *et al*, 2005) which may suggest either under reporting or under recording.

Analysis for the JAK2 V617F mutation was performed in 73 patients in the study cohort. Of these, 90% of the PV patients and 44.4% of PT patients were positive for

the mutation, in keeping with the published literature (Baxter *et al*, 2005;James *et al*, 2005;Jones *et al*, 2005;Kralovics *et al*, 2005;Levine *et al*, 2005). With regard to phenotypic variation in PT patients, those positive for the JAK2 V617F mutation had significantly higher haemoglobin and haematocrit values at diagnosis ($p<0.001$), consistent with previous reports (Campbell *et al*, 2005;Wolanskyj *et al*, 2005), but there was no difference in the values for mean cell volume. Higher leucocyte and lower platelet counts were also noted but these differences were not statistically significant.

Thrombo-haemorrhagic and haematological complications

Despite the relatively small patient numbers, the prolonged length of time from diagnosis provides a total of 419 patient years of follow-up. During this period only seven thrombotic events occurred in six patients, an incidence of 1.67 per 100 patient years, similar to although lower than, other treated patient populations (Harrison *et al*, 2005;Jensen *et al*, 2000b;Marchioli *et al*, 2005;Passamonti *et al*, 2004;Wolanskyj *et al*, 2006). As with the nature of events at presentation, the majority of these were arterial.

Unlike previous studies, we did not detect an association between older age and a history of thrombosis (Gruppo Italiano, 1995;Landolfi *et al*, 2007;Marchioli *et al*, 2005;Passamonti *et al*, 2004;Wolanskyj *et al*, 2006). In fact patients experiencing a thrombotic event at presentation were on average younger at the time. This may be explained by the earlier presentation of symptomatic patients. As would be expected with a cumulative risk, patients who had had a complicating thrombosis had a longer median follow-up from diagnosis; at 16 years this was over five times greater than those in whom no thrombotic events had occurred.

Previous thrombosis is another established risk factor for subsequent thrombosis. We distinguished a previous history of CVD, where the diagnosis was made prior to that of the MPD, from vascular thromboses occurring either at presentation (or

within the previous 12 months) or following diagnosis. No association was seen between thrombosis at presentation and previous thrombosis, CVD or CVRF. There was however a significant association between thrombosis arising after the diagnosis of PV or PT and a prior history of thrombosis or CVD combined. Complicating arterial thromboses were associated with a previous diagnosis of CVD ($p<0.001$) but not CVRF alone. We did not detect an association of any clinical variables with complicating VTE but this may well be as a result of the small numbers in the current study as VTE following diagnosis was an infrequent occurrence, recorded in only two patients.

With regard to haematological and molecular parameters only the leucocyte count at the time of diagnosis was associated with a history of thrombosis at presentation, as has previously been reported (Carobbio *et al*, 2007;Landolfi *et al*, 2007;Wolanskyj *et al*, 2005). This effect was not apparent for complicating thrombosis which may either reflect the small numbers of events, or that the effect is lost with normalisation of the leucocyte count by cytoreduction. Contrary to some previous reports we found no association between JAK2 mutation status and risk of venous thrombosis (Campbell *et al*, 2005;Kittur *et al*, 2007) although again this was an infrequent event.

Progression to post-PT MF was recorded in four patients on the basis of clinical and histological evidence. Review of the diagnostic trephine reports in three of these patients revealed the presence of atypical megakaryocytic features. According to the WHO criteria for PT, this might suggest a diagnosis of pre-fibrotic MF, which has been associated with an increased risk of progression to clinical MF (Tefferi & Vardiman, 2007;Thiele *et al*, 2002;Thiele & Kvasnicka, 2003). All three reports also described an increased degree of reticulin fibrosis at diagnosis which may be another predisposing factor. These findings support concerns about the interpretation of the results from the high risk PT-1 trial where a significantly increased risk of progression to MF was seen in the anagrelide arm but diagnostic trephines were not available (Harrison *et al*, 2005). However, we did not review the diagnostic trephines for all our patients and therefore do not know the prevalence of such findings in the cohort as a whole.

Management

The majority of PV patients were receiving venesection and cytoreductive therapy with hydroxycarbamide. At the point of entry to the study, the mean haematocrit was 0.42 and 75% of patients were below the target of 0.45 (McMullin *et al*, 2005). Cytoreduction for thrombocytosis is also recommended and the median platelet count in the PV patients was 405 x 10^9/L with 75% less than 450x10^9/L. These results may suggest less aggressive management compared to the current guidelines however in a recent study of PV patients receiving standard current management, no effect of platelet count or haematocrit level on thrombotic risk was observed (Di Nisio *et al*, 2007).

In the PT group, only four patients were not receiving cytoreductive therapy, three of whom were aged 40-60 years but had no other risk factors for thrombosis. The vast majority (52/55) of PT patients on cytoreductives were receiving hydroxycarbamide; two required dual therapy with anagrelide to achieve adequate control of the platelet count without unacceptable side effects including cytopenias and mouth ulcers. In patients on cytoreduction, at the single time point of entry to the study over 80% of the PT patients had a platelet count below 500 x10^9/L but only 50% had a platelet count below the widely accepted target of 400x10^9/L ((Barbui *et al*, 2004;Harrison, 2005b).

Although the leukaemic risk of hydroxycarbamide is likely to be low, until adequate long term follow-up data are available, the current guidelines continue to recommend that non-cytotoxic agents should be considered in high risk patients under the age of 40 years (Barbui *et al*, 2004;McMullin *et al*, 2005). Four PT patients in the study were under 40 years and all had indications for cytoreduction. However, only one of these patients received a non-cytotoxic agent in the form of anagrelide; the other three received hydroxycarbamide, one after being intolerant of interferon. Radiophosphorus and alkylating agents are currently recommended only as second line therapy in patients over the age of 60 years due to concerns regarding their leukaemogenicity (Barbui *et al*, 2004;McMullin *et al*, 2005). Radiophosphorus or

busulphan had previously been used in five patients; two of these patients were below the age of 60 when they were treated in the mid-1980s. None had experienced haematological transformation at the time of the study.

In keeping with the current guidelines, the majority of PT and PV patients were managed with aspirin or another antiplatelet agent (Barbui *et al*, 2004;McMullin *et al*, 2005). Overall over 90% of patients were on either antiplatelet or anticoagulant therapy and no haemorrhagic complications were recorded following diagnosis. Although the exact contribution of CVRF to thrombotic risk is unclear, control of these in individual patients is generally practised. As previously discussed it is likely that a minority of patients had undiagnosed hypertension. In addition, in seven of eleven non-study patients with known hypertension this was not controlled to recommended levels. Tobacco use and hyperlipidaemia appeared to be infrequent but again may have been underdiagnosed. These findings suggest that more pro-active assessment and management of CVRF may be indicated in our MPD patients.

JAK2 V617F in the diagnosis of PV

We reviewed the diagnosis of seven PV patients who were JAK2 V617F negative. A number of issues were highlighted by comparison of the diagnoses based on each of the PVSG, BCSH and WHO guidelines. Notably, despite borderline RCM results in two patients, both would meet the definition for an absolute erythrocytosis using the WHO criteria based on the haemoglobin values alone. In contrast all of the patients with haematocrit values above the cut-off in the BCSH criteria also had an elevated measured RCM. The use of elevated haemoglobin values alone to assess absolute erythrocytosis has not been validated (McMullin *et al*, 2005) and our results suggest that it may result in the inclusion of patients who do not have an elevated measured RCM.

There are also difficulties in the inclusion or exclusion and interpretation of some of features in support of a diagnosis of PV. In one case with a reduced serum Epo, the

diagnosis lies between idiopathic erythrocytosis and PV. The differentiation depends on whether the ultrasound measured spleen size of 13.5cm is judged to be "splenomegaly", highlighting the recognised difficulties of interpreting this parameter (McMullin *et al*, 2005). The exclusion of histological data from the BCSH and PVSG guidelines meant that two patients who are likely to have a myeloproliferative disorder based on their bone marrow histology, did not meet these criteria for PV.

The other three cases had borderline results in support a diagnosis of myeloproliferative disorder and probably have had insufficient investigation to confirm or refute the diagnosis of PV. This is especially true for the 2008 WHO criteria where in JAK2 wild type patients, two out of three of consistent bone marrow histology, reduced Epo levels and endogenous erythroid colony (EEC) growth must be present. Most UK haematology departments do not have access to EEC tests which are expensive and require technical expertise, making the use of these guidelines difficult. However in these cases measurement of the serum Epo level may indeed be of value in directing further investigation as it would be expected to be reduced in PV and to remain so even following venesection (McMullin *et al*, 2005;Tefferi & Vardiman, 2007).

In a clinical context, this diagnostic review of JAK2 V617F negative PV patients emphasises the importance of pursuing further investigation, both for features in support of PV or to determine an alternative explanation, in patients with a confirmed erythrocytosis who are negative for the mutation.

Summary

The demographics of our study cohort, in particular the PT patient group, were similar to but slightly younger than the local MPD population. The group had a relatively low incidence of thrombo-haemorrhagic events both at presentation and follow-up, with more arterial than venous events, in keeping with the findings of other recently published studies. There were no statistically significant differences in thrombo-haemorrhagic events between the PV and PT groups but this is likely to be affected by the relatively low numbers of patients and events. Consistent with previous reports we identified associations between thrombosis and leucocyte count at diagnosis and prior thrombotic history but in contrast found no associations with age or JAK2 mutation status. Management of the patient group as a whole was generally in keeping with the current guidelines although control of haematocrit and platelet counts was less aggressive than current recommendations and under diagnosis of CVRF was identified as a potential problem.

CHAPTER 3

MICROPARTICLE ASSAY

3.1 Introduction

Differences in the definition of microparticles and the methods used to isolate and measure them are likely to have an important influence on the results reported from individual studies. A forum addressing these issues reported that most groups define microparticles as plasma particles of less than 1 μm in diameter, bearing surface antigens of their cell of origin (Jy *et al*, 2004;Biro *et al*, 2004;Dignat-George *et al*, 2004;Hugel *et al*, 2004;Nomura, 2004;Shet *et al*, 2004;Jimenez *et al*, 2004). Some groups also used the additional criterion of Annexin V binding as evidence of the PPS rich surface of microparticles. While the use of such a consensus definition is helpful there remains significant variation between studies in the absolute numbers of microparticles reported for both patients and controls. This is likely to be due, in part, to the second issue of methodological differences.

Isolation techniques vary widely from centre to centre and microparticles may be measured in platelet poor plasma (PPP), microparticle suspensions or less commonly whole blood (Biro *et al*, 2004;Dignat-George *et al*, 2004;Hugel *et al*, 2004;Jimenez *et al*, 2004;Jy *et al*, 2004;Nomura, 2004;Shet *et al*, 2004). PPP is obtained by serial centrifugation of citrated whole blood and may also be filtered to remove particles of greater than 1μm. Alternatively, microparticles can be isolated from the PPP by ultracentrifugation, washing and resuspension of the microparticle pellet. The latter method is likely to avoid any platelet contamination but there are concerns that microparticles may be created *in vitro* by the ultracentrifugation steps. There has been no direct comparison of these methods and even within them there is wide variation in the centrifugation protocols used.

Measurement of microparticles is most commonly performed by flow cytometry but increasingly solid phase capture assays which exploit the functional properties of microparticles are used and results may be expressed as percentages, absolute concentrations or activity units. A summary of the published methods of measurement of *in vivo* levels of plasma microparticles and the reported ranges is shown in Table 4.4, Chapter 4.

Flow cytometry

Flow cytometry techniques allow both the quantitation of microparticles and identification of subtypes. Microparticles are isolated in PPP or microparticle suspensions and then labelled with fluorescently conjugated monoclonal antibodies. Annexin V binding can be used to confirm the phospholipid properties of the microparticles. Monoclonal antibodies (mAb) to specific surface antigens expressed on the cells of origin are used to identify the subtype of microparticle, for example anti-CD42 (GPIb) for identification of PMP or anti-glycophorin A for RMP. Two or three colour flow cytometry can be used to identify which microparticle subtypes express particular antigens, for example TF expression on monocyte microparticles. A variety of cell specific mAb are used in the identification of microparticles and the specificity chosen is likely to influence the results (Table 3.1). For example CD42a and CD62P (P-sel) are both platelet specific antigens but CD42a is present on all platelets while CD62P is found only on activated platelets.

Flow cytometry also permits the direct analysis of microparticle size by assessment of their forward light scatter. The identification of events of a specified size is most accurately done by comparison with using calibration beads of known diameter. Although a criteria of <1μm has been suggested for microparticle definition, in practice there is variation in the size criteria used from 0.8μm to 1.5μm Alternatively, some groups have identified microparticles as those particles of a size less than the platelet population in normal subjects (Villmow *et al*, 2002). However, this is a less standardized method as it is likely to be subject to biological variation. Further, as platelets are reported to be 2-3μm in diameter, the populations of PMP and small platelets may form a continuum in terms of size.

Absolute quantitation of microparticles by flow cytometry can be achieved using commercially produced counting beads of known concentration, which are added to the samples themselves, or used to calculate the volume of sample analysed over a standard collection time.

Activity assays

Solid phase capture assays isolate and immobilise microparticles in platelet free plasma using Annexin V binding antibodies which have a high affinity to the phospholipid surfaces and/or antibodies to specific cell surface antigens. This method of quantitation exploits the functional properties of the microparticle phospholipids by using an assay of prothrombinase activity and expresses quantity in PPS equivalents. As this technique cannot directly assess the size of the microparticles the sample can be filtered to remove particles of greater than 1μm before analysis. These assays describe activity and not absolute microparticle numbers which may not be directly equivalent.

Enzyme linked immunoassays (ELISA)

In attempt to develop easier methods for microparticle detection, commercial ELISA assays have also been investigated. These use combinations of antibodies to platelet antigens to allow PMP capture and detection in PPP and good correlation with measurement by standard flow cytometry methods in samples of *in vitro* induced PMP has been demonstrated (Osumi et al, 2001). More recently there has been publication of a clinical study where *in vivo* levels of microparticles were measured using this method (Inoue *et al*, 2006) and this may prove to be a useful method of detection.

We have designed a flow cytometry assay for the measurement of microparticles in PPP with identification of endothelial, platelet, red cell and leucocyte microparticles by size and antigen expression. The assay is based on previously published methods but we have aimed to optimise and standardise the sample preparation techniques. We have also devised a suitable method to allow the absolute quantitation of microparticles in our samples.

MICROPARTICLE SUBTYPE	ANTIGEN	COMMENTS	REFERENCES
ENDOTHELIAL	CD31 (CD42-)	PECAM-1	1-7
	CD31 (CD41-)		8
	CD62E	E-selectin	1- 4, 9
	CD144	VE Cadherin	8-14
	CD51 /$\alpha_v\beta_3$	Vitronectin receptor	1, 2, 7, 15- 18
	CD146	MelCAM	6, 11, 19
	CD105	Endoglin	13, 14
	CD54	ICAM-1	13, 14
PLATELET	CD42a	GPIX	20, 21
	CD42b	GPIb	5, 6, 22, 23
	CD42	GPIbIX	7
	CD41	GPIIbIIIa	8, 10, 11, 16, 18
	CD61	GPIIIa	9, 19, 22, 24
	CD62P	P-selectin - activation	20,23
MONOCYTE	CD14	Endotoxin receptor	9, 10, 16, 20, 21, 23
LEUCOCYTE	CD45	Common leucocyte antigen	
ERYTHROCYTE	CD235	Glycophorin A	8, 9 22

Table 3.1 Specificities of monoclonal antibodies used in microparticle identification

References (1) Jimenez et al 2001,(2) Jimenez et al 2003, (3) Gonzalez-Quintero et al 2003, (4) Gonzalez-Quintero et al 2004, (5) Chirinos et al 2005, (6) Mallat et al 2000, (7) Bernal-Mizrachi et al 2003, (8) Amabile et al 2005, (9) VanWijk et al 2002, (10) Shet et al 2003, (11) Faure et al 2006, (12) Koga et al 2005, (13) Simak et al 2004, (14) Simak et al 2006, (15) Combes et al 1999, (16) Sabatier et al 2002, (17) Dignat-George et al 2004, (18) Bretelle et al 2003, (19) Pereira et al 2006, (20) Nomura et al 2005, (21) Ogata et al 2006, (22) Hugel et al 1999, (23) Villmow et al 2002, (24) Harlow et al 2002

3.2 Identification of microparticles

3.2.1 Antibody selection and titration

Directly conjugated fluorescent mAb were used for microparticle identification and the choice of specificity was based on the published literature and local laboratory experience. EMP and PMP were identified using phycoerythrin (PE) labelled CD31 (PECAM) and fluorescein isothiocyanate (FITC) labelled CD42 (GPIbIX) (both AbD serotec). RMP were identified using FITC labelled CD235a (Glycophorin A) (BD Biosciences) and LMP were identified using FITC labelled CD45 (AbD serotec). Samples were also stained with the appropriate negative isotype controls; Mouse IgG1 PE, Mouse IgG1 FITC and Mouse IgG2a FITC (all AbD serotec).

According to published methods, 25 µl of PPP were incubated with 2µl of each mAb or isotype control for 1 hour at room temperature in the dark. Phosphate buffered saline (PBS) was then added to a total volume of 1ml prior to analysis.

All mAb were titrated to determine saturating concentrations. PPP and platelet rich plasma (PRP) samples were used for the titration of CD31 and CD42 antibodies and a dilution of 1 in 4 in PBS was found to be optimal. Due to low numbers of microparticles positive for CD45 and glycophorin A, mAb to these antigens were titrated using lysed whole blood and an optimal antibody dilution of 1:4 was determined. An example titration is shown in Figure 3.1. In practice when using the CD45 antibody on PPP samples a consistent right shift in the "negative" peak was noted in the specific mAb labelled samples as compared to the negative isotype control. To investigate whether this may be due to true events or to antibody excess we performed a number of samples with a further dilution of antibody to 1:8. This resulted in a loss of this phenomenon suggesting antibody excess.

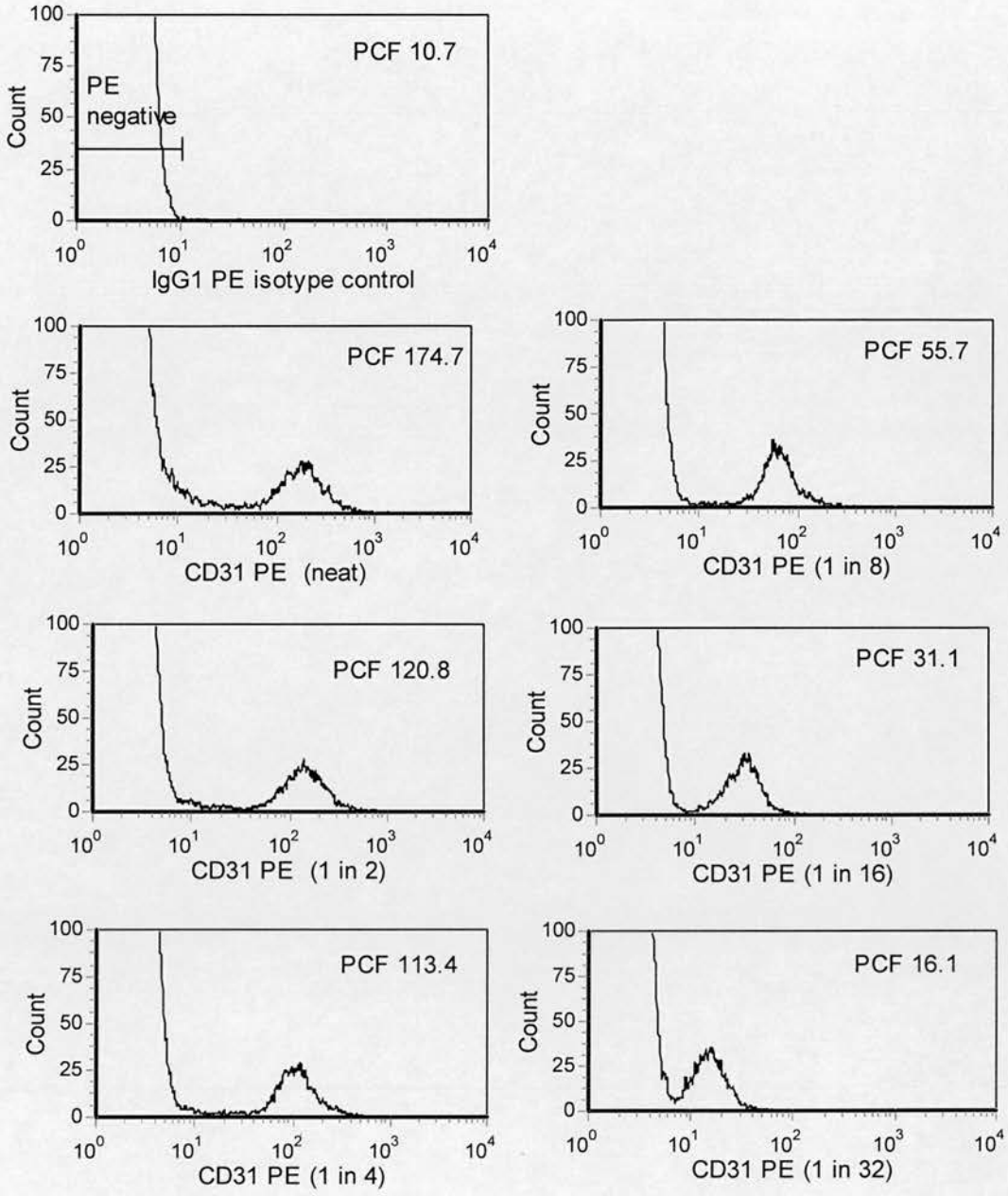


Figure 3.1 Example antibody titration.

Example of antibody titration using the CD31 PE antibody. Fluorescence histograms for the isotype control, neat antibody and serial dilutions are shown. Values for peak channel fluorescence (PCF) are shown for each dilution. With serial dilution of the antibody the PCF reduces towards that of the isotype control. At an antibody dilution of 1 in 4, a one-log difference in PCF is preserved compared to the isotype control.

3.2.2 Size calibration

Calibrated 1μm beads (Duke Scientific Corporation) were diluted 1:100 with PBS and 0.5% bovine serum albumin. The flow cytometer instrument settings were set to those used for microparticle collection (Appendix 3). The data was acquired on a contour plot and the bead population was used to identify a region with forward scatter of up to 1μm (used to set the 1μm gate) (Figure 3.2).

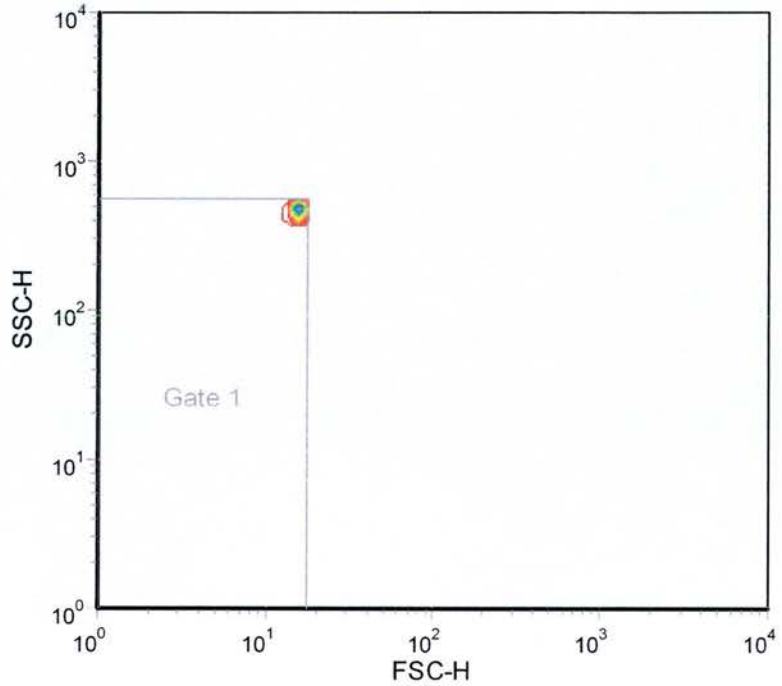


Figure 3.2 Size calibration beads

Contour plot illustration the use of size calibration beads to set the 1μm gate by forward scatter (FSC-H), for determination of microparticles by size

3.2.3 Flow cytometer sample collection

All flow cytometric experiments were carried out using a Fluorescence Activated Cell Scanner (FACScan) benchtop flow cytometer using FACS Flow^{TM} optimised sheath fluid (Becton Dickinson Immunocytometry Systems). The flow cytometer was regularly calibrated using calibrite beads (BD biosciences). Flow cytometer settings were optimised to measure events of less than 1μm and to minimise non-specific electronic noise (Appendix 3). Samples were collected for two minutes on medium flow and all events were collected.

3.2.4 Flow cytometry analysis

Analysis was performed using both the Macintosh driven Cell Quest software for the preliminary work and FCS express software (DeNovo Software) for the majority of the analysis.

3.2.4.1 Determination of microparticles by size

Based on the forward scatter of the calibrated size beads it was possible identify events less than 1μm in size. We also considered other described methods of size analysis including "less than platelets". However we were unable to clearly distinguish the platelet and microparticle populations by forward and side scatter as these formed a continuous population. Furthermore, by analysis of samples of PRP using a contour plot we could identify a region of highest density of events which had a size consistent with platelets: the lower forward scatter limit of this region was less than 1μm in size. (Figure 3.3).

For the remainder of the analysis, a gate of less than 1μm was set using the calibrated size beads as described and this was applied to all histograms and plots.

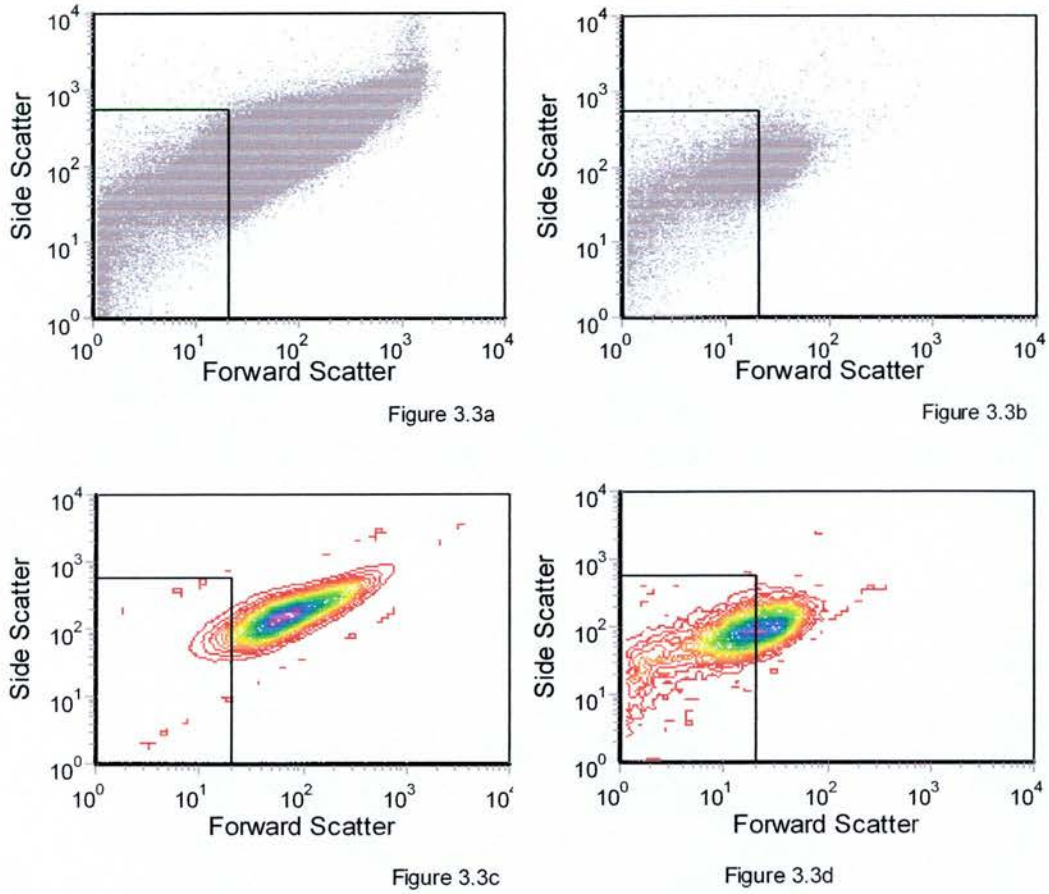


Figure 3.3 Comparative analysis of platelet rich plasma and platelet poor plasma

Forward and side scatter profiles of platelet rich plasma and platelet poor plasma samples (PRP, PPP). The residual PPP platelet count in this sample was 3 x 10^9/L. The size region of <1μm is superimposed onto the plots. The dot plots of (a) PRP and (b) PPP show that platelets and microparticles (<1μm) did not form two distinct populations. The contour plots show the areas of highest density of events and their size distribution in the (c) PRP and (d) PPP samples. The lower size limit of the area of events in the PRP lies below 1 μm.

3.2.4.2 Determination of microparticle subtype by antibody specificity

Fluorescence gates were set by forward gating of samples stained with specific mAb on histograms plots using negative isotype control samples for comparison to exclude non-specific background staining (Figures 3.4, 3.5, 3.6)

For EMP and PMP the CD31 PE positive gate was set by using the double labelled CD31 PE / CD42 FITC sample in comparison to the CD42 FITC / IgG1 PE isotype control sample. The CD42 FITC positive gate was set using the CD31 PE / CD42 FITC sample in comparison to the CD31 PE/ IgG1 FITC isotype control sample (Figure 3.4).

EMP were defined as CD42 negative, CD31 positive events less than 1μm. To correct for non-specific staining, the number of CD42 negative, PE positive events in the isotype control sample could then be subtracted from this (Figure 3.4g).

PMP were defined as CD31 and CD42 double positive events less than 1μm. The number of non-specifically labelled events in the isotype control samples was <1% of the CD31/42 positive events therefore a correction was not performed.

LMP were defined as CD45 positive events less than1μm. The positive gate was set using the CD45 FITC labelled sample in comparison to the IgG2a FITC isotype control (Figure 3.5). To correct for non-specific staining, the number of FITC positive events in the isotype control sample was subtracted from the CD45 positive events.

RMP were defined as Glycophorin A positive events less than 1μm. The positive gate was set using the Glycophorin A FITC labelled sample in comparison to the IgG1 FITC isotype control (Figure 3.6). To correct for non-specific staining, the number of FITC positive events in the isotype control sample was subtracted from the Glycophorin A positive events.

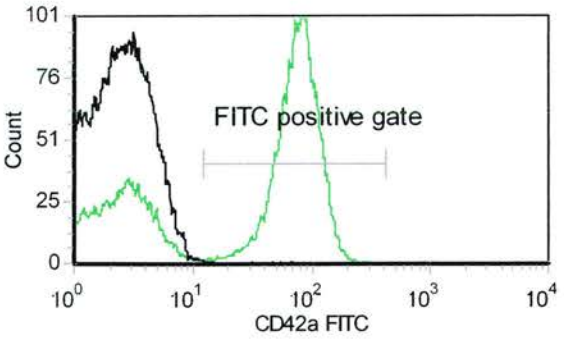


Figure 3.4a

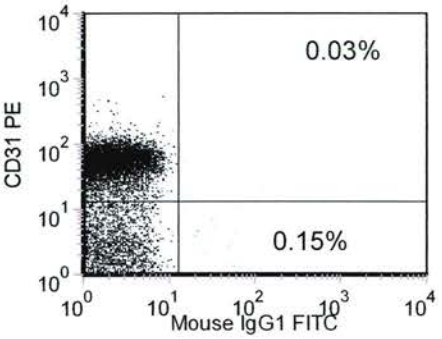


Figure 3.4c

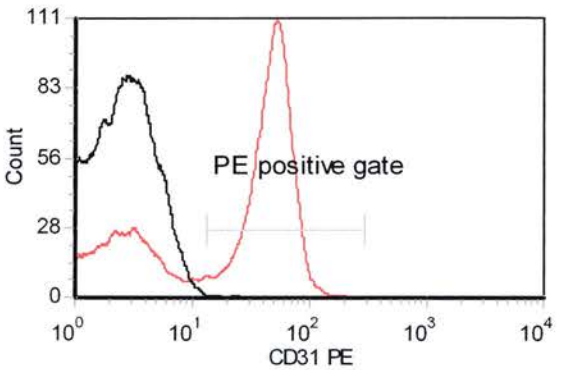


Figure 3.4b

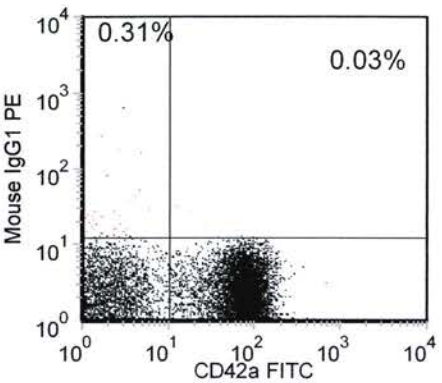


Figure 3.4d

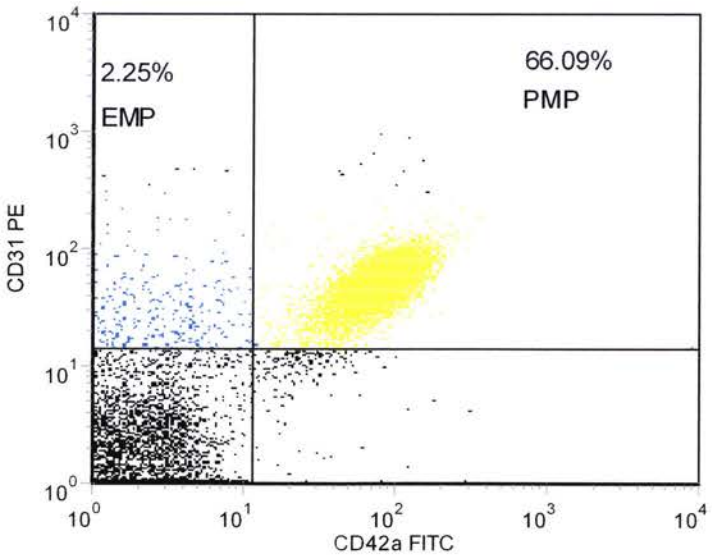


Figure 3.4e

Sample	Gates applied	Number of events
PPP CD31 PE / CD42 FITC	<1μm, PE +ve, FITC +ve	10174
PPP CD31 PE/ IgG1 FITC isotype	<1μm, PE+ve, FITC +ve	4
PPP IgG1PE isotype / CD42 FITC	<1μm, PE+ve, FITC +ve	6
		PMP corrected = 10164

Figure 3.4f

Sample	Gates applied	Number of events
PPP CD31 PE / CD42 FITC	<1μm, PE +ve, FITC -ve,	380
PPP IgG1PE isotype / CD42 FITC	<1μm, PE+ve, FITC -ve	39
		EMP corrected = 341

Figure 3.4g

Figure 3.4 Analysis of platelet and endothelial cell microparticles

Analysis of platelet and endothelial cell microparticles. (a) Histogram showing FITC staining of CD42a labelled sample (green line) and IgG1 isotype control sample (black line) and setting of FITC positive gate. (b) Histogram showing PE staining of CD31 labelled sample (red line) and IgG1 isotype control sample (black line) and setting of PE positive gate. (c and d) Dot plots of FITC positive (green) and negative (black) events in the FITC IgG1 isotype control sample (c) and PE positive (red) in the PE IgG1 isotype control sample (d); less than 1% of the isotype control events are positive. CD42 FITC /CD31 PE double positive events (PMP - yellow) and CD31 PE positive / CD42 FITC negative events (EMP - blue) are shown in (e). PMP numbers are calculated by subtraction of FITC/PE double positive events in the isotype control samples from the CD42 FITC/ CD31 PE labelled sample (f). EMP numbers are calculated by subtraction of FITC negative /PE positive events in the isotype control sample from the CD42 FITC/ CD31 PE labelled sample (g).

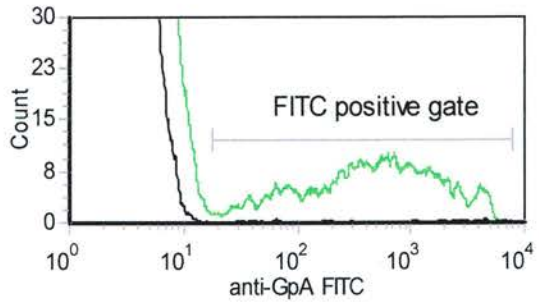


Figure 3.5a

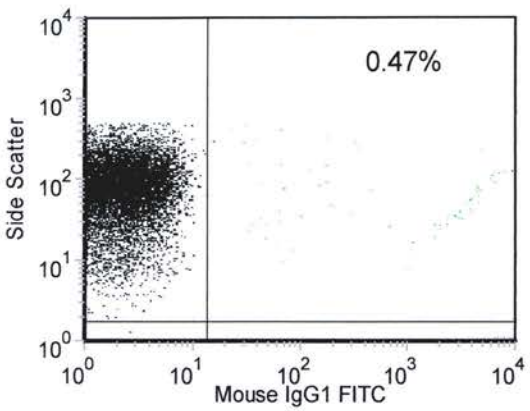


Figure 3.5b

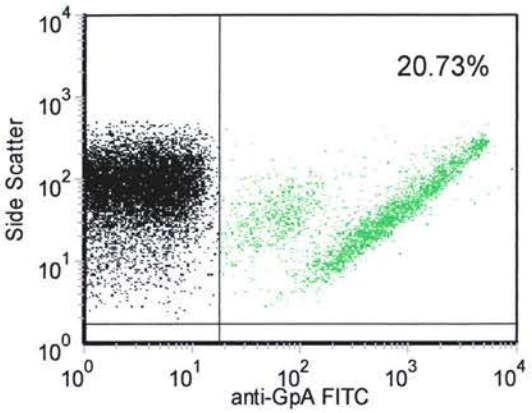


Figure 3.5c

Sample	Gates applied	Number of events
PPP Glycophorin a FITC	<1μm, FITC +ve	3428
PPP IgG1 FITC isotype	<1μm, FITC +ve	68
		RMP corrected = 3360

Figure 3.5d

Figure 3.5 Analysis of red cell microparticles

Analysis of red cell microparticles. (a) Histogram showing FITC staining of Glycophorin A labelled sample (green line) and IgG1 isotype control labelled sample (black line) and setting of FITC positive gate. (b and c) Dot plots of FITC positive (green) and negative (black) events in the IgG1 isotype control labelled sample (b) and in the Glycophorin A FITC labelled sample (c); less than 1% of the isotype control events are positive. RMP numbers are calculated by subtraction of FITC positive events in the isotype control sample from the Glycophorin A FITC labelled sample (d).

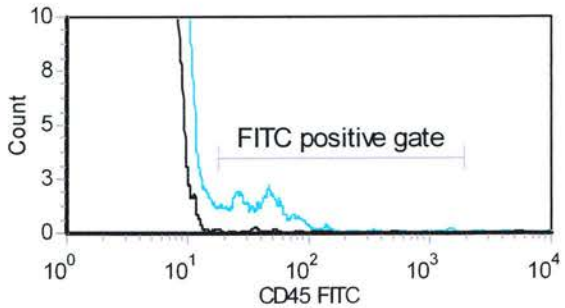


Figure 3.6a

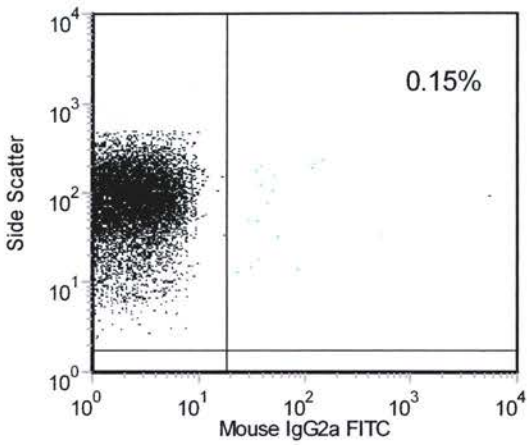


Figure 3.6b

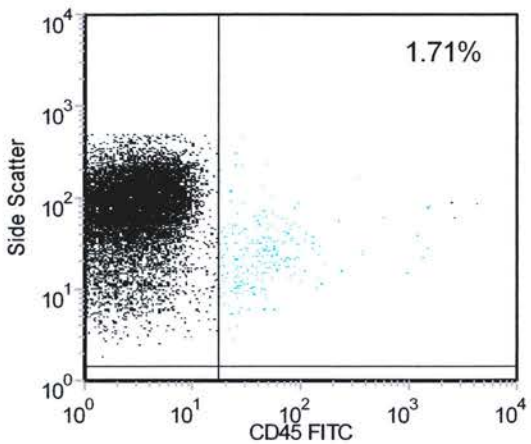


Figure 3.6c

Sample	Gates applied	Number of events
PPP CD45 FITC	<1μm, FITC +ve	265
PPP IgG2 FITC isotype	<1μm, FITC +ve	19
		LMP corrected = 246

Figure 3.6d

Figure 3.6 Analysis of leucocyte microparticles

Analysis of leucocyte microparticles. (a) Histogram showing FITC staining of CD45 labelled sample (pale blue line) and IgG2 isotype control labelled sample (black line) and setting of FITC positive gate. (b and c) Dot plots of FITC positive (blue) and negative (black) events in the IgG2 isotype control labelled sample (b) and in the CD45 FITC labelled sample (c); less than 1% of the isotype control events are positive. LMP numbers are calculated by subtraction of FITC positive events in the isotype control sample from the CD45 FITC labelled sample (d).

3.3 Quantitation of Microparticles

Quantitation of events in flow cytometry is often expressed as a percentage of a population of cells. However this method is not suitable for quantitation of microparticles due to the cell poor nature of PPP. In order to allow comparison between samples from subjects collected at different times, a method of absolute quantitation is required. This can be achieved using commercial counting beads of known quantitation which can be "spiked" into the flow cytometry sample. However, many of these beads are significantly larger than the microparticle size which poses difficulties in analysis using instrument settings optimised for events of less than 1μm. Further, the beads are identified using a fluorescence trigger and this appeared to skew the microparticle populations collected in double labelled samples.

We therefore devised a method whereby a sample of counting beads at a known concentration was collected for a defined time period, allowing determination of the volume sampled in that time. This was performed prior to each microparticle collection session and all samples were collected for the same time period. We were then able to calculate the absolute microparticle concentration in the plasma sample.

Quantitation method

Trucount counting beads were used (BD biosciences) for which the exact concentration is provided for each individual vial but is approximately 1000 beads per μl. The beads are 4μm in size and are double fluorescence labelled (FITC and PE). Using the recommended reverse pipetting technique, 50μl of beads were diluted in 950μl of PBS (Cellwash, BD biosciences). Optimal flow cytometer instrument settings were determined according to the advice given with the beads (Appendix 3). After vortexing, the diluted bead sample was collected for a defined time period and the number of events within the predefined bead region was recorded (Figure 3.7). This was performed three times and the mean bead count was calculated.

The following calculation was then applied to determine the microparticle (MP) concentration in the plasma sample where 25µl of plasma was diluted to a final volume of 1000µl with PBS (Cellwash, BD biosciences).

$$\text{Final Trucount concentration (beads/µl)} = \frac{\text{Stated bead concentration (beads/µl)} \times 50\mu l}{1000\mu l}$$

$$\text{Volume sampled in X seconds (µl)} = \frac{\text{Mean bead count (in X seconds)}}{\text{Final Trucount concentration (beads/µl)}}$$

$$\text{Final sample MP concentration (MP/µl)} = \frac{\text{MP count (in X seconds)}}{\text{Volume sampled (µl)}}$$

$$\text{Original plasma MP concentration (MP/µl)} = \frac{\text{Final sample MP/µl} \times 1000\mu l}{25\mu l}$$

To assess the variation in the recorded bead event count for a single preparation of Trucount beads, 12 preparations were collected in triplicate at both the start and end of a flow cytometry session. The coefficient of variation (CV) for the bead event count for a single preparation was 2.88% (standard deviation (sd) 1.51). During this period a problem was identified with the flow cytometer that was reducing the flow rates. After resolution of this problem, the subsequent four collections had a CV of 1.68% (sd 0.45).

A new Trucount preparation was made for each flow cytometry session and for comparison the Trucount preparation from the previous session was also analysed. The difference between pairs of samples made on the same day or run on the same day was expressed as a percentage of the mean bead count for the pair. There was

greater variation between bead counts for Trucount preparations made on the same day and run on different days (mean difference (sd) 12.9% (13.0), n=9) than for preparations made on different occasions and run on the same day (3.6% (3.54), n=12). This suggests that variation in the flow rate on different days had a greater influence on the bead count than variation in the preparation. This is likely to reflect the fault in the flow cytometer that was reducing the flow rates over this time period.

3.4 Preanalytical variables

3.4.1 Centrifugation protocols

Whole blood was collected from eight healthy subjects into citrate vacutainers (Becton Dickinson) at room temperature and PPP was prepared at room temperature according to four different protocols (P1-P4) adapted from those reported in the literature (Table 3.2). In protocols P1-P3, PRP was obtained in the first step. PPP was then obtained by a second centrifugation step and the removal of the middle portion of the supernatant. In protocol 4, PPP was prepared in a single step.

EMP and PMP were measured as described and microparticles per microlitre (/μl) of plasma were calculated. Results were analysed using the paired t-test and expressed as the mean and SEM. The residual platelet count of the PPP was measured using a Sysmex analyser (1:5 dilution, capillary mode).

PMP numbers decreased with length of time of centrifugation and were lowest using Protocol 3 (P3 v P1, P2, P4, p=0.02, 0.03, 0.008 respectively). EMP numbers showed a similar trend but this was not statistically significant (Table 3.3, Figure 3.7).

Protocol	Step 1	Step 2
P1	160g x 10 mins	1500g x 6 mins
P2	160g x 10 mins	1500g x 10 mins
P3	160g x 10 mins	1500g x 15 mins
P4	1500g x 15 mins	-

Table 3.2 Summary of centrifugation protocols

Summary of centrifugation protocols showing centrifugal force applied (g) and length of centrifugation (minutes)

Protocol	**PMP per µl**	v P1	v P2	v P3	v P4
P1	1829 (268)	-	0.080	**0.024**	0.153
P2	1532 (219)	-	-	**0.031**	0.716
P3	1007 (143)	-	-	-	**0.008**
P4	1454 (143)	-	-	-	-
Protocol	**EMP per µl**	v P1	v P2	v P3	v P4
P1	463 (119)	-	0.145	0.079	0.153
P2	403 (93)	-	-	0.221	0.343
P3	374 (86)	-	-	-	0.824
P4	366 (65)	-	-	-	-

Table 3.3 PMP and EMP values by centrifugation protocol

Mean (SEM) PMP/µl and EMP/µl for each centrifugation protocol (n=8 for all). P values for comparison of protocols are given with statistically significant results in bold

Protocol	PPP platelet count (mean)	Range	v P1	v P2	v P3	v P4
P1	9.6	0-20	-	0.348	0.076	0.367
P2	7.9	0-14	-	-	0.061	0.719
P3	3.9	0-10	-	-	-	**0.003**
P4	7.3	5-10	-	-	-	-

Table 3.4 Residual platelet count in PPP by centrifugation protocol

Mean and range of residual platelet count in PPP for each centrifugation protocol (n=8 for all). P values for comparison of protocols are given with statistically significant results in bold

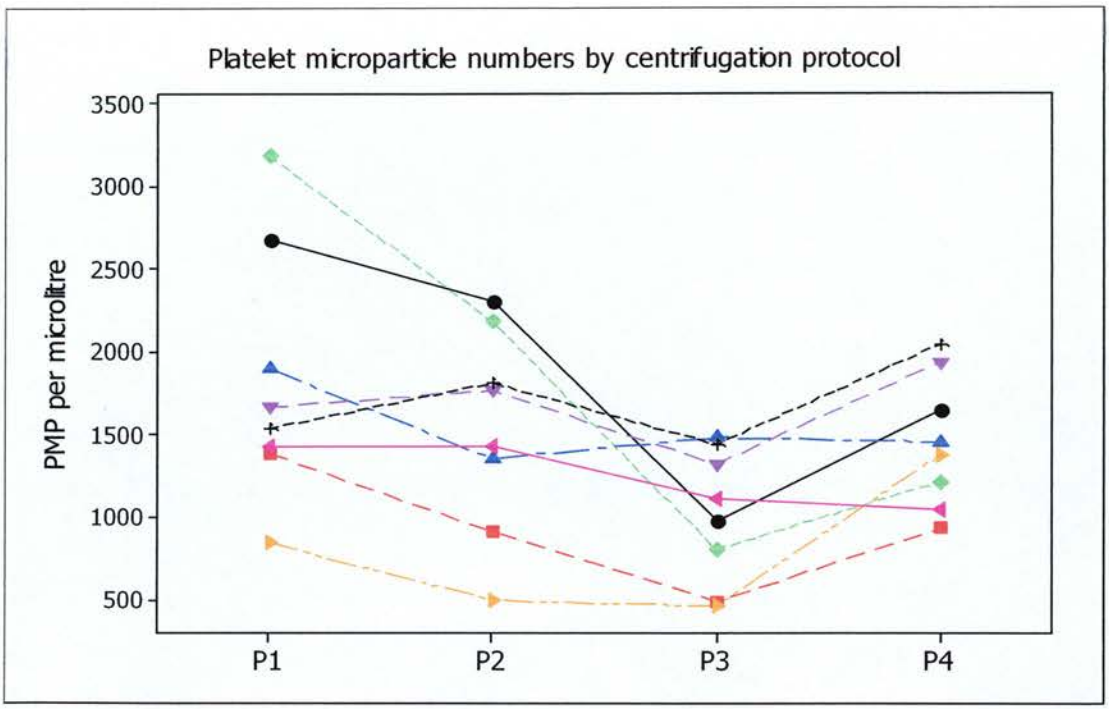


Figure 3.7a

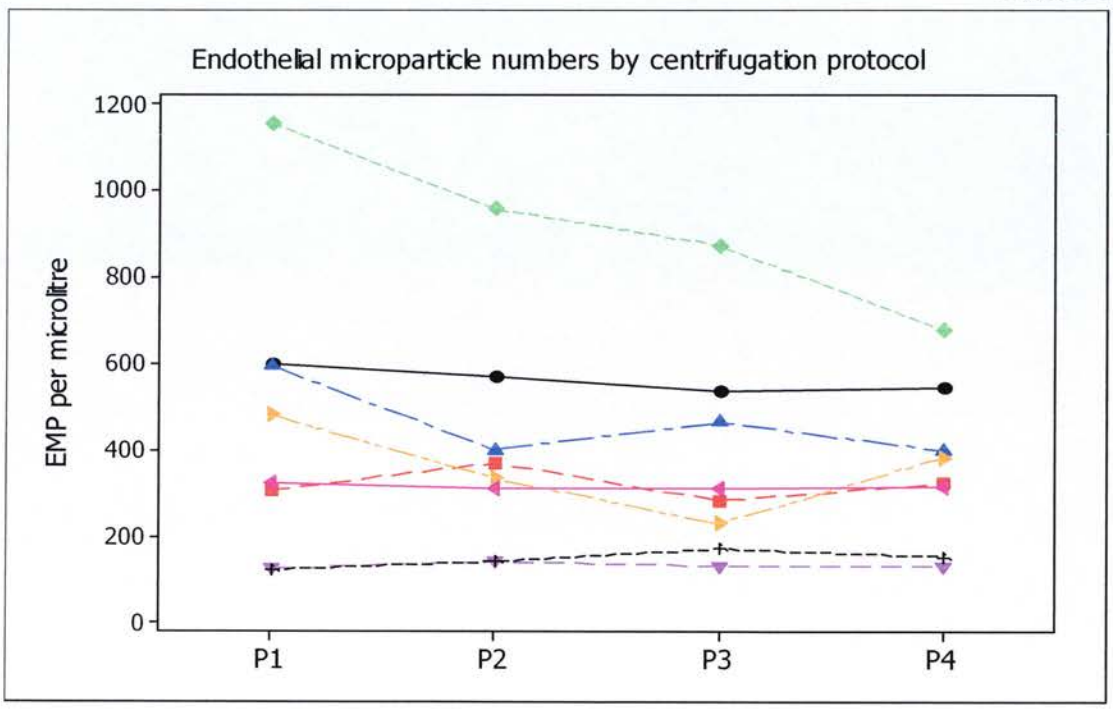


Figure 3.7b

Figure 3.7 PMP and EMP per microlitre by centrifugation protocol

Results for (a) PMP and (b) EMP per microlitre are shown for each centrifugation protocol P1-P4. Each coloured line represents the samples from one subject. PMP numbers were significantly lower for protocol 3 compared to the other protocols. EMP numbers showed a similar trend but this was not statistically significant.

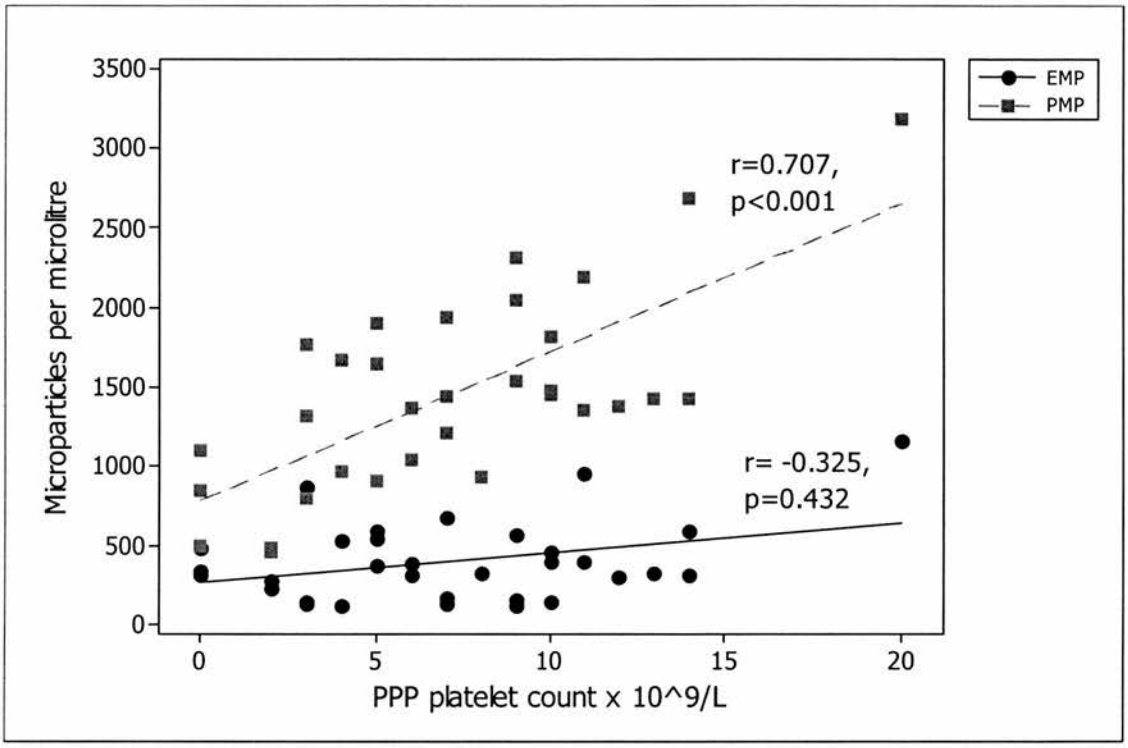


Figure 3.8 Association of residual platelet count with PMP and EMP

Association of PPP residual platelet count with PMP and EMP results. There was a significant strong correlation between residual platelet count and measured PMP (Pearson correlation).

The mean PPP platelet count also decreased according to the centrifugation protocol used and was lowest for P3 (Table 3.4). The differences only reached statistical significance for P3 v P4. For protocols 1 and 2, maximum platelet counts of greater than ten were obtained. The PPP platelet count correlated with PMP ($r=0.707$, $p<0.001$, Pearson correlation) but not EMP numbers ($r= -0.325$, $p=0.432$), (Figure 3.8)

There was also a trend towards Protocol 3 giving the highest percentage of events of less than 1μm compared to all events however the ranges were wide and this was not statistically significant.

3.4.2 Temperature and media

Whole blood was collected from seven healthy volunteers into citrate and CTAD (citrate, theophylline, adenosine and dipyridamole) vacutainers (Becton Dickinson) and PPP was prepared at 4°C and room temperature, using centrifugation protocol 3 (Table 3.2).

EMP and PMP were measured and microparticles per microlitre of plasma were calculated. Duplicate samples from each subject were compared i.e. citrate 4°C v CTAD 4°C; citrate 20°C v CTAD 20°C; citrate 4°C v citrate 20°C; CTAD 4°C v CTAD 20°C. Results were analysed using the paired t-test.

Overall there was no significant difference in mean EMP or PMP numbers according to collection media or temperature although differences were seen in some individual subjects (Table 3.5, Figure 3.9 and 3.10)

Medium	Citrate	CTAD	Citrate v CTAD P value
PMP/μl Mean (sd)	1132 (1260)	801 (666)	0.177
EMP/μl Mean (sd)	231 (134)	254 (138)	0.307
Temperature	**4°C**	**20°C**	**4°C v 20°C P value**
PMP/μl Mean (sd)	810 (729)	1123 (1128)	0.164
EM P/μl Mean (sd)	261 (136)	223 (134)	0.28

Table 3.5 PMP and EMP values by collection media and temperature

Comparison of PMP and EMP according to collection media (citrate or CTAD) and processing temperature (4°C v 20°C). Values for the mean and sd are given, P values for statistically significant differences are shown in bold.

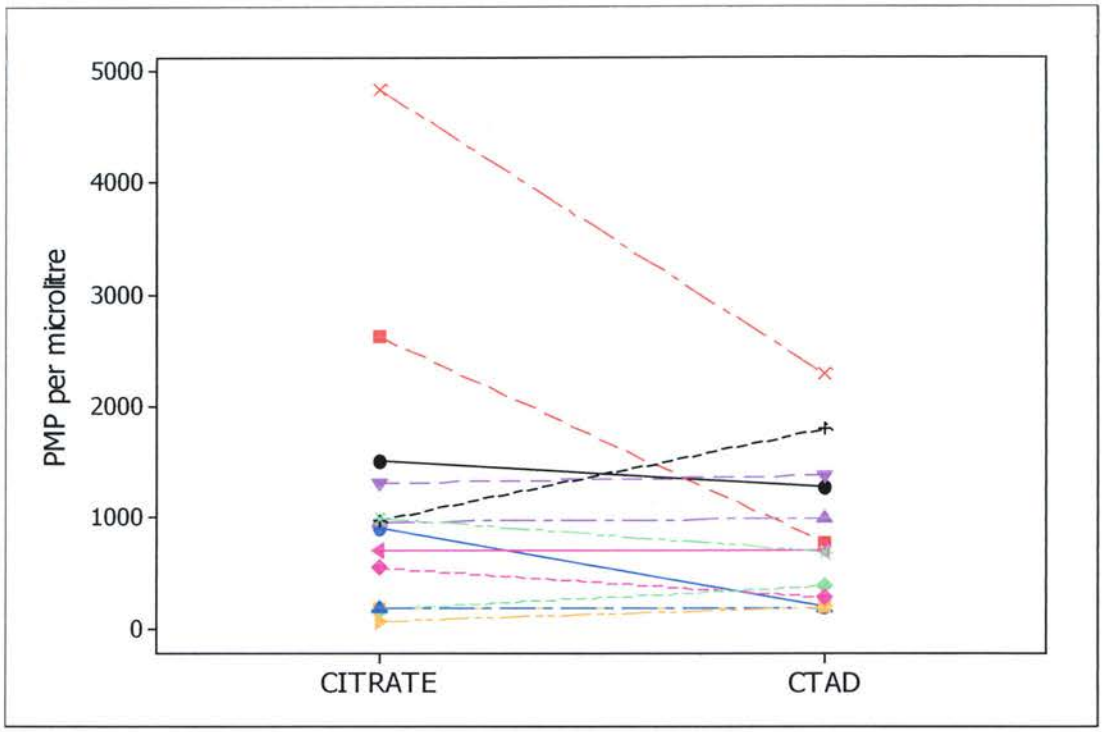


Figure 3.9a

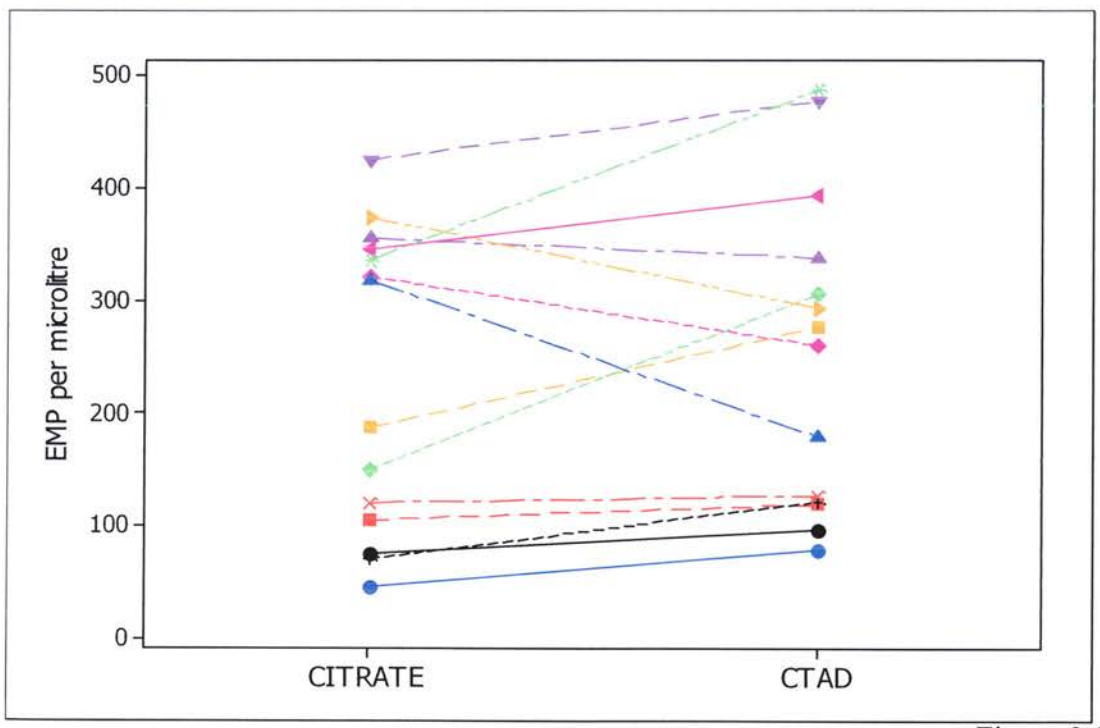


Figure 3.9 b

Figure 3.9 Comparison of PMP and EMP according to sample media

Comparison of (a) PMP and (b) EMP according to sample preparation in citrate or CTAD. Each subject is shown in a different line colour and there are two sets of samples for each. There were no significant differences according to preparation medium.

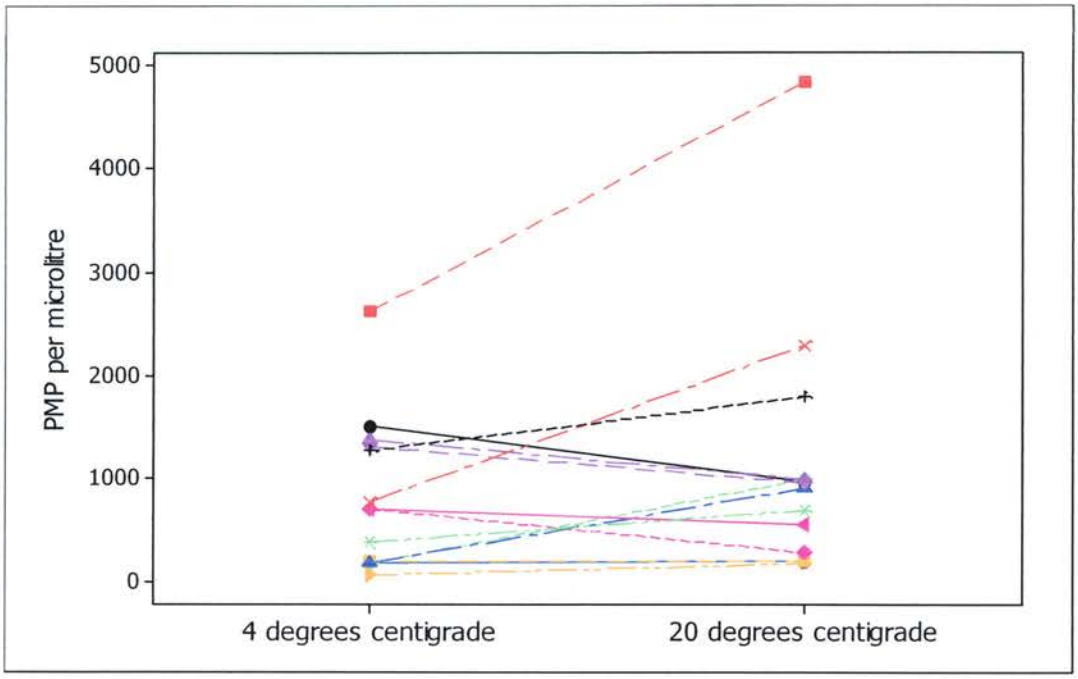


Figure 3.10 a

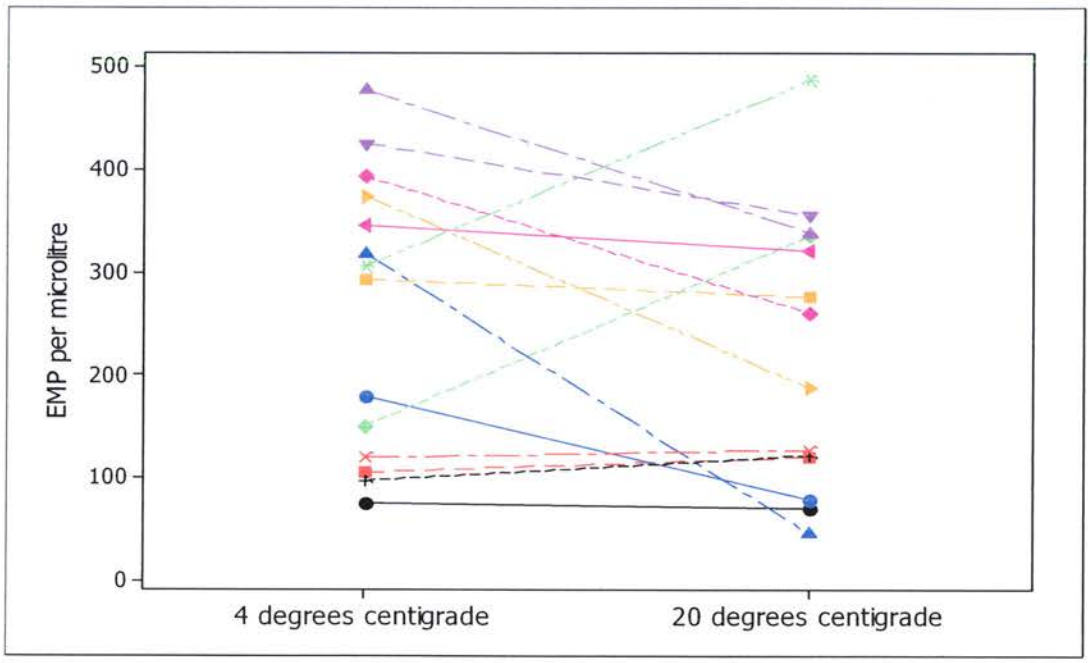


Figure 3.10 b

Figure 3.10 Comparison of PMP and EMP according to sample temperature

Comparison of (a) PMP and (b) EMP according to sample preparation at 4°C and 20°C. Each subject is shown in a different line colour and there are two sets of samples for each. There were no significant differences according to preparation temperature.

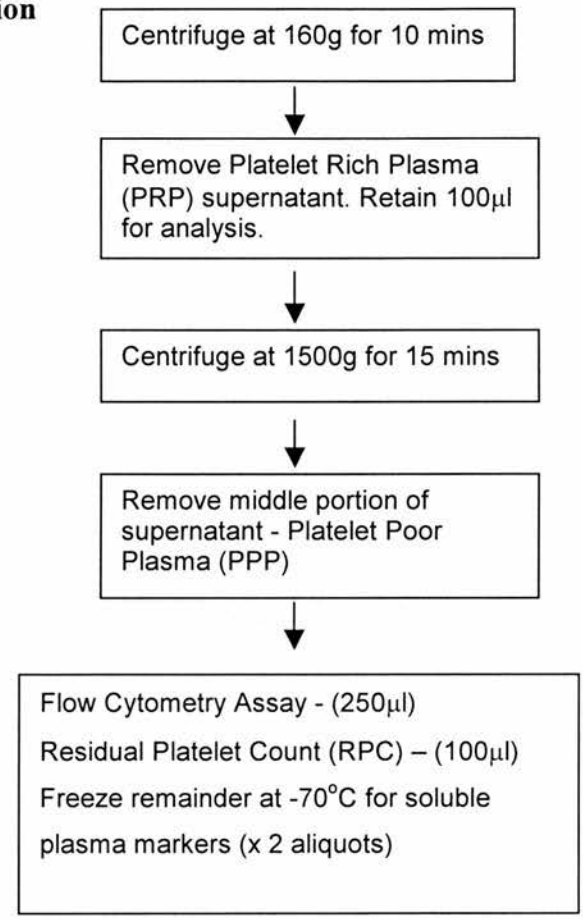
3.5 Final protocol

Sample collection

20 ml citrated whole blood collected via 12g needle

Centrifuge within 1 hour

Sample preparation



Labelling for Flow Cytometry Assay

Isotype controls

25µl PPP	2µl of 1:4 mAb -	IgG1 PE IgG1 FITC IgG2 FITC IgG1 PE + CD42 FITC IgG1 FITC + CD31 PE

Specific antibodies

25µl PPP	2µl of 1:4 mAb-	CD31 PE + CD42 FITC Glycophorin A FITC CD45 FITC

Incubate for 30 minutes at room temperature in the dark

Add PBS to a total volume of 1ml

Analyse within 4 hours

Flow Cytometer Set Up

Clean thoroughly prior to use as per manufacturer's protocol

Run through distilled water on high flow until flow rate <1000 events/ sec

Trucount beads

50µl of high concentration Trucount beads in 950µl of PBS

Set Trucount instrument settings (fluorescent trigger FL = 180)

Acquire for 120 seconds on medium flow x 3

Samples

Set on microparticle instrument settings (Forward scatter trigger =10)

Vortex immediately prior to analysis

Acquire samples for 120 seconds on medium flow rate (no gate applied)

3.6 Discussion

At the time of initial design of our microparticle assay, the main information on assay methods was from the forum reported in the Journal of Thrombosis and Haemostasis and the papers published by these groups, in particular the Miami group (Jy *et al*, 2004). Subsequently there has been growing interest in the measurement of microparticles and more extensive data has been presented and published on the effects of preanalytical variables, method of analysis and storage conditions.

Preanalytical variables – sample collection

Our results suggest that overall there was no significant difference in microparticle numbers depending on the temperature used in preparation or whether CTAD or citrate medium was used. This result may be surprising as we might have expected any *in vitro* platelet activation and consequent PMP formation to be reduced by processing the samples at a lower temperature. However, our results are in keeping with other presented data (Shah *et al*, 2007) which found no temperature effect and no difference between acid-citrate-dextrose and citrate media. The use of steps to reduce *in vitro* platelet activation including good and consistent venepuncture technique and the rapid processing of samples may have helped to minimise the effects of the processing conditions. Recently the effect of high fat meals increasing the total numbers of circulating microparticles has been reported (Tushuizen *et al*, 2006) and it is possible that other such physiological variables, for example, exercise may influence microparticle numbers in healthy individuals.

Preanalytical variables – centrifugation and platelet contamination

We measured EMP and PMP and residual platelet count in samples prepared using four different centrifugation protocols. With increasing length of centrifugation in protocols 1-3, the number of PMP was reduced in tandem with the residual platelet count. The fall in the number of PMP with increasing centrifugation may reflect

either or both of two possibilities: removal of microparticles with longer centrifugation or reduced platelet contamination with longer centrifugation.

The American laboratory standard for production of PPP is centrifugation at 1500g for at least 15 minutes to produce plasma with a platelet count of less than 10 x 10^9/l. Protocol 3, which included such a step, consistently produced PPP with a platelet count of less than or equal to 10. Further, protocol 3 had the highest ratio of events less than 1μm versus greater than 1μm. However the average proportion of events that were <1μm in size was still only 40%. This suggests that although our PPP had low residual platelet count, this platelet presence may still be numerically significant in comparison to the microparticle numbers.

The results for EMP showed a similar but non-significant trend to reduced numbers with longer centrifugation, in keeping with the theory that microparticles may be lost with increasing centrifugation. It would be useful to examine the effect of increasing centrifugation on RMP and LMP numbers.

Comparing the results for PRP and PPP, the numbers of events of less than 1μm could be up to 10 times higher in the PRP than PPP. This may be in part due to the inclusion of the smaller end of the platelet population within the 1μm gate (Figure 3.3c). However, even when a smaller size gate was set, a higher number of events were present in this region in the PRP, again in keeping with the theory that microparticles may be lost during the centrifugation.

Not all studies have employed an initial gentle centrifugation step to produce PRP prior to isolation of PPP. The proposed reasoning behind the use of this step is to avoid the creation of microparticles by more rigorous centrifugation but as it produces PRP prior to the 2nd centrifugation step it seems unlikely that it would reduce any artefactual creation of PMP. We addressed this by comparing protocol 3 to 4 and found that PMP numbers were indeed higher when this initial step was not used but the residual PPP platelet count was also higher.

Our results are supported by similar data presented recently which show that those protocols using PPP which have the most rigorous centrifugation steps produce lower

microparticle numbers and less platelet contamination than those using more gentle centrifugation (Weltermann *et al*, 2007). Therefore, when flow cytometry is used for microparticle detection, the choice of centrifugation process may result in the underestimation any of the microparticle subtypes or overestimation of the number of PMP due to platelet contamination.

The presence of residual platelets may also be a significant factor in methods which exploit the phospholipid properties of microparticles, such as prothrombinase capture or functional coagulation assays. In particular, in samples frozen for future analysis, the greater the platelet contamination the more phosphatidylserine exposure occurs on the platelet surface during the freeze-thaw process. It was demonstrated that compared to fresh samples, levels of Annexin V binding were increased in freeze-thawed samples and the increase was greatest in those with the most platelet contamination (Weltermann *et al*, 2007). It was suggested that for samples being frozen prior to analysis for Annexin V binding, prothrombinase capture or thrombin generation, a double-spin centrifugation protocol may be more suitable to reduce platelet contamination.

Quantitation of microparticles

The results for our devised method for absolute counting of microparticles confirm low intra-assay variation in the bead count and low variation in the flow rate over a single flow cytometry session. They also confirm the reliability of the bead preparation technique with low variability between preparations. They suggest however that there can be significant variation in the rate of flow of the flow cytometer over a time period. In view of this we elected to run a Trucount bead preparation pre and post each session to determine the volume sampled and allow comparison of microparticle samples run on different days. A similar method has been validated and employed by other groups (pers comm, M. Shah)

Analysis of microparticles - antigen detection

Our choice of antibodies was based on those used in published clinical studies. It may have been useful to have had *in vitro* models of microparticle production for each of the cell types to confirm their detection of the respective microparticle species. We used PPP and PRP or lysed whole blood samples to perform the titrations for each of the antibodies. The use of whole blood may have led to an antibody excess as numbers of antigenic sites might be expected to be lower in PPP.

We chose CD31 (PECAM), a shared platelet and endothelial antigen, to identify EMP as it is constitutively exposed on resting endothelial cells in comparison to antigens which in *in vitro* studies are only expressed on a subset of EMP following specific stimuli (Abid Hussein *et al*, 2003). CD31 and CD42 double staining has been employed in numerous *in vivo* studies to identify EMP (CD31+, CD42-) and PMP (CD31+, CD42+) (Table 3.1). In practice we identified some difficulties with the use of a shared endothelial and platelet antigen, to identify EMP in a situation where EMP were significantly rarer events than PMP. The IgG1 PE isotype control for the CD31 frequently showed significant, person specific, background staining. Correction of PMP and EMP numbers by subtraction of excess positive events in the isotype control may therefore have resulted in an absolute underestimation of both EMP and PMP. The significant difference in absolute EMP and PMP meant that in relative terms this had a much greater effect on EMP numbers and often produced negative values. In future, we would consider the use of more specific endothelial antigens, such as CD144, bearing in mind that these may only identify subsets of EMP released from activated endothelial cells (Abid Hussein *et al*, 2003).

In an attempt to address the problem of excess isotype staining we reanalysed the data using the values obtained for EMP before subtraction of the isotype control numbers. This resulted in an upward shift of all the EMP results in absolute terms but as it was applied to all the samples, relative differences were retained. This process also removed the negative values which are not biologically plausible and allowed more valid statistical comparisons to be made.

The glycophorin A antibody adequately stained red cells in whole blood and detected events less than 1μm. Similarly the CD45 antibody detected leucocytes in whole blood, however very few CD45 positive events of less than 1μm were detected in PPP. During analysis of the study samples, a consistent shift in the "negative" peak for the specific antibody was noted. We considered whether this may be due to true events especially as the mean fluorescence intensity of microparticle events is likely to be low due their size. However it is more likely that this was due to antibody excess as we performed a number of samples with a further 1:2 dilution of antibody which resulted in a loss of this phenomenon.

Another reason that we considered for the lack of CD45+ events in PPP was the sample preparation. It was proposed to us that the initial gentle centrifugation may not adequately separate leucocyte MP (pers comm, Amsterdam group), however this theory would suggest that they are a different size from other microparticles. In order to investigate this we prepared duplicate samples for a number of study subjects in which the initial centrifugation step was omitted. There was no significant difference in the results although the numbers were small.

We only used antibodies against cell specific antigens and therefore we did not assess the phospholipid properties of the microparticles. Annexin V binding is not universally employed as a criterion for microparticles (Jy *et al*, 2004) and some studies have suggested that it is not exhibited by all microparticles (as defined by size and antigen specificity) (Shet *et al*, 2003). However, as Annexin V binding would be expected over all microparticle subtypes, its use would have allowed us to assess the total number of microparticles. With the current method we were unable to do this satisfactorily due to difficulties distinguishing true events which were not stained with specific antibody, from electronic noise and debris at the lower limits of detection of the flow cytometer.

Analysis of microparticles – size discrimination

We analysed microparticle size using commercial size calibrated beads of 1μm. A cut off of 1μm is generally accepted but some groups use 0.5μm-1.5μm and others use comparison with the platelet population (see Table 4.4, Chapter 4). In our assays the events collected formed a continuous population of less than and greater than 1μm. In this case it would seem that an upper limit of 1μm is a somewhat arbitrary cut-off however the absence of clear separation of the populations made this the most consistent choice of method. It does however raise the question of what biological differences exist between the larger microparticles and smaller platelets in this crossover region.

Another "size issue" is whether the presence of exosomes can be excluded from the microparticle population as defined by size and antigen expression. This distinction may be important as exosomes differ from microparticles in their formation and constitution and are therefore likely to have different functional properties (Thery *et al*, 2002). Exosomes are generally considered to be 50-100 nm in size and standard flow cytometers are unable to resolve events of this size, however the presence of exosomes might affect functional assays dependent on their specific properties. Currently our understanding of the biology of exosomes remains limited and as more becomes known it will be important to consider their influence on our study of microparticles.

Summary

We have designed a flow cytometry assay for the detection and quantitation of plasma microparticles (platelet, endothelial, red cell and leucocyte) according to their antigenic phenotype and size. We found that the pre-analytical variable with the most significant influence on the assay results was the centrifugation technique used for the preparation of the platelet poor plasma. The effect of this and the resultant degree of platelet contamination was most important for the measurement of platelet microparticles in our assay, particularly because we did not identify a clear size

distinction between the microparticle and platelet populations. Further, the effect of even a small degree of platelet contamination such as was present in our samples may be significant if measuring the functional properties of microparticles in a coagulation assay using stored frozen samples. In the future, further optimisation of the assay by investigating the use of double-spun samples, alternative specific endothelial antibodies and double staining for Annexin V would be valuable.

CHAPTER 4

MICROPARTICLES IN MYELOPROLIFERATIVE DISORDERS

4.1 Introduction

Microparticles have diverse functions in coagulation and cellular interactions and numbers are elevated in a variety of conditions where vascular dysfunction and inflammation are important pathophysiological mechanisms. Quantitation of microparticles and identification of their cell of origin can therefore provide information about the state of the vasculature and disease mechanisms. In the MPD evidence exists for platelet, endothelial, leucocyte and coagulation abnormalities. However the pathogenesis of the prothrombotic state in MPD is not comprehensively established and normalisation of the abnormal peripheral blood counts does not abolish the thrombotic risk associated with these disorders. A more detailed review of the literature on microparticles and vascular abnormalities in MPD is provided in Chapter 1. The investigation of microparticles in MPD has been very limited to date and further study may enhance our understanding of the vascular abnormalities in MPD and may suggest potential pathogenic mechanisms to direct future investigation.

We have therefore measured platelet, endothelial, leucocyte, and red cell microparticles in patients with PV and PT compared to control subjects and have examined their associations with clinical and haematological parameters.

4.2 Materials and methods

4.2.1 Subjects

Recruitment of 19 PV and 59 PT patients and clinical data collection was carried out as detailed in Chapter 2. One patient was excluded from analysis due to pancytopenia as a result of transformation to myelofibrosis and in one patient no samples could be obtained. 30 healthy volunteers aged over 40 years and not on regular medications were recruited by advertisement across the hospital sites.

4.2.2 Sample collection and processing

Subjects attended on a single occasion and 15ml of blood was obtained by venepuncture with a 14G needle into citrate vacutainers (Becton Dickinson). Samples were processed within one hour and PPP was prepared. Measurement and analysis of microparticles was performed according to the protocol described in Chapter 3. Microparticle analysis failed in one patient. Microparticles results are reported as microparticles per microlitre of plasma (/μl).

4.2.3 Statistical analysis

Normally distributed data is reported as mean values and SEM. Between group comparisons were made by the student's t-test. Microparticle values and some haematological parameters were not normally distributed and values are therefore reported as median and IQR. Distribution of haematological parameters and microparticles is shown in Figures 4.1 and 4.2. Between group comparisons were made using the non-parametric Mann Whitney U tests. No correction was made for multiple comparisons. Associations between variables were analysed used Spearman's rank correlation. Multivariant analyses were by multiple regression analysis of data log transformed to a normal distribution. P values of <0.05 were considered to be statistically significant. All calculations and analyses were performed using Minitab 15 software (Minitab Inc, USA).

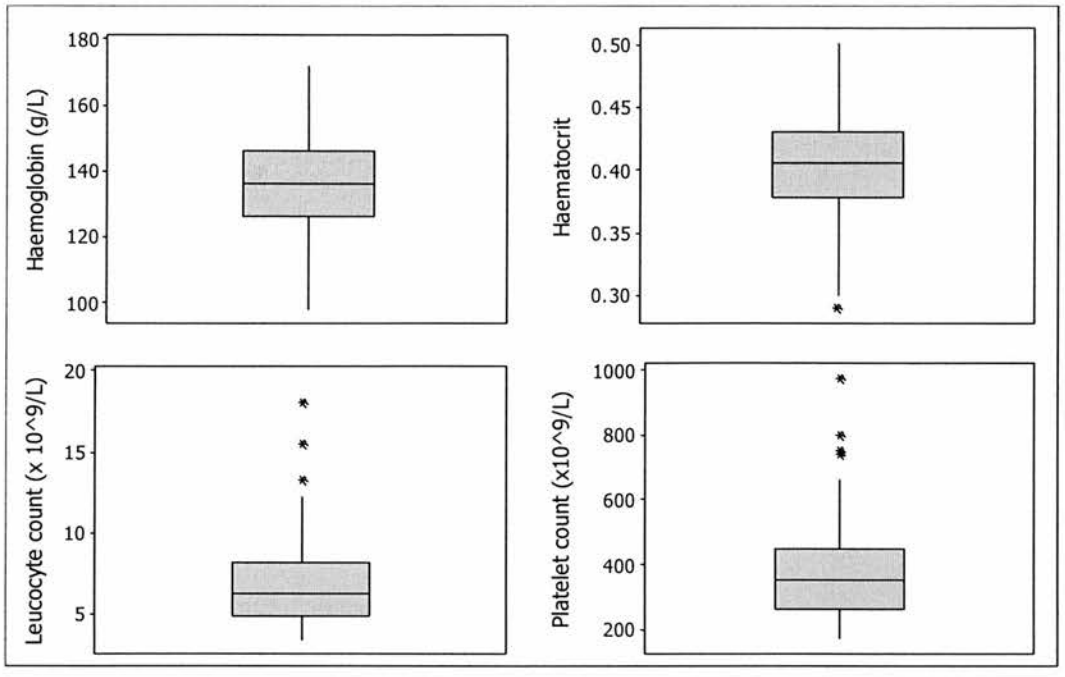


Figure 4.1 Distribution of haematological parameters

Box plots illustrating the normal distribution of haemoglobin and haematocrit values and the skewed distribution of leucocyte and platelet count values for the whole study cohort. * denotes outlier values

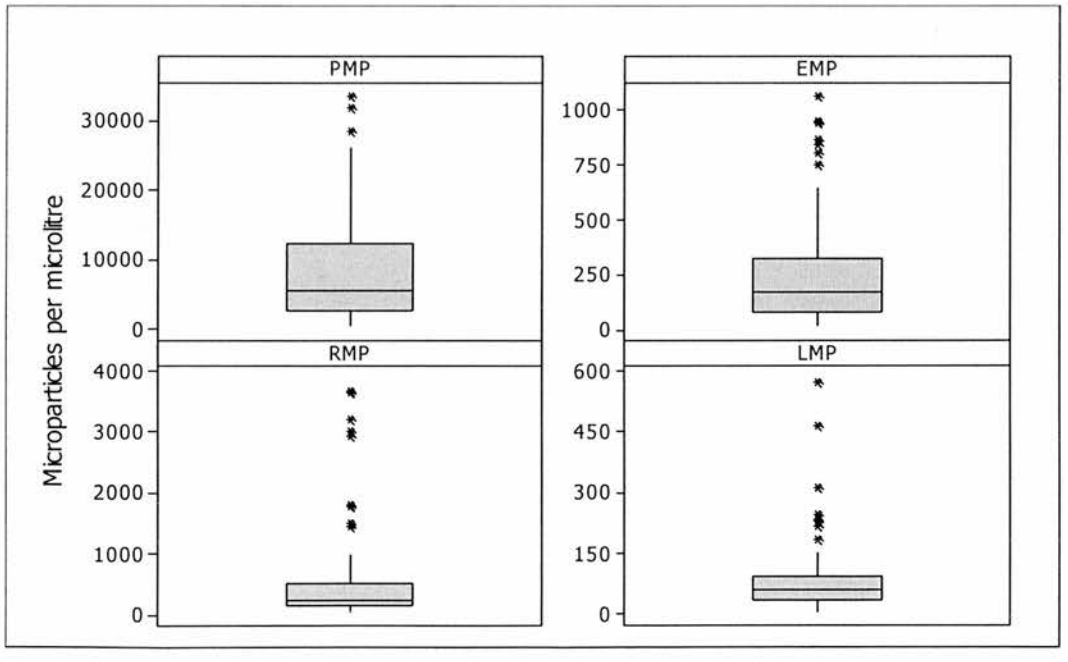


Figure 4.2 Distribution of PMP, EMP, LMP and RMP values

Box plots illustrating the skewed distribution of PMP, EMP, LMP and RMP, for the whole study cohort, in microparticles per microlitre. * denotes outlier values.

4.3 Results

4.3.1 Baseline characteristics

The demographic characteristics and haematological parameters of the study subjects are summarised in Table 4.1. MPD patients were significantly older than controls and had higher platelet counts. A comparison of the PV and PT patients is shown in Tables 2.2 and 2.3, Chapter 2; PT subjects were slightly older than PV subjects (66.0 v 64.3 years, p=0.663) and PV patients had a higher haematocrit (0.42 v 0.38, p=0.002) and leucocyte count (8.4 v 5.6 x 10^9/L, p= 0.018).

4.3.2 Microparticle results by diagnosis

Microparticle results are summarised in Tables 4.2a and 4.2b and Figure 4.3. PMP were significantly elevated in patients compared to controls. EMP were also significantly higher in patients than controls, by either method of EMP analysis (with or without subtraction of isotype control values, as discussed in Chapter 3). LMP were higher in patients than controls (p=0.061) but this difference only reached statistical significance for the PT patients (p=0.048). There was no difference in RMP between patients and controls. There was no significant difference between the PV and PT patients for any of the microparticle subgroups.

	Control subjects n = 30	MPD n = 75	P value
Male n (%)	9 (30.0)	32 (41.6)	0.379
Age (years) Mean (SEM)	49.4 (1.0)	65.0 (1.7)	**<0.001**
Haemoglobin (g/L) Mean (SEM)	137.4 (2.2)	135.1 (1.9)	0.431
Haematocrit Mean (SEM)	0.41 (0.005)	0.40 (0.006)	0.096
Leucocyte count x 10^9/L Median (IQR)	5.9 (5.0-7.0)	6.3 (4.9-8.6)	0.142
Platelets x 10^9/L Median (IQR)	237 (206-269)	397 (304-464)	**<0.001**

Table 4.1 Demographic and haematological parameters

Demographics and haematological parameters at study entry for patients and control subjects. Statistically significant differences are highlighted in bold.

	Controls n= 30	MPD n = 75	MPD v Controls P value
PMP/μl Median (IQR)	2069 (1122, 2980)	7974 (5041, 14438)	**<0.001**
EMP/μl Median (IQR)	66 (43, 117)	230 (133, 417)	**<0.001**
EMP (sub)/μl Median (IQR)	37 (21, 53)	132 (70, 288)	**0.002**
RMP/μl Median (IQR)	230 (155, 391)	283 (176, 553)	0.241
LMP/μl Median (IQR)	48 (31, 70)	64 (39, 108)	0.061

Table 4.2a

	PT n = 56	PV n = 19	PT v PV P value
PMP/μl Median (IQR)	8032 (5112, 14800)	7285 (3758, 13022)	0.345
EMP/μl Median (IQR)	245 (134, 447)	216 (118, 283)	0.235
EMP (sub)/μl Median (IQR)	162 (77, 403)	85 (25, 187)	0.096
RMP/μl Median (IQR)	272 (187, 550)	300 (129, 568)	0.478
LMP/μl Median (IQR)	66 (39, 112)	60 (27, 110)	0.646

Table 4.2b

Table 4.2 PMP, EMP, LMP and RMP quantitation

Results for quantitation of PMP, EMP with (EMP sub) and without subtraction of isotype control values, LMP and RMP in (a) patients compared to control subjects and (b) PT compared to PV patients. Statistically significant results are shown in bold.

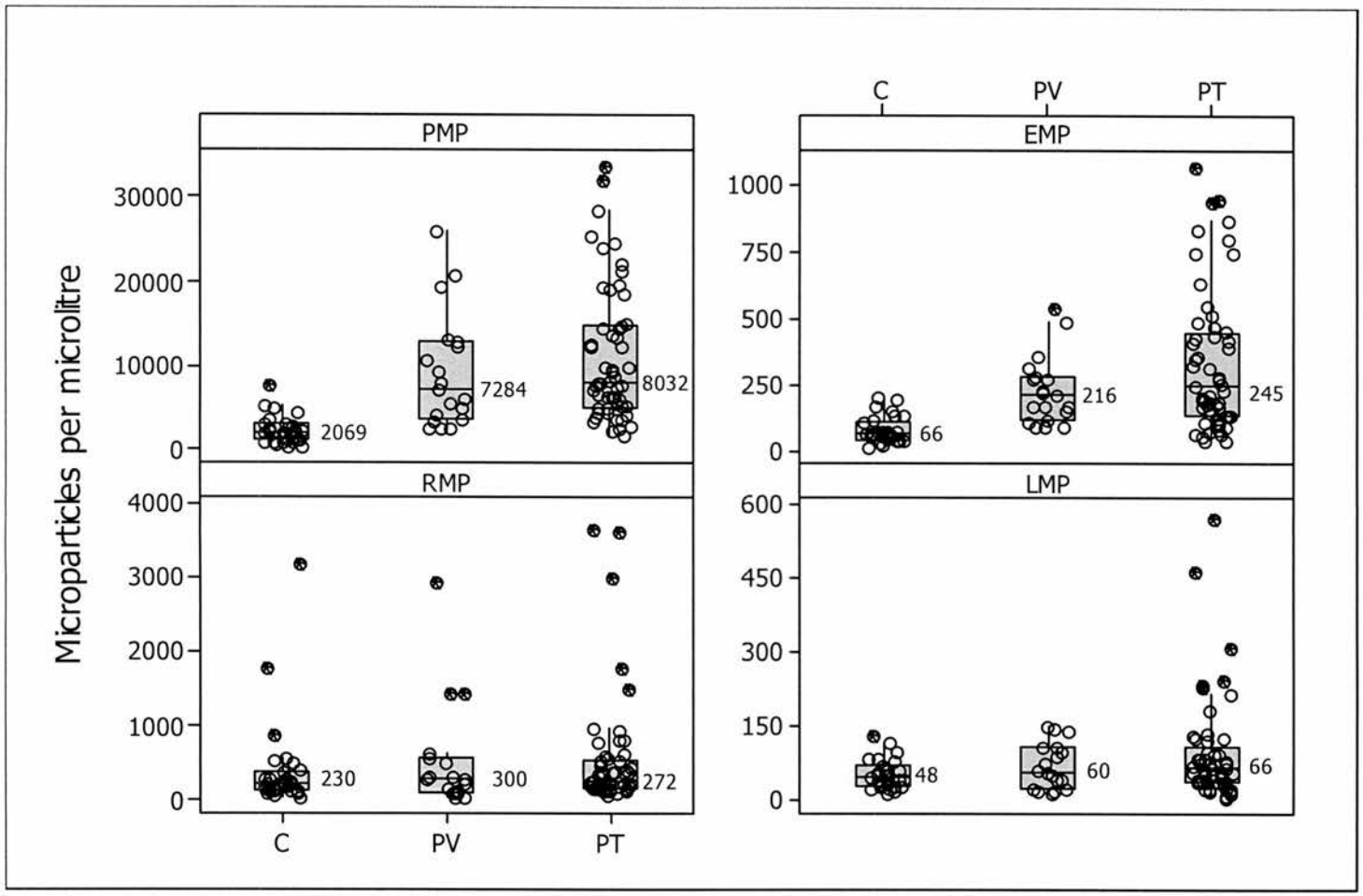


Figure 4.3 PMP, EMP, RMP and LMP in patients and controls

Box plots of PMP, EMP, RMP and LMP per microlitre in control subjects (C), PV and PT patients. Median values are shown. Circles denote individual values and *denotes outlier values.

4.3.3 Microparticles, gender and age

For the cohort as a whole, there were no significant differences in microparticle subgroups according to gender. The MPD patients were significantly older as a group than the control subjects by 15.2 years (95% CI 11.3-19.1 years) (Table 4.1). No significant correlations of microparticles with age, for either the control subjects or the patients, were detected by Spearman's rank testing (Figure 4.4). However, as the control group were all under 60 years, we divided the MPD group into those patients above (n=50) and below 60 years (n=27). The mean age (SEM) in the younger MPD group was 47.7 (1.7) years, similar to the control group 49.4 (1.0) years, (p=0.397). We then compared microparticle levels between the three groups (Figure 4.5).

PMP remained significantly higher in the younger MPD patients compared to controls, 6217/μl (4138, 8731) v 2069/μl (1122, 2980) respectively, (p<0.001). PMP were even higher in the older MPD group at 9873/μl (5150, 18916), (p=0.024) compared to the younger group. EMP remained elevated in the younger MPD patients compared to controls, 222/μl (135, 441) v 66/μl (43, 112) respectively, (p<0.001), but were not significantly different from the older MPD patients at 230/μl (120, 412), (p=0.898). There were no significant differences in RMP or LMP according to age group.

4.3.4 Microparticles and haematological parameters

There was no significant difference in haemoglobin or haematocrit between MPD patients as a whole compared to controls but haematocrit was higher in the PV patients compared to PT patients (p=0.02) (Table 2.3). The platelet count was higher in patients than controls (p<0.001) but was not significantly different between PV and PT patients (Table 4.1 and Table 2.3).

There were moderate but significant correlations between the peripheral platelet count and PMP ($r = 0.61$, $p<0.001$) and with EMP ($r = 0.497$, $p<0.001$) (Figure 4.6). As the peripheral platelet count was significantly higher in patients than controls, we also compared the ratios of PMP and EMP to peripheral whole blood platelet count. The PMP and EMP to platelet ratios were significantly higher in patients compared to controls; PMP to platelet ratio, 21.7 (13.2, 35.5) v 7.7 (5.2, 13.0) respectively and EMP to platelet ratio 0.55 (0.36, 0.96) v 0.25 (0.19, 0.40) respectively, ($p<0.001$ for both). The ratios were similar in PT and PV patients. There was a weak correlation between leucocyte count and LMP, $r=0.225$. There was no correlation between haematocrit and RMP, $r= 0.05$.

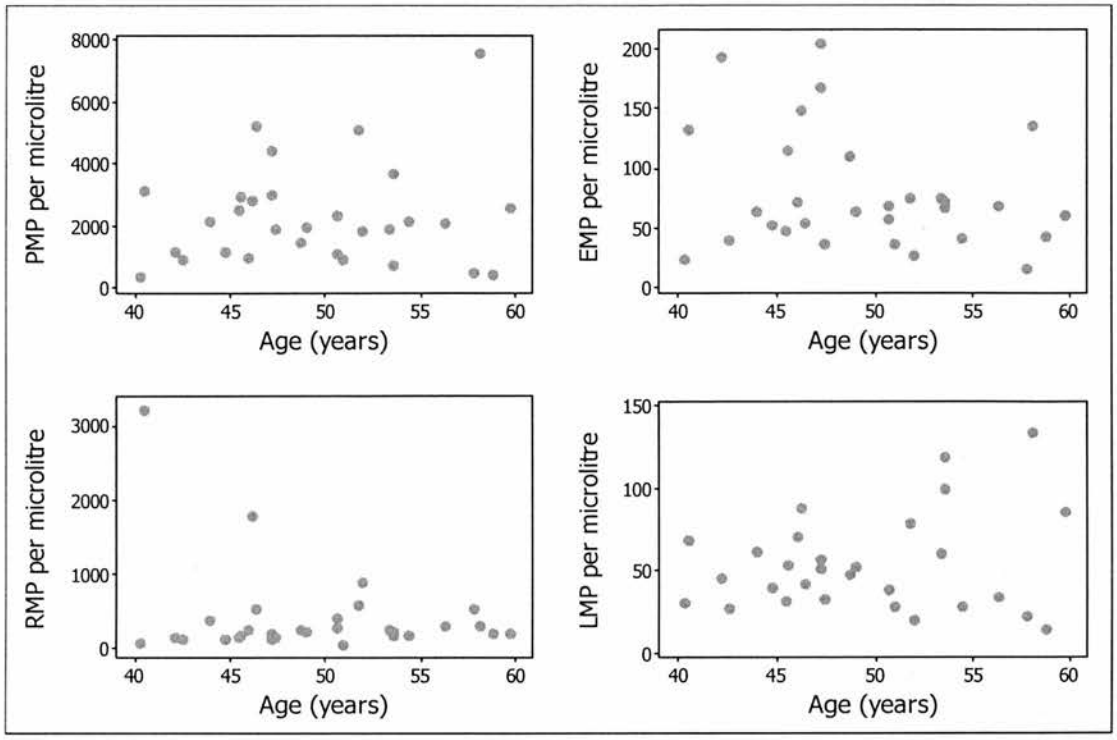


Figure 4.4a

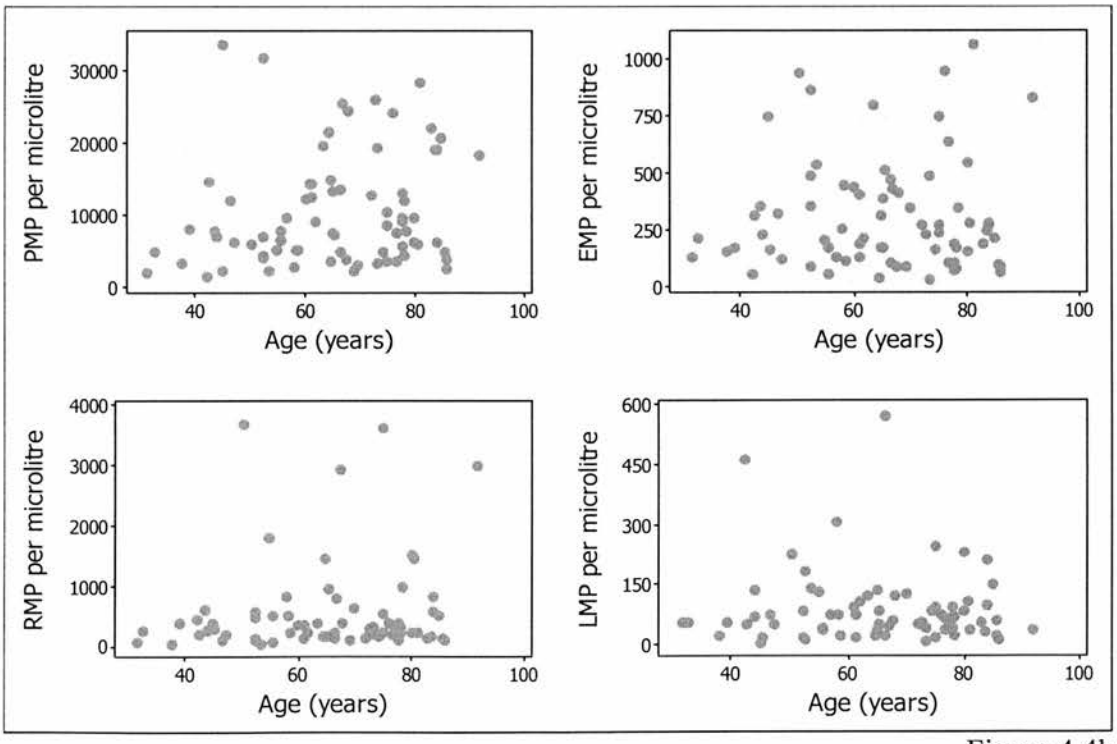


Figure 4.4b

Figure 4.4 Relationship of PMP, EMP, LMP, and RMP to age in (a) control subjects and (b) MPD patients.

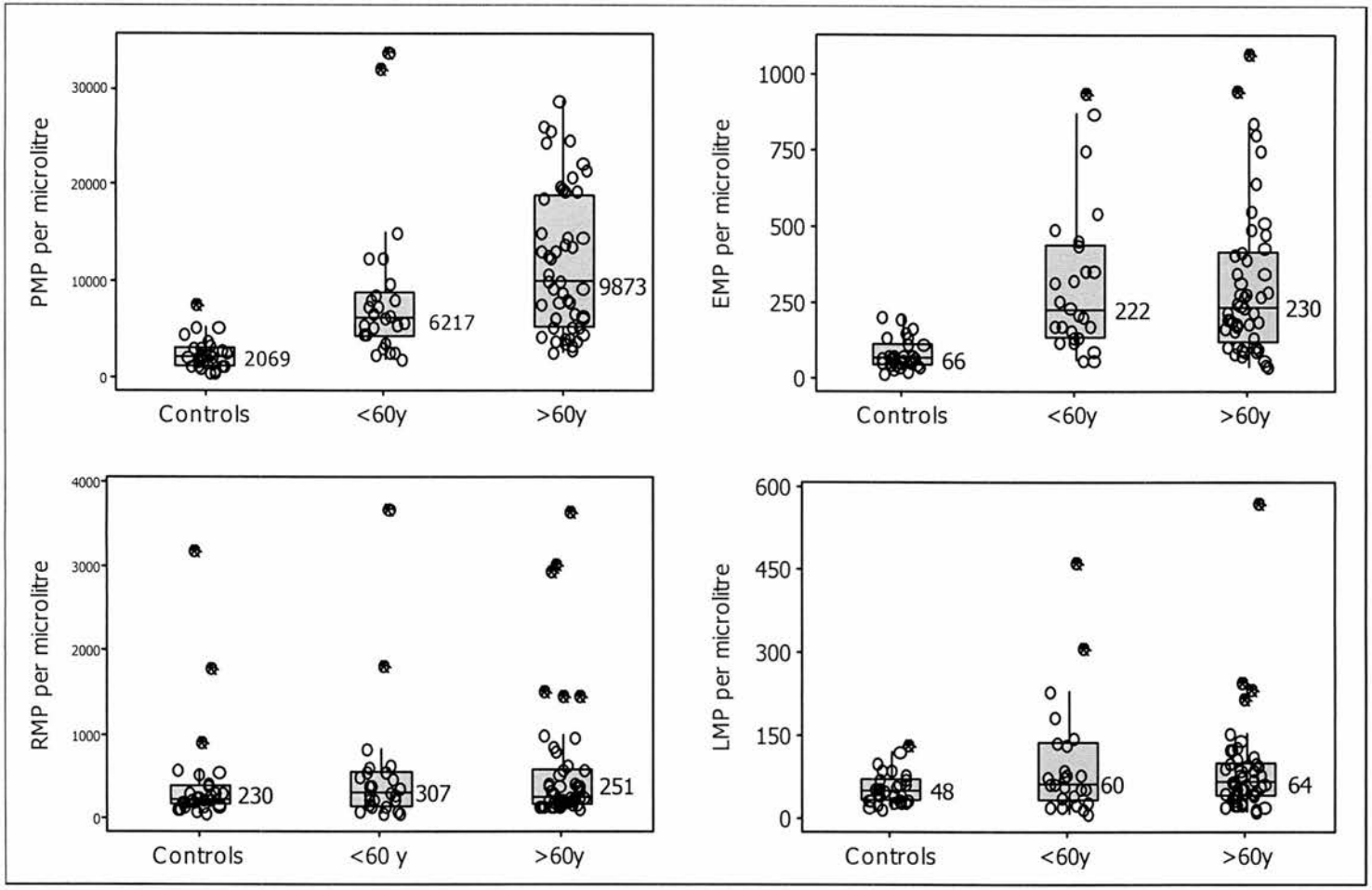


Figure 4.5 Microparticles in controls and MPD patients by age group

PMP, EMP, LMP and RMP in controls compared to MPD patients younger or older than 60 years of age. Circles denote individual values and *denotes outlier values. Median values are shown.

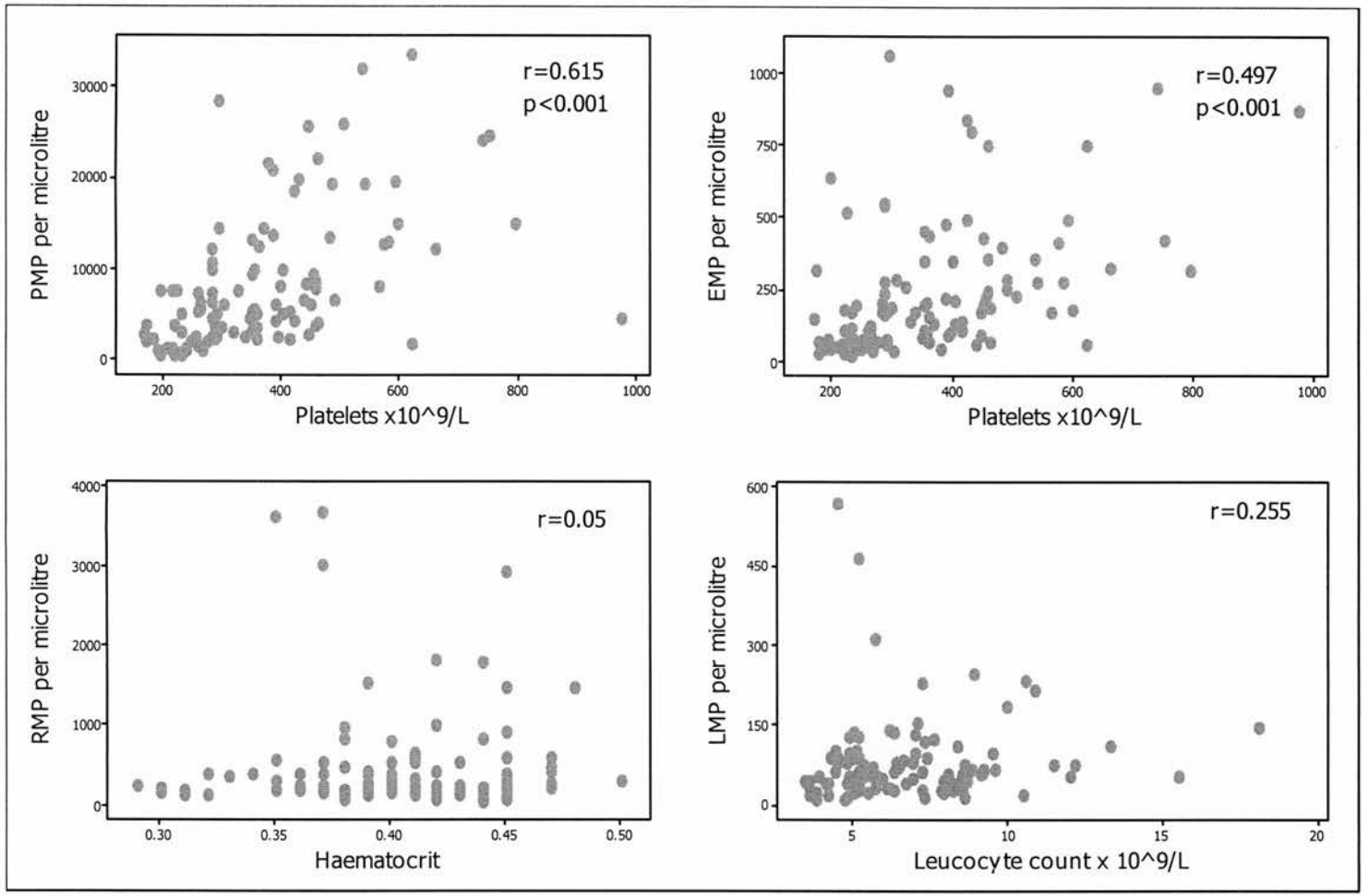


Figure 4.6 Associations between microparticles and haematological parameters

Associations between PMP and platelet count, EMP and platelet count, RMP and haematocrit and LMP and leucocyte count. r values for Spearman's rank correlation are shown.

4.3.5 Effect of residual platelet count

We also measured the residual platelet counts in the PPP. The median PPP platelet count was higher in patients than controls, 3 x 10^9/l (range 0, 23) v 1 x 10^9/l (0, 4), ($p<0.001$). There was no difference in PPP platelet counts between PV and PT patients ($p=0.91$). Comparison of the PMP to residual PPP platelet count ratios showed a higher ratio in the MPD patients compared to controls ($p=0.004$).

There were seven patient samples with a PPP platelet count of greater than or equal to 10 x 10^9/l. After exclusion of these samples, there was still a significant difference in PMP between patients and controls, 7249/μl (4447, 12825) v 2069/μl (1122, 2980), ($p<0.001$). Comparing only samples with PPP platelet counts of less than or equal to 2 x 10^9/l, the median PMP for the MPD patients was lowered at 5150/μl (3526, 8471) but remained twice that of the control group ($p<0.001$).

4.3.6 Microparticles and JAK2 V617F

JAK2 V617F status was known for 51/56 PT patients of whom 22/51 were positive for the mutation. There were no significant differences in microparticle numbers according to JAK2 V617F mutation status for any of the microparticle subgroups (Figure 4.7).

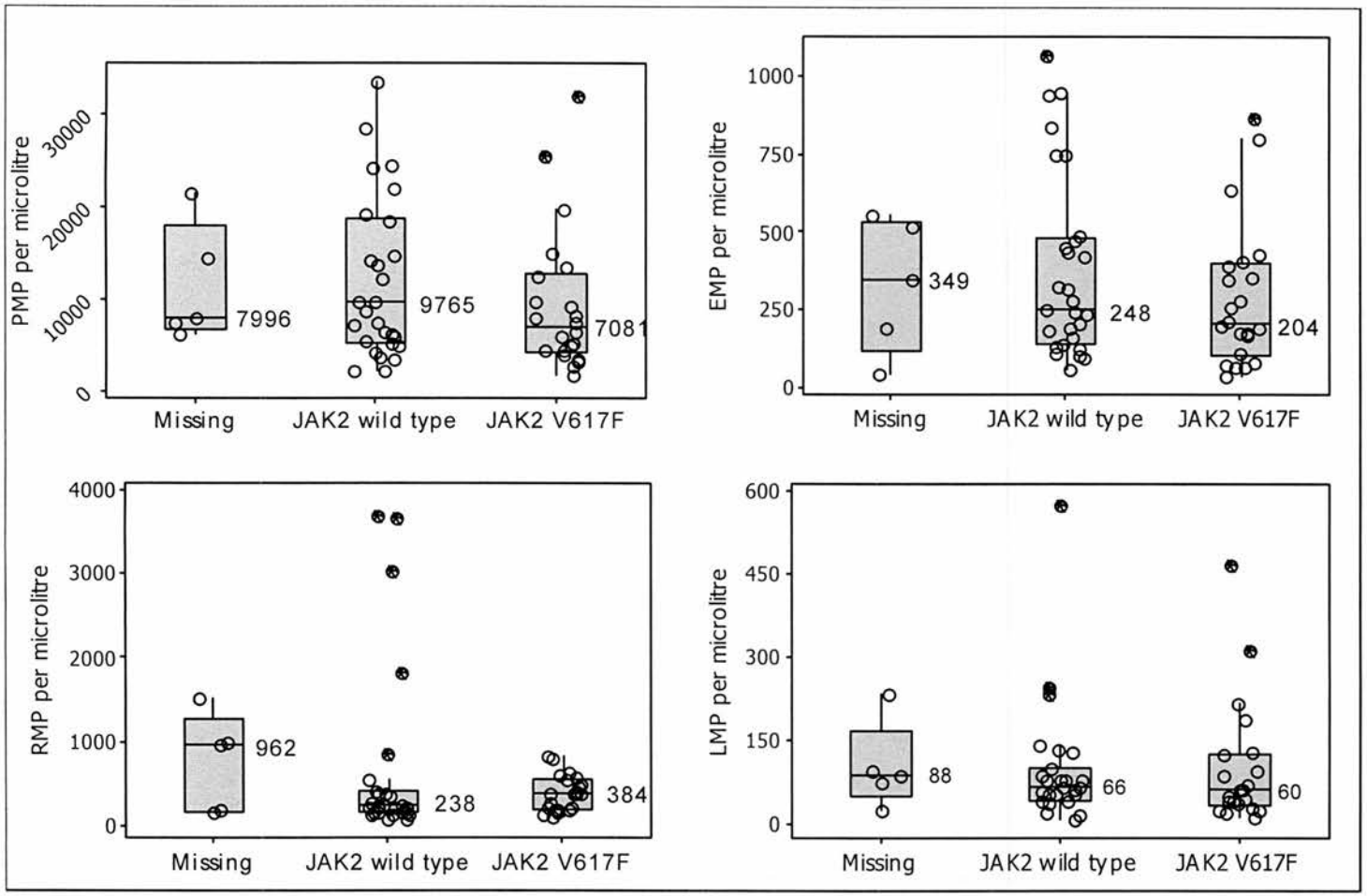


Figure 4.7 Microparticles in PT patients according to JAK2 mutation

PMP, EMP, RMP and LMP values in the PT patients according to JAK2 mutation status. No significant differences were observed. Circles denote individual values and *denotes outlier values. Median values are shown.

4.3.7 Microparticles, cardiovascular disease and thrombosis

The presence of CVRF or a history of CVD, VTE or microvascular symptoms were present in over half of the patient cohort and are discussed in detail in Chapter 2. We compared microparticle levels in the three groups detailed below in Table 4.3 and shown in Figure 4.8. PMP and EMP were higher in each of the three groups compared to controls, including PV and PT patients with no history of CVRF, CVD or thrombosis (all $p<0.001$). LMP were higher in patients with no history of CVRF, CVD or thrombosis 82/μl (45, 137) and in patients with CVRF only 76/μl (44, 128) than in controls 48/μl (31, 70), ($p=0.033$ and $p=0.058$ respectively). There was no difference for patients with a history of thrombosis or CVD compared to controls ($p=0.45$). RMP were not significantly different from controls in any of the patient groups. There were no significant differences in any of the microparticle subtypes between any of the patient groups (all $p>=0.1$).

	N
All patients	**75**
No thrombosis, CVRF or CVD	**27**
CVRF only	**15**
Thrombosis or CVD (+/- CVRF)	**33**

Table 4.3 MPD patients according to CVRF, CVD and thrombotic history

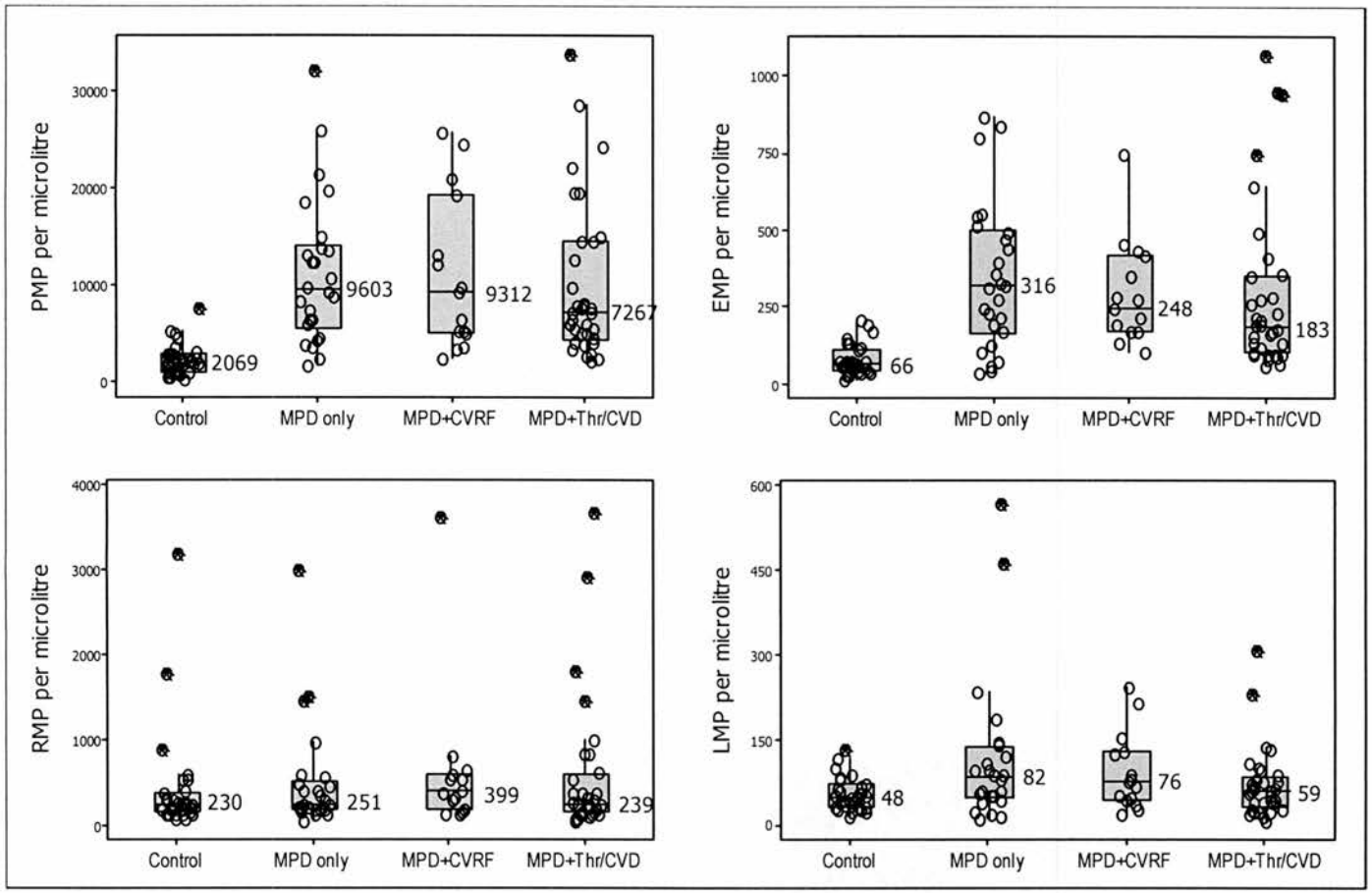


Figure 4.8 Microparticles in controls and patients according to vascular history

PMP, EMP, RMP and LMP in control subjects and patients with no history of thrombosis, CVRF or CVD (MPD only); patients with CVRF but no history of thrombosis or CVD (MPD+CVRF); and patients with a history of thrombosis or CVD (MPD+Thr/CVD). Circles denote individual values and *denotes outlier values. Median values are shown.

4.3.8 Microparticles and treatment

Drug therapy was known for 75 patients of whom 66 were treated with hydroxycarbamide. PMP were significantly higher in the patients treated with hycdroxycarbamide compared to not 9019 (5350, 14931) v 4368 (2417, 6037) /μl, (p=0.0017). There was no significant difference in peripheral platelet count between the two groups (p=0.85) but the treated group were significantly older with a mean age (SEM) of 67.1 (1.7) compared to 47.9 (3.8) years, (p=0.001). There were no significant differences in EMP, RMP or LMP (all p>0.2).

PMP were higher in patients on antiplatelet therapy compared to not, 8556/μl (5112, 14789) v 5391/μl (3723, 7974) but this was not statistically significant (p=0.095). There was no difference in microparticle levels for the 15/59 patients on statin therapy (all p>0.5).

4.3.9 Multivariant analysis

On multivariant analysis of the group as a whole, the presence of disease, platelet count and age were all significant independent predictors of PMP (p<0.001, p<0.001 and p=0.011 respectively). Disease was the strongest predictor for EMP (p<0.001) but platelet count was also of borderline significance (p=0.050). Within the patient group, platelet count was the strongest predictor of PMP (p<0.001) but age was also significant (p=0.012).

There were 11 patients below the age of 60 years with no history of vascular events or CVRF. Comparison of this group to the age matched control subjects revealed that both PMP and EMP were significantly elevated at 6566/μl (4368, 12439) v 2069/μl (1122, 2980) and 325/μl (122, 488) v 66/μl (43, 112) respectively both p<0.001). The platelet count was greater in the patients compared to controls, 445 x 10^9/L (362, 662) v 239 x 10^9/L (213, 271), (p<0.001) but on multivariate analysis the presence of disease was the only significant predictor of PMP and EMP.

4.4 Discussion

We have observed significantly elevated levels of PMP and EMP in patients with MPD compared to healthy controls. LMP levels were also higher but this was statistically significant in PT patients only and we found no difference in RMP from controls. The presence of MPD was an independent predictor of PMP and EMP; age and platelet count were also independent predictors for PMP. We found no correlation between microparticle levels and thrombotic history. These results are consistent with previous observations of platelet activation and endothelial disturbance in MPD and of elevation of microparticles in prothrombotic disorders where they may have a pathological role.

Microparticle levels in normal subjects

We observed similar proportions of microparticle subtypes in our healthy control subjects compared to previously published clinical studies with PMP accounting for the majority (70-80%) and lower numbers of RMP, EMP and LMP. The absolute PMP and EMP numbers in our healthy control subjects were intermediate compared to previously reported values (Table 4.4). There are a number of possible methodological differences which may account for this variation in absolute numbers including the mAb specificities used, the size criterion applied, quantitation method and sample preparation. In general, lower PMP and EMP values were reported from studies using either ultracentrifugation methods or which prepared PPP at higher centrifugation speeds compared to ours. In contrast, studies using slower or shorter centrifugation protocols reported higher EMP values. This difference could be confounded by their use of a larger size limit of 1.5μm but we found that changing the size limit had a minimal effect on EMP numbers. With regard to PMP, one study using slower, shorter centrifugation for PPP preparation, reported higher PMP numbers but in the study by Preston and colleagues, which used a protocol similar ours, PMP levels comparable to our own were observed.

Study	Preparation method	PMP per microlitre		EMP per microlitre	
		Marker	Median (IQR) or Mean (±sd)	Marker	Median (IQR) or Mean (±sd)
Berckmaans, 2001	Ultracentrifugation	CD61+	237 (116, 565)	CD62E+	64 (16, 136)
Peirera, 2006	Ultracentrifugation	CD61+	517 (±72)	CD146+	0.8 (±0.5)
Faure, 2005	1500g x15m, 13000g x 2m	CD41+	275 (±28)	CD144+	10 (±1.3)
Choudry, 2007	2000g x 20m	CD42+	680 (3360, 1170)	-	-
Tan, 2005	1500g x 20m	CD61+/42+	109 (53, 146)	-	-
Current study	**160gx10m, 1500g x 15m**	**CD31+/42+**	**2069 (1122, 2980)**	**CD31+/42-**	**66 (43, 117)**
Preston, 2005	1500g x 10m	CD31+/42+	2000	CD31+/42-	800
Chirinos, 2005	160 x10m, 1000g x 8m	CD31+/42+	5395	CD31+/42-	383 (24, 2043)
Gonzalez, 2004	160g x 10m, 1500g x 6m	-	-	CD31+/42-	6119 (±3592)
Jimenez, 2001	160g x 10m, 1500g x 6m	-	-	CD31+/42-	734 (±227)
Minagar, 2001	160g x 10m, 500g x 5m	-	-	CD31+/42-	870 (±270)

Table 4.4 Comparison of PMP and EMP results with published studies

Summary of absolute concentrations reported in various clinical studies for PMP and EMP in healthy subjects in comparison to the results from the current study. The method of sample preparation and the centrifugation protocol used for preparation of platelet poor plasma is shown along with the antibody specificities used. PMP and EMP per microlitre are reported as either median and IQR or mean and sd where available.

Platelet and endothelial microparticles

We observed around four-fold higher levels of PMP and EMP in MPD patients compared to controls, with similar levels in PT and PV patients. As microparticles are released following activating or injurious stimuli, these observations would be consistent with a number of reports of platelet activation and endothelial abnormalities in MPD including one study which measured PMP (Arellano-Rodrigo *et al*, 2006;Bellucci *et al*, 1993;Falanga *et al*, 2000;Jensen *et al*, 2000a;Musolino *et al*, 2002;Robertson *et al*, 2007;Villmow *et al*, 2002). However we also considered the effect of confounding factors in our study group including methodological factors. The patient group was significantly older than the control group, had a higher peripheral platelet count and approximately half of the patients had known cardiovascular comorbidities.

PMP were significantly higher in the older MPD patients (>60 years) compared to the younger group (<60 years) consistent with an effect of age on PMP. However both PMP and EMP remained significantly higher in the younger MPD group compared to the control subjects (age matched) excluding the possibility that difference between patients and controls was an effect of age alone.

We also considered the additional effect of cardiovascular co-morbidities in the patient group such as hypertension and IHD which are associated with increased microparticles (Bernal-Mizrachi *et al*, 2003;Mallat *et al*, 2000;Preston *et al*, 2003). We found no difference amongst the MPD patients dependent on a history of such co-morbidities. Further, an elevation in PMP and EMP compared to the age-matched healthy controls, was still present in younger MPD patients with no history of thrombosis, cardiovascular disease or risk factors (n=11). These results imply that the presence of PV or PT per se results in increased PMP and EMP independent of advancing age and cardiovascular co-morbidities. An increase of PMP with age has not been previously described and it would be useful to perform a comparison of microparticles in healthy older and younger people. That PMP were even higher in

older MPD patients may be consistent with the association of increased age with thrombotic risk.

PV and PT patients had similar platelet counts but these were significantly higher than in the control subjects. It may therefore be postulated that the increase in circulating PMP could be accounted for by similar levels of production but from a greater number of platelets. Consistent with this theory, PMP were significantly correlated with peripheral platelet count however the ratio of PMP to whole blood platelet count was significantly higher in patients than controls. Although overall platelet mass is therefore likely to be a contributing factor, these results would suggest a qualitative difference in the release of PMP from platelets in MPD patients perhaps due to increased platelet activation. Concomitant measurement of microparticle P-sel levels may have provided additional useful information in this respect.

Another explanation might be sampling differences. We treated the control and patients samples in exactly the same ways in order to minimise any such differences. However it was noted that despite identical sample handling, the measured residual platelet count in the PPP samples was significantly higher in patients than controls. As we observed a continuum between PMP (<1μm) and small platelets (>1μm), greater numbers of small platelets included in the PMP gate might explain the difference in PMP between patients and controls. To investigate this further we analysed the PMP to PPP platelet count ratios and these were higher in patients than controls. Further, when PPP samples with residual platelet counts of over 10 or even 2×10^9/l were excluded from the analysis the median PMP levels remained higher in patients than controls although the median values were lowered. This suggests that differences in PPP quality do not fully account for the differences in PMP. However, even using the widely accepted size criterion of <1μm, residual platelets in the PPP sample may influence PMP results and this may be even more significant in studies using slower centrifugation protocols.

EMP levels were also correlated with platelet count. A number of possible explanations for this might be considered. A higher platelet count particularly with platelet activation may promote more platelet-endothelial cell interactions with resultant endothelial cell activation or damage and microparticle release (Burger & Wagner, 2003). Similarly PMP have themselves been reported to induce endothelial cell changes *in vitro* (Barry *et al*, 1997;Barry *et al*, 1998) and the correlation of PMP with platelet count may therefore be a confounding factor. Methodological issues may also be important; we measured EMP by CD31 (PECAM) positivity which is an adhesion molecule constitutively expressed by both endothelial cells and platelets. In addition we used CD42 negativity to differentiate EMP from PMP. It is possible that use of this shared marker in a situation where PMP comprised the vast majority of events detected may have artefactually influenced EMP detection. Against this, in one patient (excluded from the analysis) who had developed clinical myelofibrosis with severe thrombocytopenia, EMP numbers were significantly higher than PMP. In future the use of an exclusive endothelial antigen such as CD144 may clarify this.

Although the effect of these potential confounding factors should be borne in mind, multivariate analysis of our data supported the evidence that disease was independently associated with both PMP and EMP, and additional independent associations of age and platelet count on PMP.

Leucocyte microparticles

In view of more recent work reporting increased leucocyte activation and PLA in MPD and the association of leucocyte count with thrombosis, we also investigated LMP in MPD patients (Carobbio *et al*, 2007;Falanga *et al*, 2000;Falanga *et al*, 2005;Jensen *et al*, 2001;Landolfi *et al*, 2007). In the control patients LMP were observed at similar levels to EMP. We found that LMP were elevated in MPD patients compared to controls with similar levels in the PV and PT patients. However the difference from controls was only significant in PT patients, probably due to the combined effects of a relatively small difference and smaller numbers of

PV patients. There was no correlation between LMP and age but a weak correlation with leucocyte count was observed. LMP were highest in patients with no history of cardiovascular disease, risk factors or thrombosis although this did not reach statistical significance compared to patients with such a history. We considered that this difference might be explained by higher leucocyte counts in low risk patients not treated with hydroxycarbamide. Both the leucocyte count and LMP were higher in the non-hydroxycarbamide treated group but these differences were not statistically significant.

Leucocyte and in particular monocyte microparticles are recognised to be an important source of TF and their recruitment to areas of vascular damage or aggregates of activated platelets may contribute to localised thrombin generation (Falati *et al*, 2003;Shet *et al*, 2003). Further investigation of these findings in a larger patient cohort would be useful to clarify whether a significant difference exists. It would also be of interest to measure LMP in high risk patients at presentation and serially following commencement of hydroxycarbamide therapy. We measured LMP using the pan-leucocyte antigen CD45 in order to detect both monocyte and neutrophil microparticles but specific antibodies for each and double staining for TF may be more informative.

Red cell microparticles

We observed no difference in RMP between patients and controls or according to clinical or haematological parameters. In contrast a study, published only in abstract form, reported higher levels of all microparticle subtypes including RMP in MPD compared to controls with PMP accounting for the majority (Fontana *et al*, 2006). They also observed higher levels of RMP in patients with a history of thrombosis compared to without and levels were associated with increased red cell distribution width and length of disease. We did not collect data on red cell distribution width but did not identify a positive correlation of RMP with length of disease. A number of our patients and controls had RMP results significantly outwith the interquartile

range. In one case this was associated with sample haemolysis but this did not appear to affect any of the other samples and the reason for these outlying results is unclear.

Other factors influencing microparticle numbers

We observed no positive correlation between any microparticle subgroup and either thrombotic history or the presence of cardiovascular disease or risk factors – one or more of which were present in around two-thirds of the MPD patients. Blood pressure has previously been correlated with increased PMP and EMP but the difference from controls was statistically significant in severe hypertension only and not in mild disease (Preston *et al*, 2003). Similar to the current observations, patients with CRF have elevated microparticle levels compared to controls but no difference was observed between patients according to history of previous vascular events (Faure *et al*, 2006). It may be that additional increments in circulating microparticles above those associated with steady state disease only occur in the acute setting. This would be supported by the finding of higher microparticle numbers in acute coronary syndrome compared to stable angina (Bernal-Mizrachi *et al*, 2003;Mallat *et al*, 2000). In addition, the lack of a detectable correlation of microparticle numbers with previous thrombosis may not be surprising given the variable correlation reported for other markers of platelet and endothelial activation in MPD. Clearly, the small numbers in this study and others may be insufficient to detect relatively small differences.

We did not detect any difference in microparticle numbers, of any subtype, in PT patients in relation to JAK2 V617F mutation status. No clear predictive value of JAK2 V617F in thrombotic risk has been established although some studies have shown an association with venous thrombosis (Campbell *et al*, 2005;Kittur *et al*, 2007). Further, the differences in haematological parameters at diagnosis associated with JAK2 V617F, such as increased leucocyte count and elevated haematocrit, are generally attenuated by treatment effects. Thus in treated patients we might not

expect to see a differential effect of JAK2 V617F on parameters of vascular activation. However, two recent studies have reported an association between JAK2 V617F and increased markers of platelet activation (Arellano-Rodrigo *et al*, 2006;Robertson *et al*, 2007). In contrast we did not find an association with PMP, which are also likely to reflect platelet activation. Differences in study design and the small numbers involved may account for this discrepancy with as neither did we detect such differences in other platelet activation markers (see Chapter 5).

Microparticle binding to other cellular elements is a further factor which may affect the measurement of microparticles by reducing the numbers circulating in cell free samples. Platelet binding to activated leucocytes is now well established in MPD and in other vascular disorders (Falanga *et al*, 2005;Jensen *et al*, 2001). It is therefore possible that PSGL-1 positive leucocyte microparticles may also bind activated platelets or endothelium, as are observed in MPD, thus reducing the circulating numbers. Similarly, leucocyte positivity for platelet antigens may in fact be partly due to PMP binding and this has been shown by electron microscopy in patients on cardio-pulmonary bypass (Chung *et al*, 2007). In acute DVT, where a marked increase in EMP was seen there was also noted to be increased leucocyte activation and EMP-monocyte conjugates (Chirinos *et al*, 2005).

Summary

We have observed elevated PMP, EMP and LMP in patients with MPD even in the absence of co-morbid cardiovascular disease. These findings support the existing evidence for platelet, endothelial and leucocyte abnormalities in MPD. Microparticles may therefore be a useful marker for in vascular disturbance in MPD. In light of the known prothrombotic effects of microparticles the results reported may be a newly recognised mechanism contributing to thrombotic risk in these diseases. PMP can increase platelet aggregation and both PMP and EMP may induce endothelial dysfunction and increased expression of endothelial molecules. All microparticles may promote thrombin generation by providing a suitable

phospholipid surface and microparticle TF may also contribute to coagulation activity. Current treatment of MPD reduces but does not remove the associated prothrombotic risk and it is of note that markers of platelet, endothelial and coagulation activation remain elevated in treated patients. The current observation of elevated microparticles in MPD patients despite standard treatment may suggest an additional therapeutic target for reduction of thrombotic risk.

CHAPTER 5

VASCULAR MARKERS IN MYELOPROLIFERATIVE DISORDERS

5.1 Introduction

The pathogenesis of thrombosis in MPD is has not been comprehensively elucidated but previous studies have identified platelet activation, endothelial dysfunction and coagulation activation as potential contributing factors (Arellano-Rodrigo *et al*, 2006;Falanga *et al*, 2000;Musolino *et al*, 2000;Robertson *et al*, 2007). Correlation of these parameters with patient characteristics, in particular thrombotic history, has however been inconsistent. In other prothrombotic disorders correlation between levels of platelet and endothelial microparticles and more established markers of platelet, endothelial and coagulation activation have been reported (Koga *et al*, 2005;Nomura *et al*, 2005;Ogata *et al*, 2006;Shet *et al*, 2003).

The selectins are cell adhesion molecules which mediate leukocyte, platelet and endothelial interactions via binding with specific carbohydrate ligands. Their biology is comprehensively reviewed by Polgar (P-selectin) and Roldan (E-selectin) (Polgar *et al*, 2005;Roldan *et al*, 2003). P-sel is contained in the membrane of platelet α-granules and in the Weibel-Palade bodies of endothelial cells. On platelet activation, the α-granules fuse with the cell membrane and release their contents and P-sel is translocated to the platelet surface. Its major receptor is PSGL expressed mainly by leucocytes. P-sel - PGSL interactions have a key role in leucocyte rolling on endothelium and dimeric or oligomeric forms of P-sel may induce cross-linking of leucocyte PSGL and subsequent signal transduction. PSGL is also expressed by platelets to a lesser extent and, along with the VWF receptor GPIbα, another counter-receptor for P-sel, can mediate platelet rolling on activated endothelium. Soluble P-sel can also be detected in plasma and is elevated in prothrombotic disorders and cardiovascular diseases (Polgar *et al*, 2005). There appear to be a number of mechanisms for the production of sP-sel including secretion of an alternatively spliced molecule lacking the transmembrane domain and cleavage of surface P-sel from activated platelets (Ishiwata *et al*, 1994;Michelson *et al*, 1996).

E-sel is expressed specifically on the surface of stimulated endothelial cells unlike other soluble endothelial adhesion molecules such as VCAM and ICAM-1 which can

be expressed on a variety of cells including leucocytes. Its counter receptors are PSGL and endothelial selectin ligand 1 (ESL-1) and thus, like P-sel, it is involved in leucocyte rolling on endothelium. Soluble E-sel is most likely generated by enzymatic cleavage from its membrane insertion, consequent on cytokine stimulation of endothelial cells. From *in vitro* studies of cytokine activated HUVEC, sE-sel correlates with endothelial surface expression of E-sel. It is therefore postulated that *in vivo,* sE-sel levels reflect the presence of either systemically or locally activated/damaged endothelium. Elevated levels of sE-sel are reported in some clinical studies of patients with cardiovascular risk factors and overt disease but there is a lack of consensus, with other studies reporting no differences from controls. Whether sE-sel has any active pathogenic role in these disorders is unclear (Roldan et al, 2003).

The degree of activation of the coagulation system can be measured *in vivo* using a number of components of the system as markers (Boisclair et al, 1990). During the generation of thrombin by activated Factor X, prothrombin is cleaved to produce activation fragments (PF 1+2) which can be measured in plasma as an indication of *in vivo* thrombin generation. Following the activation of thrombin it becomes irreversibly bound to the proteinase inhibitor antithrombin. Levels of these thrombin-antithrombin (TAT) complexes therefore reflect thrombin production and inhibition *in vivo*. Activation of the fibrinolytic system can be monitored by measurement of specific fibrin degradation products such as D-dimers, which are released following lysis of cross-linked fibrin by plasmin. Each of these by-products of coagulation activation or fibrinolysis have been reported to be elevated in hypercoagulable states such as disseminated intravascular coagulation and VTE (Bozic *et al*, 2002;Levi *et al*, 2002).

We have measured sP-sel, sE-sel and PF 1+2 in MPD patients and controls and correlated these with clinical and haematological parameters in the MPD patients. We have also assessed the relationship of these parameters to plasma microparticle levels.

5.2 Materials and Methods

Patient recruitment and data collection were performed as detailed in Chapter 2.

5.2.1 Sample collection and processing

Subjects attended on a single occasion and 15ml of whole blood was obtained by venepuncture with a 14G needle and collection into citrate vacutainers (Becton Dickinson). Samples were processed within one hour and PPP was prepared according to the protocol described in Chapter 3. Samples were aliquoted and frozen at -80^{o}C until analysis.

5.2.2 Assays

Soluble P-sel (CD62P) and E-sel (CD62E) were measured by enzyme immunoassay (Quantikine assay, R and D Systems, Minneapolis). PF1+2 were measured by enzyme immunoassay (F1+F2 microassay, Sysmex).

5.2.3 Statistical analysis

Normally distributed data is reported as mean values and SEM. Between group comparisons were made by the student's t-test. sP-sel, sE-sel and PF 1+2 results and some haematological parameters were not normally distributed and values are therefore reported as median and IQR. Between group comparisons were made using the non-parametric Mann Whitney U tests. No correction was made for multiple comparisons. Associations between variables were analysed used Spearman's rank correlation. Multivariant analysis was by multiple regression analysis of data log transformed to a normal distribution. P values of <0.05 were considered to be statistically significant. All calculations and analyses were performed using Minitab 15 software (Minitab Inc, USA).

5.3 Results

5.3.1 Normal ranges and control subjects

The results for sP-sel, sE-sel and PF 1+2 in the control subjects are shown in Table 5.1. The normal range quoted by the manufacturers for sP-sel is 18-40ng/ml. The upper limit for our control group was 91.2ng/ml, with 16/30 controls above 40ng/ml. Our normal range based on the control subjects was 28.4-66.3ng/ml (mean ± 2sd). The manufacturer normal range for sE-sel is based on serum samples and a lower normal range would be expected from citrated plasma, consistent with our results. We observed a similar range for PF 1+2 in our control samples to that quoted by the manufacturer (76-293 and 69-229 pmol/L respectively).

5.3.2 sP-selectin, sE-selectin and PF 1+2 by diagnosis

The results for sP-sel, sE-sel and PF 1+2 in the MPD patients are shown in Table 5.1 and Figure 5.1. sP-sel was elevated in all MPD and each of the PV and PT groups compared to controls (all $p<0.001$) and was significantly higher in the PV group compared to the PT group ($p=0.020$). 71/73 patients had sP-sel levels greater than 40ng/ml and 54/73 patients had sP-sel levels above the upper limit of the normal range for the control subjects. There was no significant difference in sE-sel levels in the MPD group compared to controls but sE-sel was higher in the PV patients compared to PT patients ($p=0.037$). PF 1+2 were elevated in the MPD patients compared to controls ($p<0.001$).

	Controls n = 30	MPD n = 74	MPD v Controls P value
sP-selectin (ng/ml)	42.5 (34.1, 53.6)	86.5 (65.3, 107.0)	**<0.001**
sE-selectin (ng/ml)	25.1 (20.0, 29.7)	24.7 (20.1, 31.5)	0.802
PF 1+2 (pmol/L)	151.5 (115.8, 203.5)	238.5 (151.0, 330.3)	**0.002**

Table 5.1a

	PT n = 56	PV n = 18	PT v PV P value
sP-selectin (ng/ml)	82.1 (64.8, 99.9)	106.0 (71.2, 134.8)	**0.020**
sE-selectin (ng/ml)	24.2 (19.6, 28.5)	30.0 (22.0, 40.4)	**0.037**
PF 1+2 (pmol/L)	251.5 (147.0, 352.3)	212.0 (144.3, 251.0)	0.176

Table 5.1b

Table 5.1 sP-sel, sE-sel and PF 1+2 results for patients and controls

Results (median and IQR) for sP-sel, sE-sel and PF 1+2 in (a) patients compared to control subjects and (b) PT compared to PV patients. Statistically significant results are shown in bold.

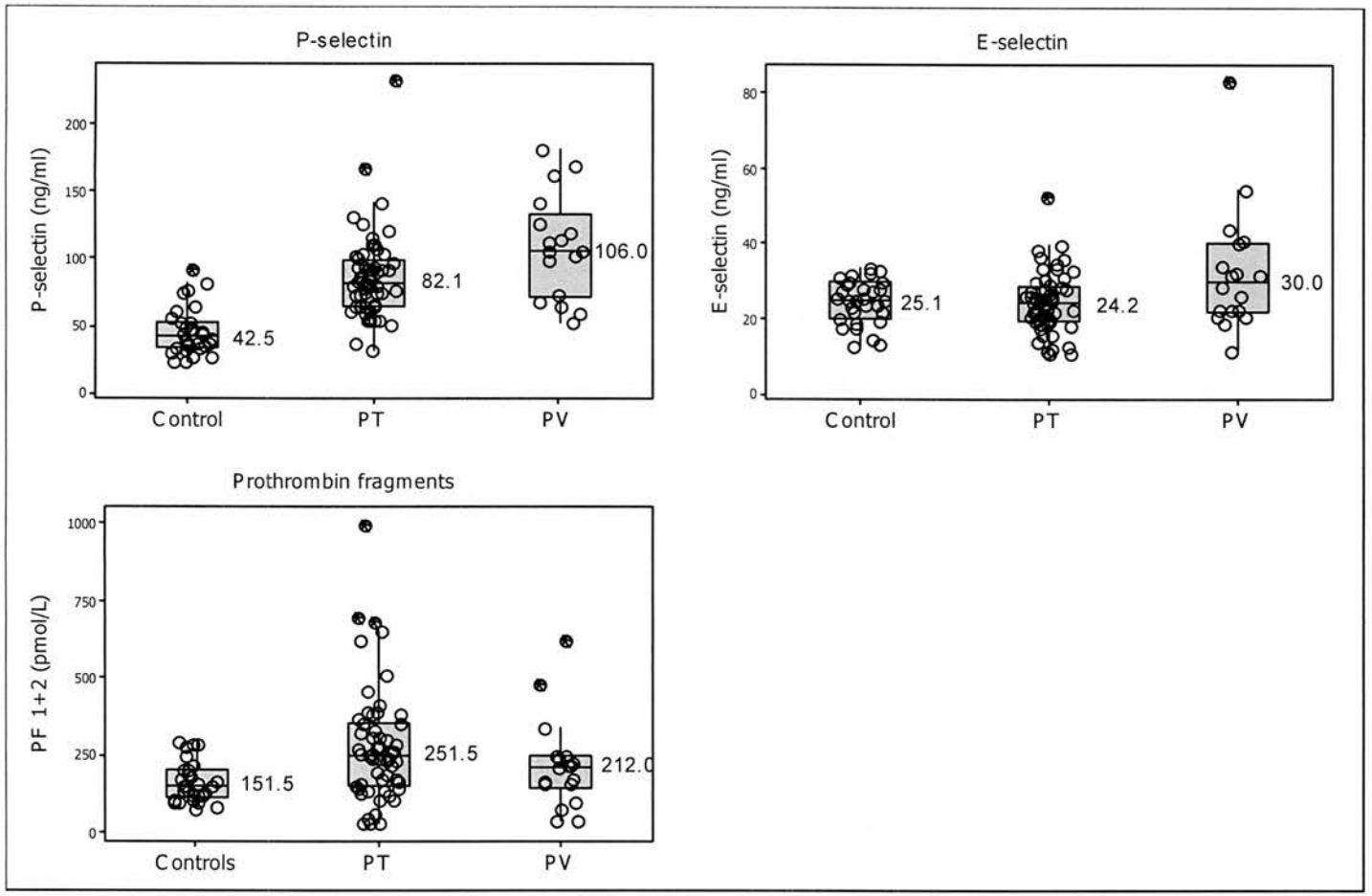


Figure 5.1 sP-sel, sE-sel and PF1+2 results for patients and controls

sP-selectin, sE-selectin and PF1+2 results in control subjects and patients with PT or PV. Circles denote individual values and *denotes outlier values. Median values are shown.

5.3.3 Vascular markers, age and gender

A sex specific difference was apparent in PF1+2 results with higher levels being observed in women than men, 238.0 (152.0, 320.3) v 160.0 (106.0, 233.0) pmol/L, (p=0.004). There were no significant differences according to gender between sP-sel (p=0.422) or sE-sel (p=0.331).

The MPD patients were significantly older than the control subjects by 15.2 years (p<0.001) (Table 4.1, Chapter 4). There were moderate correlations between age and both sP-sel and PF1+2 (r =0.357, p=0.001 and r =0.413, p<0.001) but not sE-sel (r= -0.156, p=0.116). As the control group were all under 60 years, we divided the MPD group into those patients above (n=50) and below (n=27) 60 years. The mean age (SEM) in the younger MPD group was comparable to the control group 47.7 (1.7) years v 49.4 (1.0) years, (p= 0.397).

Comparing the MPD patients above and below 60 years of age there was no difference in sE-sel or sP-sel levels (p=0.224 and p=0.742) (Figure 5.2). sP-sel levels were significantly higher in each age group compared to controls (p<0.001 for both). PF1+2 were significantly higher in the older patients compared to those below 60 years, 251.0 (3173.0, 357.3) v 167.0 (112.8, 277.3) pmol/L (p=0.040). There was no significant difference in PF1+2 levels between the younger patients and the control group (151.5 (115.8, 203.5) pmol/L), (p=0.352) (figure 5.2).

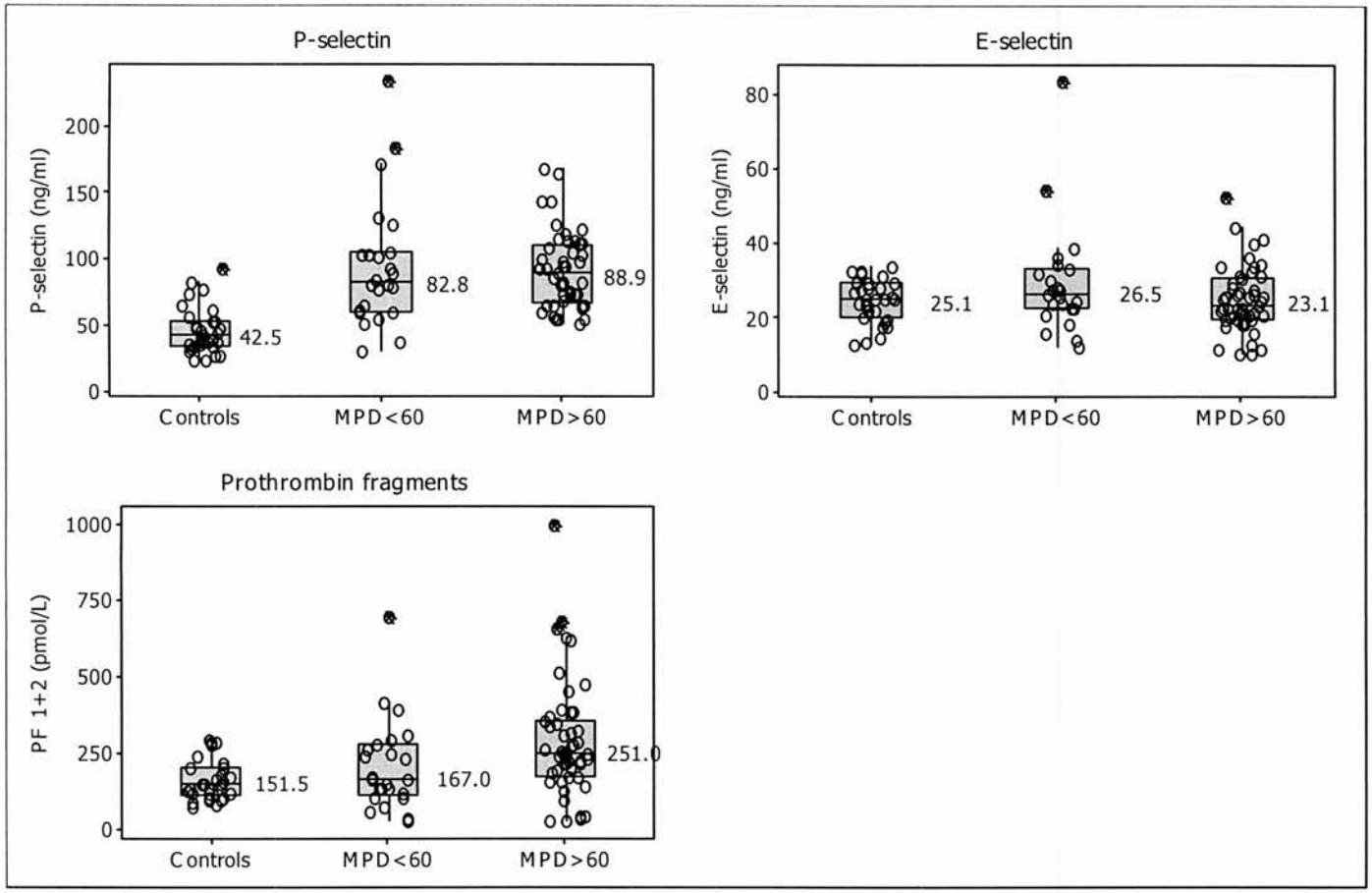


Figure 5.2 sP-sel, sE-sel and PF1+2 in controls and MPD patients by age

sP-sel, sE-sel and PF1+2 results in control subjects and MPD patients older or younger than 60 years. Circles denote individual values and *denotes outlier values. Median values are shown.

5.3.4 Vascular markers and haematological and molecular parameters

There were weak but significant correlations between leucocyte count and both sP-sel ($r = 0.279$, $p=0.005$) and sE-sel ($r = 0.275$, $p=0.005$). There was a moderate correlation between the platelet count and sP-sel levels ($r = 0.474$, $p<0.001$). The ratio of sP-sel to platelet count was higher in PV patients compared to controls ($p=0.002$) or PT patients ($p=0.004$) but there was no significant difference between PT patients and controls ($p=0.293$), (Figure 5.3). There were no statistically significant differences in sP-sel, sE-sel or PF 1+2 between JAK2 V617F positive and negative PT patients (Table 5.2).

5.3.5 Vascular markers, cardiovascular disease and thrombosis

Within the MPD patients there was no significant difference between sP-sel, sE-sel or PF1+2 according to history of CVRF, CVD or other thrombosis (all $p>0.1$) (Table 5.3 and see Table 4.3, Chapter 4 for breakdown of patient groups).

5.3.6 Vascular markers and treatment

Current treatment was known in 73 patients. 60 patients were on aspirin and a further three patients were on clopidogrel. There was no significant difference in sP-sel levels according to aspirin or any antiplatelet therapy ($p=0.34$ and $p=0.11$ respectively) (Figure 5.4). There were no significant differences in sP-sel or sE-sel in patients currently treated with hydroxycarbamide (64/73) or not ($p>0.2$). PF1+2 were lower in patients not on hydroxycarbamide ($p=0.037$) but this group were of a younger age (mean 47.9 v 67.1 years, $p<0.001$) (Figure 5.4).

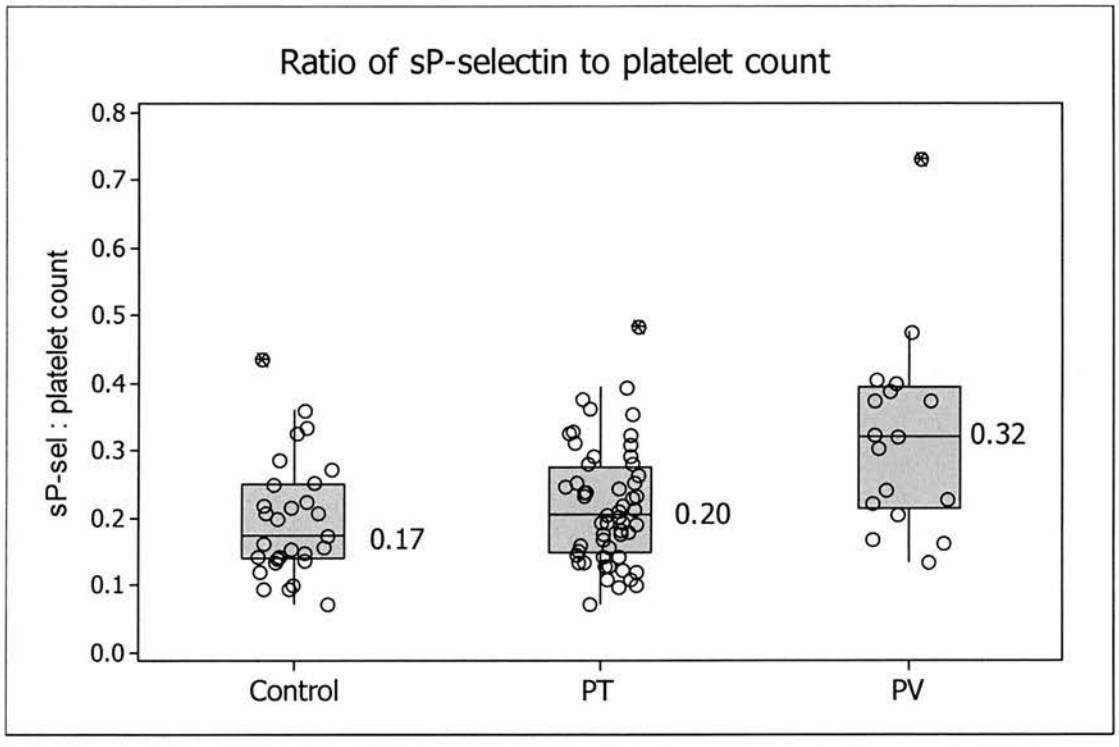


Figure 5.3 Ratio of plasma sP-sel to whole blood platelet count

Ratio of plasma sP-sel to whole blood platelet count in control subjects, PT and PV patients. Circles denote individual values and *denotes outlier values. Median values are shown.

	JAK2 wild type n=29	JAK2 V617F n= 22	P value
sP-selectin (ng/ml)	77.0 (64.2, 94.1)	84.7 (70.8, 104.0)	0.227
sE-selectin (ng/ml)	23.1 (19.6, 29.1)	24.3 (20.5, 30.4)	0.747
PF 1+2 (pmol/L)	277.0 (162.5, 403.0)	202.0 (122.5, 325.8)	0.082

Table 5.2 sP-sel, sE-sel and PF 1+2 in PT patients according to JAK2 mutation

sP-sel, sE-sel and (PF 1+2) in PT patients with the JAK2 V617F mutation compared to wild type. Median (IQR) values and P values for the comparison are shown.

	Controls n= 30	MPD only n=27	MPD + CVRF n=14	MPD + Thr/CVD n=33
P-selectin (ng/ml)	42.5 (34.1, 53.6)	88.9 (74.2, 105.8)	74.0 (59.9, 96.4)	95.0 (64.2, 114.9)
E-selectin (ng/ml)	25.1 (20.0, 29.7)	26.1 (16.0, 30.1)	22.8 (19.6, 32.0)	24.9 (20.5, 31.6)
PF 1+2 (pmol/L)	151.5 (115.8, 203.5)	251.0 (153.0, 337.0)	194.5 (130.0, 231.3)	251.0 (162.5, 385.5)

Table 5.3 sP-sel, sE-sel and PF 1+2 according to vascular history

sP-sel, sE-sel and PF 1+2 (median and IQR) in control subjects, patients with no history of thrombosis, CVRF or CVD (MPD only), patients with CVRF but no history of thrombosis or CVD (MPD+CVRF), and patients with a history of thrombosis (including prior cardiovascular events) (MPD+Thr/CVD). There were no statistically significant differences between the three patient groups (all $p>0.1$).

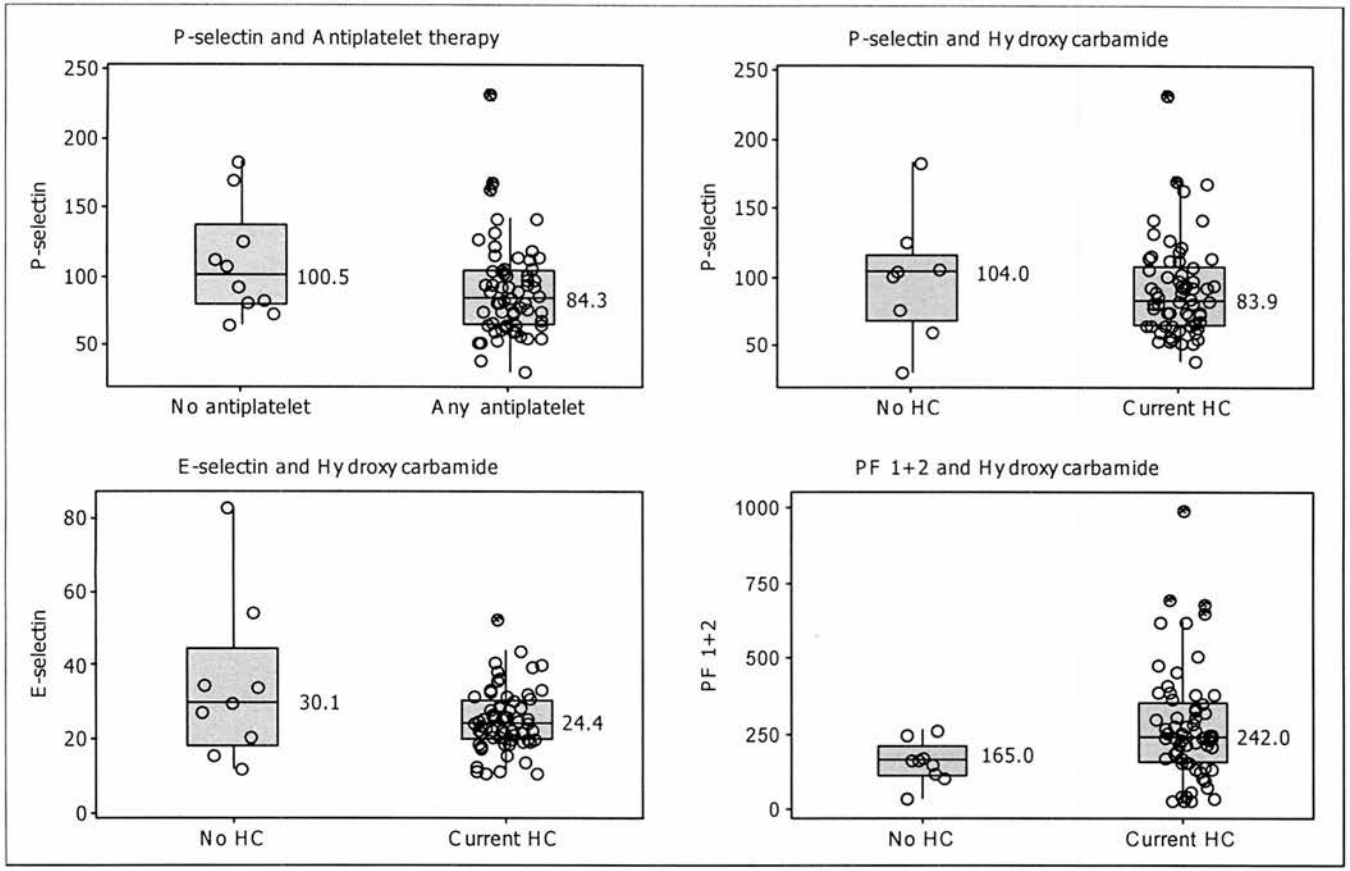


Figure 5.4 sP-sel, sEsel and PF 1+2 according to treatment

sP-sel levels according to treatment with antiplatelet agents or hydroxycarbamide (HC) and sE-sel and PF1+2 according to treatment with hydroxycarbamide. Circles denote individual values and *denotes outlier values. Median values are shown. PF 1+2 were lower in patients not treated with HC (p=0.037), there were no other significant differences between treatment groups.

5.3.7 Multivariant analysis

For the group as a whole, only the presence or absence of disease was a significant predictor for sP-sel ($p<0.001$). Age, platelet count and leucocyte count were not significant. For the patient group only, specific diagnosis (PV v PT) and platelet count were both significant predictors of sP-sel ($p=0.006$ and $p=0.023$ respectively). Age was the only significant predictor of PF1+2 ($p=0.004$ v $p=0.807$ for disease).

5.3.8 Vascular markers and microparticles

There were moderate but significant correlations between sP-sel and both PMP and EMP ($r =0.50$ and $r =0.52$, $p <0.001$ for both). There was no correlation between EMP and sE-sel ($r =-0.02$, $p=0.882$) (Figure 5.5). PF1+2 were weakly correlated with PMP ($r =0.298$, $p=0.002$) (Figure 5.5) but not with any other microparticle subgroup.

We examined the addition of these results to the multivariant analysis for PMP and EMP. For PMP the presence of disease and platelet count remained the strongest predictors. sP-sel was predictive of PMP independent of platelet count (both $p<0.001$) but not independent of disease ($p=0.120$). Similarly, disease remained the strongest predictor for EMP ($p<0.001$) however sP-sel levels were also predictive ($p=0.018$).

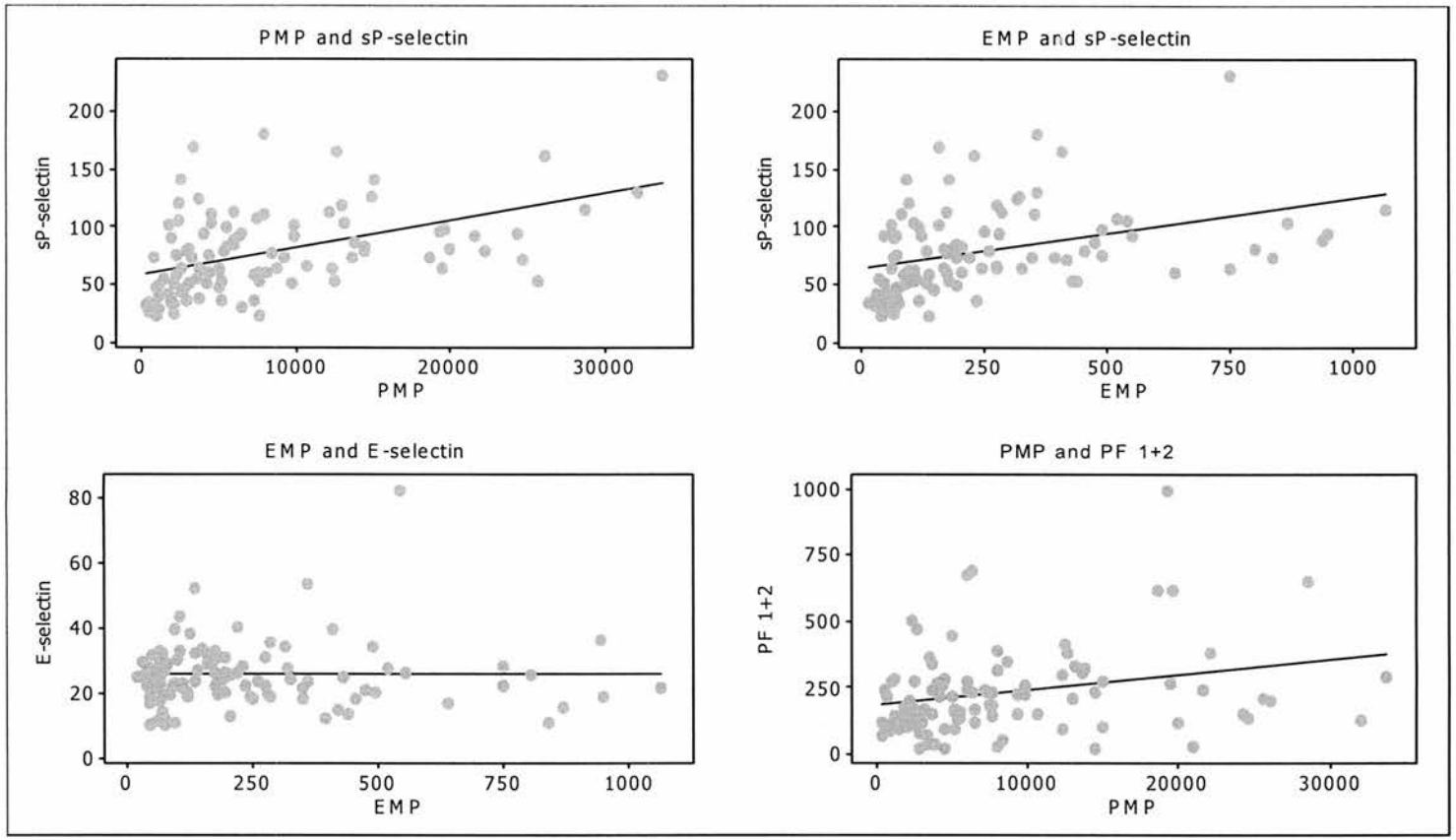


Figure 5.5 Correlation of microparticles and vascular markers

Dot plots showing the associations between sP-sel and PMP and EMP(r =0.50 and r =0.52, p <0.001 for both), sE-sel and EMP (r =-0.02, p=0.882) and PMP and PF1+2 (r =0.298, p=0.002).

5.4 Discussion

We have found elevated levels of the platelet activation marker sP-sel but not the soluble endothelial molecule sE-sel in MPD patients compared to controls. The coagulation activation marker PF1+2 was elevated in the MPD patients as a whole but this could be attributed to the older age of the patients compared to controls. sP-sel levels showed a moderate correlation with PMP consistent with their release following platelet activation and were also independently predictive of EMP.

Normal ranges

We used commercial immunoassays to measure sP-sel, sE-sel and PF1+2. The sP-sel levels in our control group were higher than the manufacturer's range which was based on a similar number of samples. Half of the control samples but almost all of the patient samples were above the manufacturer upper limit. This may be explained by different sample preparation, in particular, different protocols for the preparation of PPP. Our control range (10.8-81.8 ng/ml) was similar to that reported in other control groups (14-104 ng/ml, n=127) (Robertson *et al*, 2007). The manufacturer control range for sE-sel is based on serum samples and as expected we obtained a lower range in plasma samples. The range of PF1+2 results in the control patients was similar to that quoted by the manufacturer.

sP-selectin and sE-selectin

We found elevated levels of sP-sel in both PV and PT patients compared to controls, consistent with previous studies of sP-sel (Robertson *et al*, 2007;Musolino *et al*, 2000) and of platelet membrane P-sel measured by flow cytometry (Villmow et al, 2002;Arellano-Rodrigo et al, 2006;Jensen et al, 2000a;Falanga et al, 2005). sP-sel levels showed a moderate correlation with age but this could not fully account for the elevated levels in MPD patients which were equivalent in older and younger MPD

patients and greater in both than in controls. Similar to other groups we noted a moderate correlation between peripheral blood platelet count and sP-sel levels (Robertson *et al*, 2007). On multiple regression analysis, overall only the presence of disease was significant for sP-sel levels. However within the patient group, the diagnosis of PV compared to PT, and platelet count were also significant factors.

To further investigate the effect of platelet count, we determined the ratio of sP-sel to whole blood platelet count. This remained significantly higher in PV patients compared to controls but in PT patients the ratio was no different to controls and was significantly lower than PV patients. In three studies which measured the proportion of platelets positive for P-sel by flow cytometry, one did not differentiate PT from PV patients (Jensen et al, 2000a), one found equally elevated P-sel expression in both compared to controls (Villmow et al, 2002) and one found more P-sel positivity in the PV patients compared to PT patients (Falanga et al, 2005). In a study of sP-sel in PT patients, although levels were higher in comparison to healthy controls there was no difference from controls with secondary thrombocytosis (Musolino et al, 2000). Our findings, along with those of some of the previous studies (Musolino et al, 2000;Falanga et al, 2005), may suggest that different mechanisms contribute to elevated sP-sel in PV and PT patients, with the platelet mass being a more significant influence in PT patients.

Unlike platelet activation markers, results for markers of endothelial activation or damage in MPD have been less consistent. In separate studies sE-sel, vWF antigen and sTM have each been reported to be either elevated or no different from controls (Bellucci *et al*, 1993;Falanga *et al*, 2000;Musolino *et al*, 2000;Robertson *et al*, 2007). No clear explanation for these differences has been offered but methodological factors such as patient group or sample preparation may have some effect. In the current study we found no difference in sE-sel levels between patients and controls.

We did not observe any difference in sP-sel or sE-sel according to treatment with either hydroxycarbamide or aspirin in keeping with previous studies (Arellano-Rodrigo *et al*, 2006;Falanga *et al*, 2000;Robertson *et al*, 2007). There has been a

lack of consensus in previous studies with regard to the association between P-sel, sE-sel and thrombotic history (Table 5.4). Both sP-sel and sE-sel levels in our study were no different between patients with or without a history of cardiovascular risk factors, cardiovascular disease or other thrombosis. The current study and all others published to date are limited by relatively small cohorts in this respect.

More recently there has been interest in the relationship of the presence of the JAK2 V617F and thrombosis. Leucocyte count at diagnosis has been found to be an independent risk factor for thrombosis (Gangat *et al*, 2007;Landolfi *et al*, 2007) and PT patients with the mutation are observed to have higher leucocyte counts at diagnosis (Campbell *et al*, 2005;Wolanskyj *et al*, 2005). An association between JAK2 V617F and venous thrombosis has been reported in two studies (Campbell *et al*, 2005;Kittur *et al*, 2007) but has not been observed in others (Carobbio *et al*, 2007;Wolanskyj *et al*, 2005). Robertson and colleagues reported significantly higher sP-sel levels in patients with the mutation than wild type but they did not differentiate PV and PT patients (Robertson *et al*, 2007). Their JAK2 V617F group was therefore dominated by PV patients. We observed that PV patients had significantly higher sP-sel levels than PT patients, despite equivalent platelet counts, which may explain the association in that study. However, in 49 PT patients of whom 22 had a previous history of thrombosis, platelet P-sel expression was correlated with both JAK2 V617F positivity and thrombotic history (Arellano-Rodrigo *et al*, 2006).

Coagulation activation

Similarly to endothelial abnormalities, evidence of coagulation activation in MPD has been variable and even within single studies, measurement of the different markers, D-dimer, TAT and PF1+2, have produced differing results (Bellucci *et al*, 1993;Falanga *et al*, 2000;Robertson *et al*, 2007). We found elevated levels of PF1+2 in patients compared to healthy controls. It has been suggested that coagulation activation may be a non-specific finding associated with age and cardiovascular co-

morbidities (Robertson *et al*, 2007). In a comparison of MPD patients with age matched hypertensive controls who are at increased risk of sub-clinical vascular disease, no significant increase in D-dimer, TAT or PF1+2 was observed (Robertson *et al*, 2007). We found no difference in PF1+2 levels between patients with or without cardiovascular risk factors, overt cardiovascular disease or other thrombosis. However, PF1+2 did correlate with age and levels were only elevated in the older MPD patients compared to the younger MPD patients whose levels were not significantly different to the control group. The lower PF1+2 levels in patients who were not on hydroxycarbamide are likely to reflect the younger age of the untreated group.

Relationship of microparticles to established vascular markers

We observed a modest correlation between sP-sel and CD42+ve PMP, a relationship which has been reported previously in a cohort of patients with peripheral vascular disease (Tan *et al*, 2005). In contrast, no association with platelet P-sel was observed in patients with VTE or sP-sel in patients with atrial fibrillation (Chirinos *et al*, 2005;Choudhury *et al*, 2007). As the mechanisms of PMP formation include platelet activation and P-sel-PSGL interactions (Hrachovinova *et al*, 2003), one might indeed predict an association between sP-sel and PMP. It is also possible that some of the "soluble" P-sel measured in PPP is in fact PMP bound. However PMP formation can also be induced by specific receptor binding interactions not involving P-sel, for example GP1b-vWF (Reininger *et al*, 2006). Interestingly, in the PT subgroup the ratio of sP-sel to whole blood platelet count was similar to controls but the ratio of PMP to platelets was significantly higher than in controls. This would support the theory that more than one mechanism of PMP formation is involved. The simultaneous measurement of platelet P-sel, CD42 and P-sel positive PMP and soluble P-sel in both PPP and microparticle free plasma might provide useful information in this regard.

In contrast, we observed no association between EMP, which were elevated in MPD patients, and sE-sel which was not. One reason for this may be that we identified EMP by the constitutive antigen CD31, while sE-sel is an endothelial activation antigen. Although constitutive microparticle release has been observed *in vitro*, whether this translates to the *in vivo* situation is unclear. Alternatively, increased CD31+ve EMP release following apoptotic stimuli has been observed *in vitro* and appeared to correlate with the *in vivo* situation (Jimenez *et al*, 2001;Jimenez *et al*, 2003b). Our results are at variance to previous studies which have reported variable correlation of EMP with sE-sel and other markers of endothelial activation. In post-stem cell transplant patients, sE-sel, VCAM-1 and EMP were all elevated (Nomura *et al*, 2005) whilst in hypertensive patients, CD31+ve EMP correlated with V-CAM and VWF but not sI-CAM (Preston *et al*, 2003).

Differences in the endothelial antigens measured may therefore explain this discrepancy but as with PMP, an alternative explanation is that the EMP were induced by different mechanisms to the endothelial adhesion molecules. EMP may also be a more sensitive or general marker of endothelial activation/damage than specific adhesion molecules. In patients with Type II DM both VE-cadherin positive EMP and soluble ICAM levels were elevated compared to controls and were higher still in patients with underlying CAD. However, only EMP were independently predictive for the presence of CAD (Koga *et al*, 2005).

A moderate correlation between EMP and sP-sel has previously been reported (Preston *et al*, 2003) and we also observed such an association. It may suggest either concomitant activation of platelets and endothelial disturbance, perhaps by a shared mechanism, or that activated platelets or PMP can induce endothelial cell changes as has been demonstrated *in vitro* (Barry *et al*, 1997;Burger & Wagner, 2003).

The phospholipid properties of microparticle membranes and the presence of TF on a proportion of monocyte and also endothelial microparticles can contribute to thrombin generation. We therefore considered whether there was any association between microparticles and PF 1+2 which are released during the activation of

prothrombin. Such an association between total and TF bearing microparticles and PF1+2 as well as TAT and D-dimer have been reported in sickle cell disease (Shet *et al*, 2003;Switzer *et al*, 2006). We observed a weak but statistically significant correlation for PF1+2 and PMP but none of the other microparticle subtypes. However this association was confounded by the effect of age on both PF1+2 and PMP. The prothrombotic properties of microparticles may be more relevant in the response to local tissue damage than to measures of systemic coagulation activity.

Summary

We have measured established markers of platelet, endothelial and coagulation activation in MPD compared to microparticles which are thought to provide similar information on the state of the vasculature. In keeping with previous studies we observed evidence of platelet activation in MPD with elevated sP-sel levels. sP-sel correlated moderately with and was a significant predictor of PMP but our results suggested that platelet activation may not be the sole mechanism of PMP formation in MPD. Unlike previous studies associating platelet activation with the JAK2 V617F mutation we did not correlate sP-sel with the JAK2 V617F mutation in the PT patients.

We did not see an increase in sE-sel but did observe elevated EMP which correlated with sP-sel. This may be due to methodological reasons, in particular disparity between the markers used, and previous studies measuring endothelial markers in MPD have also reported variable results. Alternatively our results may indicate non-activating endothelial damage in MPD perhaps due to the effect of activated platelets or PMP. EMP may also prove to be a more sensitive marker for endothelial abnormalities than the measurement of specific adhesion molecules.

PF1+2 levels were only elevated in older patients, suggesting that the systemic coagulation activation which has been observed in MPD patients may reflect more

general age related changes. Microparticles did not appear to be independently associated with this systemic marker of thrombin activity and may be more important in coagulation at the local level. Specific investigation of the prothrombotic properties of microparticles in MPD, for example by the measurement of thrombin generation in PPP, would help to elucidate this issue.

Study	Patients (n)	Controls (n)	Platelet activation markers	Endothelial markers	Coagulation activation markers	Thrombosis Associations
Arellano-Rodrigo 2006	49 PT	49	Platelet P-sel - elevated	-	-	Platelet P-sel
Bellucci 1993	16		BTG - elevated	sTM - n.s vWF antigen – n.s	D-dimer - n.s.	-
Falanga 2000	34 PV 37 PT		-	sTM - elevated in PV vWF antigen - elevated	D-dimer - elevated in PV TAT - elevated PF 1+2 - elevated	-
Falanga 2005	34 PV 46 PT	50	Platelet P-sel - elevated	-	-	none
Jensen 2000	50 MPD 17 PT 15 PT	30	Platelet P-sel – elevated Platelet TSP- elevated	-	-	TSP - all patients
Musolino 2000	14 PV 24 PT	15	sP-sel - elevated	sE-sel - elevated sTM - elevated	-	sP-sel – PT only sE-sel – all patients
Robertson 2007	51 PV 63 PT	127	sP-sel - elevated	sE-sel - n.s.	D-dimer - elevated TAT - n.s. PF1+2 - n.s.	none
Villmow 2003	47 MPD 13 PV 12 PT	16	Platelet P-sel - elevated PMP - elevated	-	-	none

Table 5.4 Summary of results of published studies of vascular markers in MPD

Summary of results of published studies of markers of platelet, endothelial and coagulation activation in MPD patients and observed associations with history of thrombosis. Abbreviations: platelet and soluble (s) P-selectin; thrombospondin (TSP); platelet microparticles (PMP); soluble thrombomodulin (sTM); soluble E-selectin (sE-sel); von Willebrand (vWF) antigen; thrombin-antithrombin complexes (TAT); prothrombin fragments (PF1+2); results not significantly different from controls (n.s.).

CHAPTER 6

PLATELET α-GRANULE CONTENTS AND BONE MARROW FIBROSIS

6.1 Introduction

Bone marrow fibrosis is the prominent finding in PMF which carries the worst prognosis of the MPD (Reilly, 1997). In PV and PT, fibrosis is rarely marked at the initial presentation (Bain *et al*, 2001), but during the course of the disease it may progress to a histologically higher grade or to overt post-PV or PT myelofibrosis. Transformation to fibrosis is reported at rates of between 3 and 20% and the risk of transformation is associated with increasing time from diagnosis (Alvarez-Larran *et al*, 2007;Harrison *et al*, 2005;Marchioli *et al*, 2005;Passamonti *et al*, 2004;Tefferi *et al*, 2001;Wolanskyj *et al*, 2006).

Marrow studies in PMF demonstrate a marked increase in neovascularisation of the stromal tissue together with an increased deposition of ECM components. This is a reactive phenomenon mediated by cytokines, including TGFβ and PDGF (Reilly, 1992). TGFβ has a number of profibrotic actions (Miyazono & Takaku, 1989) and has been shown to be critical in the development of MF in murine models (Chagraoui *et al*, 2002). In addition, elevated levels have been observed *in vivo* in patients with PMF or fibrosis secondary to other haematological disorders (Shehata *et al*, 2004;Rameshwar *et al*, 1998).

Bone marrow megakaryocytes and platelets are a major source of TGFβ and PDGF, being contained within their α-granules (Fava *et al*, 1990). Platelet α-granules also contain BTG and PF4, intraplatelet levels of which are reported to be reduced or normal in MPD (Lev *et al*, 2002;Katoh *et al*, 1988), while plasma and urine levels are elevated (Lev *et al*, 2002;Gersuk *et al*, 1989;Sacchi *et al*, 1986). These observations suggest a mechanism whereby defects of α-granule storage or release in MPD could result in elevated levels of TGFβ and PDGF in the plasma and/or bone marrow.

In order to investigate whether such a generalised abnormality of platelet α-granules might be responsible for elevated plasma levels of profibrotic cytokines, we have assessed plasma levels of the α-granule proteins BTG, PF4, TGFβ and PDGF, in

patients with MPD compared to controls. We have also investigated the association of levels of these proteins with disease related factors including specific diagnosis, haematological and molecular parameters and their association with the degree of bone marrow fibrosis. The reproducibility of assessment of bone marrow fibrosis for use in investigating such an association was also considered.

6.2 Materials and Methods

6.2.1 Participant recruitment and ethical approval

Investigation of plasma levels of platelet α-granules in MPD was performed using a different group of MPD patients and control subjects from those involved in the work previously discussed.

Patients with a documented diagnosis of MPD (PV, PT or PMF) or myeloproliferative disorder not otherwise specified (MPD NOS) were recruited from the haematology out-patient service at the Royal Infirmary of Edinburgh. These diagnoses were based on the PVSG or BCSH criteria (Appendix 1). Two different control groups were recruited. Patients with a diagnosis of secondary polycythaemia or reactive thrombocytosis (based on the above diagnostic guidelines) were recruited from the haematology out-patient service at the Royal Infirmary of Edinburgh. Control subjects without haematological disease were recruited from the orthopaedic pre-assessment clinic.

For all groups, participant information sheets and consent forms (Appendix 2) were sent out at least one week prior to their pre-arranged clinic appointment and for those wishing to participate, informed consent was obtained on the day of attendance. Ethical approval was obtained from the Lothian Local Research Ethics Committee.

6.2.2 Data collection

Clinical history and haematological parameters at diagnosis, molecular data and follow-up information on haematological and thrombo-haemorrhagic complications and management were obtained by retrospective review of patient case records. Vascular events at presentation included those which occurred up to six months prior to the diagnosis of MPD. Peripheral blood count, renal function and liver function tests were also performed at the time of sample collection.

6.2.3 Sample collection and processing

We aimed to collect duplicate patient samples on two separate occasions. For practical reasons control samples were obtained on one occasion only. Whole blood was collected by venepuncture with a 21G needle into 2 x 3.5ml CTAD Vacutainers (Becton Dickinson) to improve platelet stability. Samples were immediately placed on ice and were processed within one hour of collection. PPP was obtained by double centrifugation at 2500g for 20 minutes with removal of the middle third of plasma. The obtained PPP samples were aliquoted and stored at -80°C until analysis.

6.2.4 Cytokine assays and data analysis

Plasma BTG and PF4 were measured using the Asserachrom enzyme immunoassay (Diagnostica Stago). The normal ranges provided by the company were, BTG 10-50 IU/ml and PF4 0-10 IU/ml but it is recommended that each laboratory determine its own normal range. Plasma TGFβ and PDGF were measured using the Quantikine enzyme linked immunoassays (R and D Systems).

Two samples taken on separate occasions were employed to reduce the likelihood of the results being influenced by *in vitro* platelet activation during sampling. Where duplicate results were available those from the episode with the lower BTG level were selected. Where only one set of results was available, results with a BTG level within the normal range (based on the control group) were accepted. For single BTG results outwith the normal range, a BTG:PF4 ratio of greater than 3.0 was used to determine whether *in vitro* platelet granule release was likely to have occurred. Impairment of renal function is known to increase BTG levels and therefore patients with creatinine greater than twice the upper limit of normal were excluded from analysis (Kaplan & Owen, 1981).

We encountered a number of problems with the PDGF assay. Initially the standard Quantikine assay was unavailable from the suppliers due to technical problems with

its performance and the company therefore supplied an alternative kit (Duoset). All plasma samples were processed with this assay but in 63/133 (47.3%) the PDGF level was below zero from the normal curve. When the Quantikine assay became available we therefore repeated the PDGF measurements on the 77 samples selected by low BTG results as above. With this assay a result of less than zero was again returned on 13 samples (16.8%). Comparing Duoset results of less than versus greater than zero, the corresponding Quantikine results (median (IQR)) were 21.0 (8.0, 51.0) v 120.0 (48.8, 180.8), ($p<0.001$) (excluding negative values).

This suggested that there was a particular problem with measurement of values at the lower end range of PDGF, particularly with the Duoset assay. Therefore, we used the results from the Quantikine assay and, in order to allow inclusion of results below the level of detection in the statistical analysis, we assigned these a value of 0.5pg/ml and added 0.5 to all other results.

6.2.5 Assessment of fibrosis

Where performed, bone marrow histopathology reports were obtained from a variety of sources – patient case notes, the computerised laboratory result system APEX and archived copies. Due to the non-standardised nature of reporting by a range of histopathologists over a long time period, reports were interpreted and categorised according to the scheme in Table 6.1 for purposes of analysis.

Where available, archived bone marrow trephine slides were obtained and were reassessed independently by the researcher and a pathologist, both blinded to the patient diagnosis and previous histopathology report. Reticulin fibrosis was graded 0-3 according to the criteria of Bauermeister (Bauermeister, 1971) (Table 1.1). Grade 4 fibrosis could not be assessed as collagen stains were not available. Grades 0-3 were then categorised A-C in accordance with Table 6.1.

6.2.6 Statistical analysis

Normally distributed data is reported as mean values and SEM. Between group comparisons were made by the student's t-test. The results for some haematological parameters, BTG, PF4, TGFβ and PDGF levels were not normally distributed (Figure 6.1) and are therefore reported as median and interquartile range IQR. Between-group comparisons were made using the non-parametric Mann Whitney U tests. No correction was made for multiple comparisons. Correlation analysis of skewed data was performed using the Spearman's rank method. Multivariate analysis was performed by multiple regression analysis of log transformed data. P values of less than 0.05 were considered to be statistically significant. All calculations and analyses were performed using Minitab 15 software (Minitab Inc, USA).

Category A	no comment on reticulin fibrosis
	reticulin fibrosis normal / not increased
	occasional fine reticulin fibres/networks
	Bauermeister Grade 0/1
Category B	mild diffuse increase in reticulin
	no coarse fibres noted
	Bauermeister Grade 2
Category C	coarse reticulin fibres present
	collagen fibres present
	Bauermeister Grade ¾

Table 6.1 Categorisation of fibrosis

Categorisation of bone marrow trephine histopathology reports according to degree of bone marrow fibrosis.

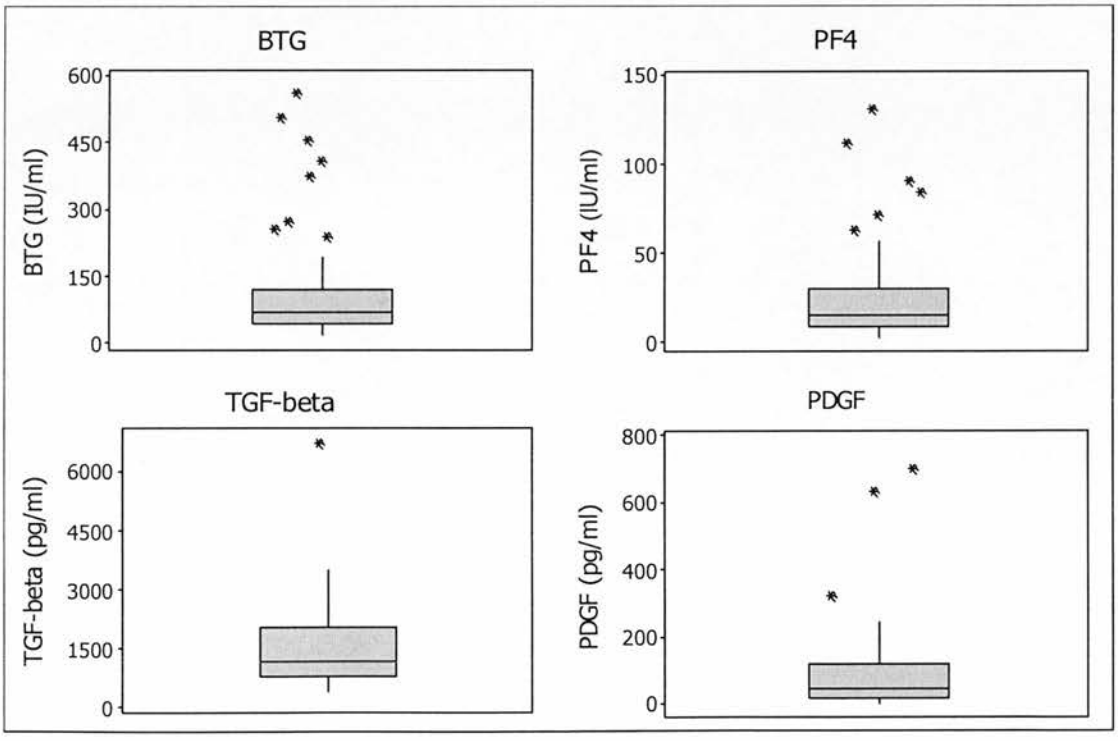


Figure 6.1 Distribution of BTG, PF4, TGFβ and PDGF values

Box plots illustrating the skewed distribution of the results for BTG, PF4, TGFβ and PDGF.

6.3 Results

6.3.1 Participant characteristics

We recruited 62 patients with MPD, including 38 PT, 16 PV, 5 PMF and 3 MPD NOS. We recruited eight controls subjects with secondary polycythaemia (5) or reactive thrombocytosis (3) (secondary controls). There were 11 non-haematological control subjects (NH controls).

The mean age of patients and controls at entry to the study was similar, 65.6 (3.1) v 60.4 (1.9) years respectively, (p=0.157). 9/19 controls were male (47.4%) compared to 25/62 patients (40.3%) (p=0.606, Fisher's exact test).

The time from diagnosis to study entry was known in 57 patients, with a median of 48 (24, 113.5) months.

6.3.2 Clinical history

Cardiovascular co-morbidities were documented in 23 patients and diagnoses included IHD (8), cerebrovascular disease (11), PVD (5), hypertension (17), Type II DM (1) and hypercholesterolemia (7). Smoking status was known in 53 patients – six smokers, nine ex-smokers and 38 non-smokers.

Thrombotic episodes occurring from six months prior to diagnosis, at the time of diagnosis or during follow-up, were recorded for 29/62 patients (46.6%). Arterial events occurred in 23 patients (40%) - seven CVA, four TIA, one myocardial infarction, four microvascular occlusive episodes and three peripheral thromboses. VTE occurred in eight patients (13.8%) – two intra-abdominal thromboses, three recurrent VTE, one PE and two DVT. There were two major and nine minor haemorrhagic episodes.

Treatment information was available for 60 patients. 43 patients were currently taking aspirin, in six it had been taken previously but discontinued due to side-effects and 11 patients had never received aspirin. Cytoreduction with hydroxycarbamide was currently prescribed in 50 patients and one further patient had had previous treatment with this agent. One patient was managed with anagrelide and a further two patients had previously received this. One patient was undergoing treatment with radiophosphorus and two had done so previously. A program of venesection was ongoing in 13/16 PV patients and three further patients (1 PV, 1 PMF and 1 PT) had previously been venesected. None of the patients with PMF had undergone splenectomy.

6.3.3 Haematological and molecular parameters

Haematological parameters at the time of the sampling are shown in Table 6.2. Haemoglobin and leucocyte count were similar in the patient and control groups. The platelet count was higher in MPD patients compared to the control group as a whole (p=0.001), but there was no significant difference for the control patients with secondary polycythaemia or thrombocytosis (p=0.423).

There was a wide range in the haematological parameters for the small group of PMF patients (n=5) but they had a significantly lower haemoglobin than either the control group or the PV and PT patients (p=0.012, 0.012, 0.018 respectively) and a significantly lower platelet count compared to the PT patients (p=0.023).

JAK2 V617F mutation status had been established in 59 patients. Of these 13/15 PV (87%), 16/35 PT (45.7%), 3/5 PMF and 2/3 MPD NOS were positive for the JAK2 V617F mutation.

	Haemoglobin (g/l) Mean (SEM)	Leucocytes (x 10^6/l) Median (IQR)	Platelets (x10^9/l) Median (IQR)
All MPD (62)	132 (2.7)	7.0 (5, 9.5)	389* (299, 440)
PMF (5)	101 (8.9)	8 (4.5, 23)	85 (52, 398)
PV (16)	138 (3.7)	11 (8, 13.8)	356 (266.5, 447.8)
PT (38)	133 (3.3)	6 (5, 7.5)	397 (333, 461.5)
MPD NOS (3)	138 (14.1)	7 (5, 8)	269 (249, 433)
All controls (19)	137 (4.7)	7.0 (6, 9)	231 (185, 265)
NH (11)	131 (5.8)	6.0 (5, 6)	225 (181, 258)
Secondary (8)	144 (7.3)	8.5 (7.3, 10.8)	242.5 (191.8, 617)

Table 6.2 Haematological parameters patients and controls

Haematological parameters in MPD patients and control subjects according to diagnosis – PMF, PV, PT, MPD NOS, NH (non-haematological controls), secondary (secondary polycythaemia or reactive thrombocytosis). Mean and SEM are shown for haemoglobin. Median and IQR are shown for leucocyte count and platelets. * The platelet count is significantly higher in patients than controls (p=0.001).

6.3.4 Plasma levels of platelet α granule contents

6.3.4.1 Sample selection

One set of results was available for each of the 11 subjects in the NH control group. All BTG:PF4 ratios were greater than 2.5. From these results we determined an in-house normal range for BTG of 12-66 IU/ml (mean +/- 2sd), similar to the suggested normal range of 10-50 IU/ml.

We considered using the BTG:PF4 ratio to select samples less likely to have been affected by *in vitro* activation; a cut-off of BTG:PF4 > 2.5 was selected based on previously published studies. We compared the BTG and TGFβ results in pairs of samples where both ratios were >2.5 (suggesting *in vitro* activation had not occurred) and in pairs where one sample had a ratio of <2.5 (suggesting significant *in vitro* activation and therefore that higher BTG and TGFβ levels might be expected). We did not find a consistent association between low BTG:PF4 ratios and higher BTG and TGFβ levels. In 13 of the 32 pairs where both BTG:PF4 ratios were >2.5, there was a two-fold or greater difference in the BTG and TGFβ levels (Figure 6.2). Therefore the BTG:PF4 ratio was not used in the sample selection process.

In the secondary control group, 6/8 had two sets of samples and the set with the lower BTG value was selected (all BTG:PF4 ratios >2.5). Two subjects had only one set of samples; in both the BTG was within the normal range and these results were included in the analysis. One secondary control subject was excluded due to the development of fulminant hepatic failure. Sample availability and selection for the MPD patients is shown in Figure 6.3. For 2/62 patients there were no suitable samples. Two sets of results were available for 50/60 patients and the set with the lower BTG value were selected. In ten patients only one set of results was available due either to haemolysed samples or incomplete follow-up. For 2/10 single samples the BTG was within our normal range and these results were included in the analysis. The remaining eight single samples where the BTG was elevated were not included in the analysis despite BTG:PF4 ratios of greater than or equal to 3.0.

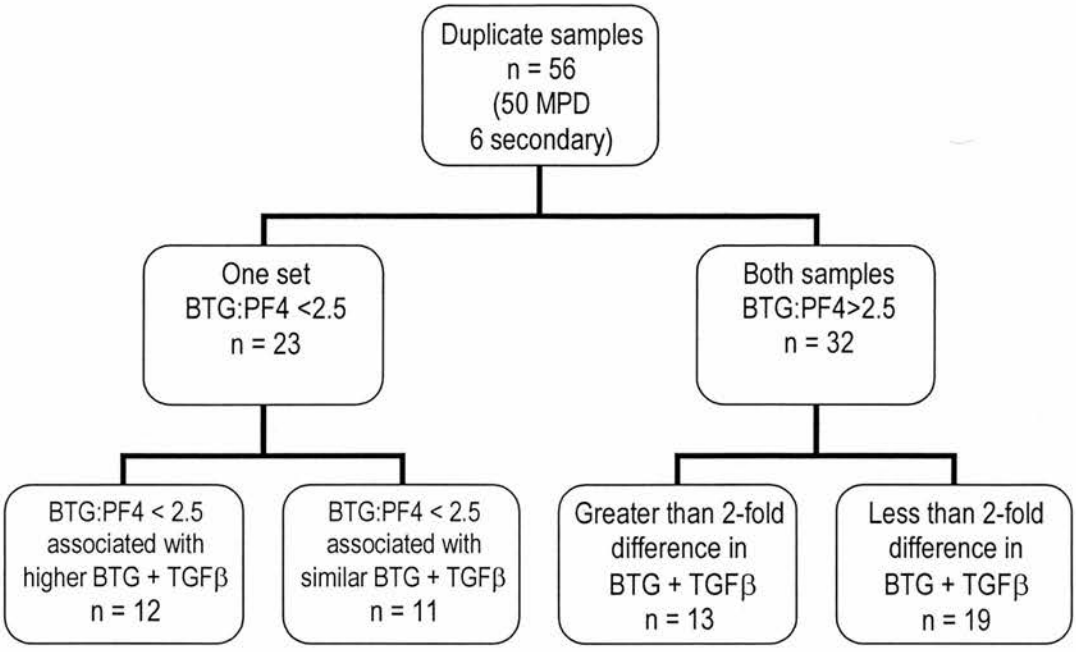


Figure 6.2 Comparison of BTG and TGFβ results by BTG:PF4 ratio

Comparison of BTG and TGFβ results in pairs of samples with BTG:PF4 ratios less than or greater than 2.5.

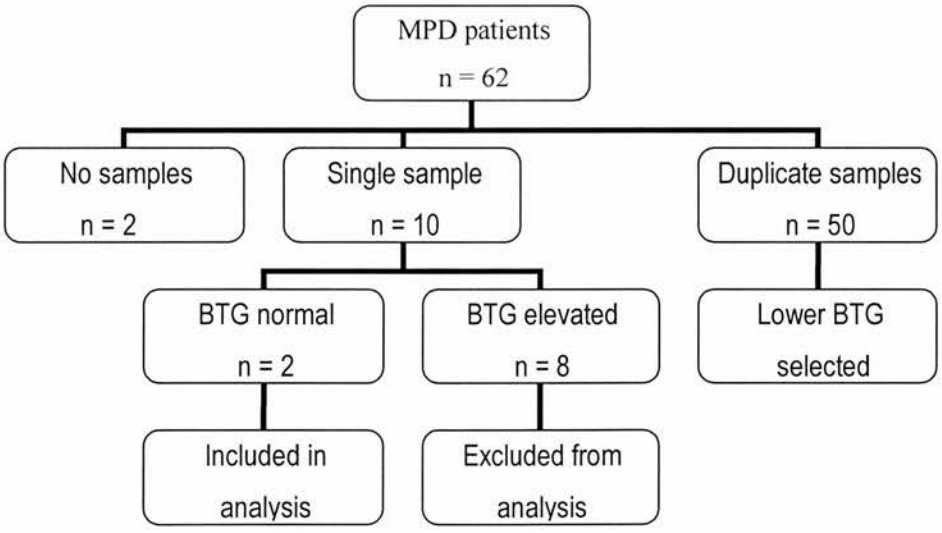


Figure 6.3 Sample availability and selection of results for further analysis in the MPD patient cohort.

6.3.4.2 BTG, PF4, TGFβ and PDGF results

The skewed distribution of the BTG, PF4, TGFβ and PDGF results is shown in Figure 6.1. Results for BTG, PF4, TGFβ, and PDGF in the patient and control groups are shown in Tables 6.3 – 6.5 and Figure 6.4(a-d).

There were significant correlations between BTG and PF4 ($r = 0.638$, $p<0.001$), BTG and TGFβ ($r = 0.746$, $p<0.001$) and BTG and PDGF ($r = 0.420$, $p<0.001$) (Spearman's rank).

6.3.4.3 Plasma levels of platelet α-granule contents by age and gender

There was no significant difference in BTG, PF4, TGFβ or PDGF according to gender. We detected no association between levels of platelet α-granule contents and age ($r = 0.145$, $r = 0.138$, $r = 0.105$, $r = 0.064$ for BTG, PF4, TGFβ and PDGF respectively, all $p>0.2$) (Spearman's rank correlation)

6.3.4.4 Plasma levels of platelet α-granule contents by diagnosis

BTG was significantly higher in all MPD patients compared to all controls and in each of the PMF, PV and PT groups compared to all controls (all $p<0.001$). BTG was highest in the PMF patients and was significantly higher in PMF and PV patients compared to the PT group ($p=0.001$ and $p<0.001$ respectively). PF4 was significantly higher in all MPD patients compared to all controls ($p=0.019$) but on analysis of the patient subgroups this remained significant only for the PV patients ($p=0.003$). The BTG:PF4 ratio was higher in patients than controls, 4.9 (3.7, 7.0) v 3.7 (2.9, 4.9), ($p=0.017$). The ratio was highest in PMF patients at 11.4 (10.2, 18.4) compared to PV patients, 4.9 (4.3, 5.9) or PT patients, 4.8 (3.4, 6.8), ($p=0.004$ and 0.014 respectively).

TGFβ was higher in all patients compared to all controls ($p<0.001$) and was highest in PMF patients ($p=0.034$ v PV patients and $p=0.014$ v PT patients). PDGF was not significantly different between patients and controls. Median PDGF was highest in the PV patients, which was of borderline statistical significance compared to PT patients ($p=0.048$) however there was marked overlap in the ranges. PDGF values of less than zero were obtained for 2/17 controls, 2/4 PMF, 1/12 PV and 6/30 PT results. By considering these as "missing values", there remained no significant difference between patients and controls ($p=0.311$).

BTG, PF4, TGFβ and PDGF were all higher in the secondary controls compared to the NH controls although none of these differences reached statistical significance (Table 6.4).

	All Controls n = 19	All MPD n = 52	Controls v MPD P value
BTG (IU/ml)	43.0 (36.0, 55.0)	81.5 (59.8, 136.5)	**<0.001**
PF4 (IU/ml)	12.0 (7.0, 15.0)	17.5 (10.0, 34.3)	**0.019**
TGFβ (pg/ml)	694.5 (552.8, 919.0)	1485.0 (1079.0, 2314.0)	**<0.001**
PDGF (pg/ml)	36.5 (14.8, 60.0)	35.0 (10.8, 122.3)	0.539

Table 6.3 BTG, PF4, TGFβ and PDGF in patients and controls

BTG, PF4, TGFβ and PDGF results for all MPD patients compared to all controls. Median and IQR values are shown and P values for statistically significant differences between the groups are shown in bold.

	NH controls n = 11	Secondary controls n = 7	NH v Secondary P value
BTG (IU/ml)	36 (27, 55)	45 (42, 56)	0.099
PF4 (IU/ml)	8 (6, 15)	12.5 (10.5, 22.3)	0.052
TGFβ (pg/ml)	586 (507, 697)	896 (760, 1133)	0.066
PDGF (pg/ml)	24 (11, 50)	56 (29, 92)	0.113

Table 6.4 BTG, PF4, TGFβ and PDGF in control groups

BTG, PF4, TGFβ and PDGF results for non haematological controls (NH) compared to controls with secondary polycythaemia or reactive thrombocytosis (secondary controls). Median and (IQR) values are shown and P values for statistically significant differences between the groups are shown in bold.

	PMF n = 4	PV n = 13	PT n = 35	P value		
				PMF v PV	PMF v PT	PV v PT
BTG (IU/ml)	241.0 (75.0, 519.0)	99.0 (68.0, 185.0)	70.5 (52.5, 104.5)	0.282	**0.001**	**<0.001**
PF4 (IU/ml)	22.0 (5.3, 45.5)	22.0 (14.5, 31.5)	14.0 (9.0, 28.5)	0.777	0.962	0.134
TGFβ (pg/ml)	3194 (1863, 5918)	1757 (1076, 2446)	1306 (1034, 1985)	**0.034**	**0.014**	0.416
PDGF (pg/ml)	18.3 (0.5, 139.5)	95.5 (21.5, 143.5)	32.5 (8.5, 106.8)	0.363	0.669	0.108

Table 6.5 BTG, PF4, TGFβ and PDGF in patient groups

Comparison of BTG, PF4, TGF and PDGF results for patients with PMF, PV and PT. Median and IQR values are shown and P values for statistically significant differences between the groups are shown in bold.

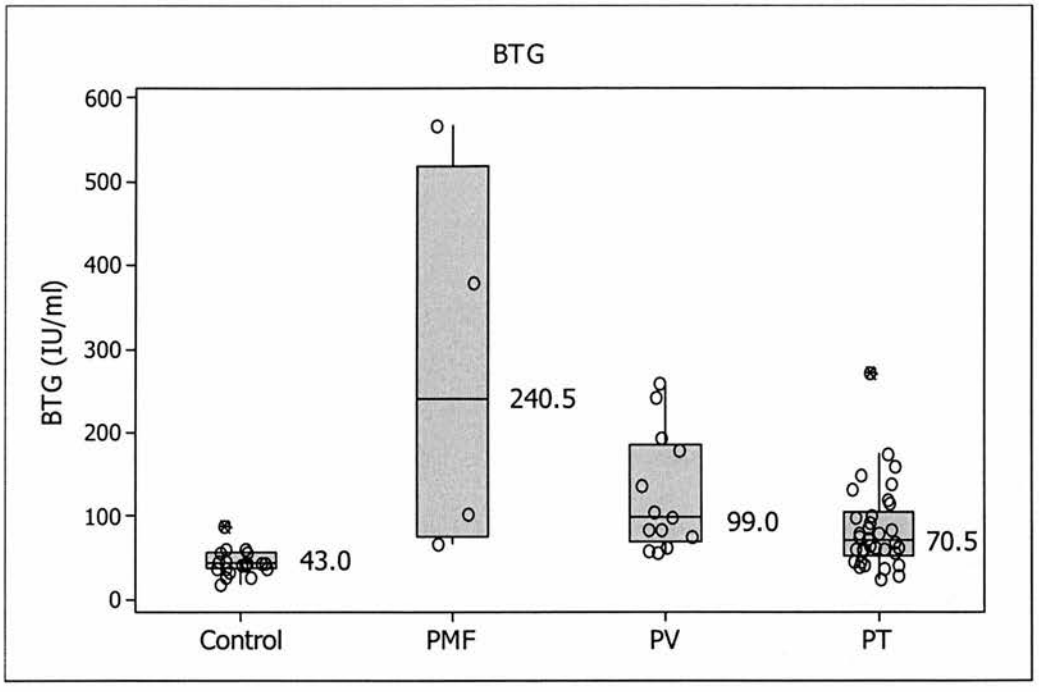


Figure 6.4(a)

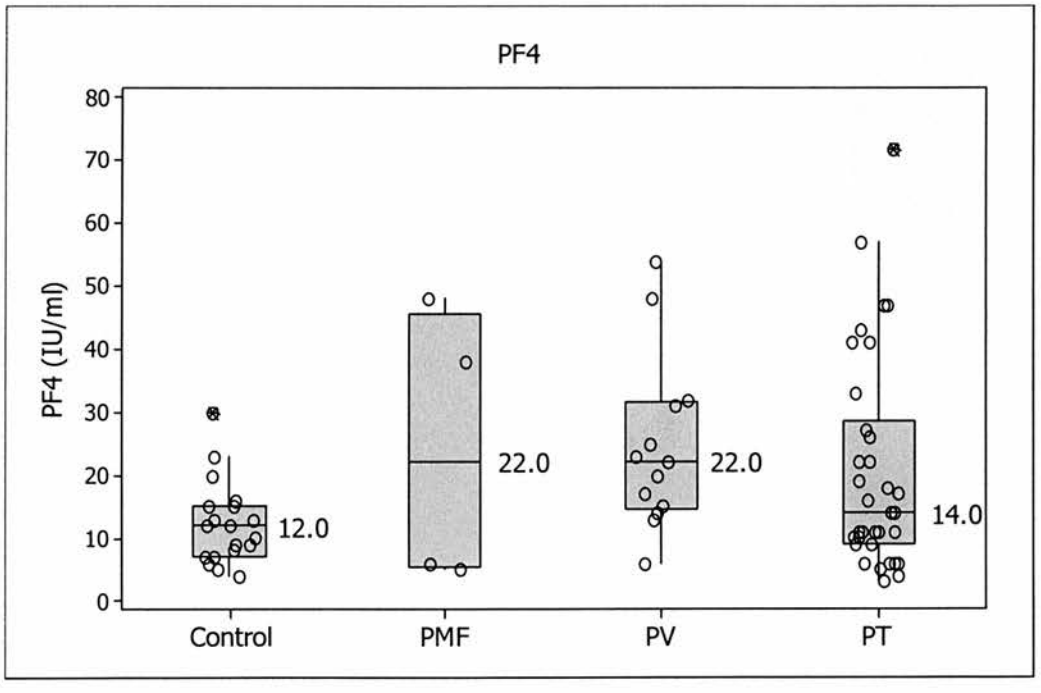


Figure 6.4(b)

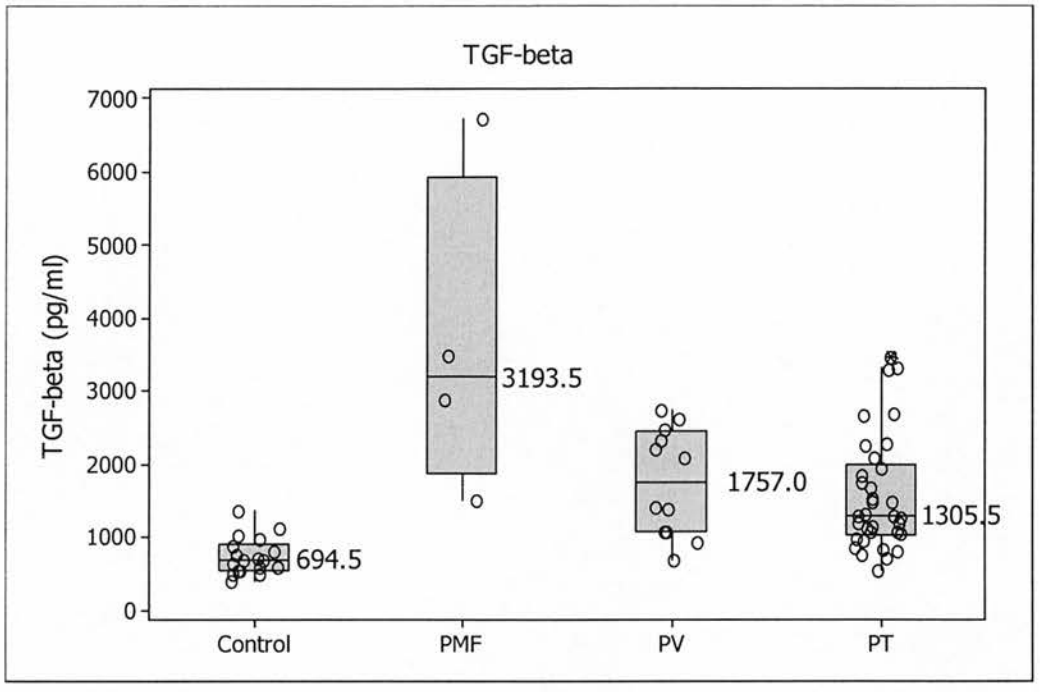


Figure 6.4 (c)

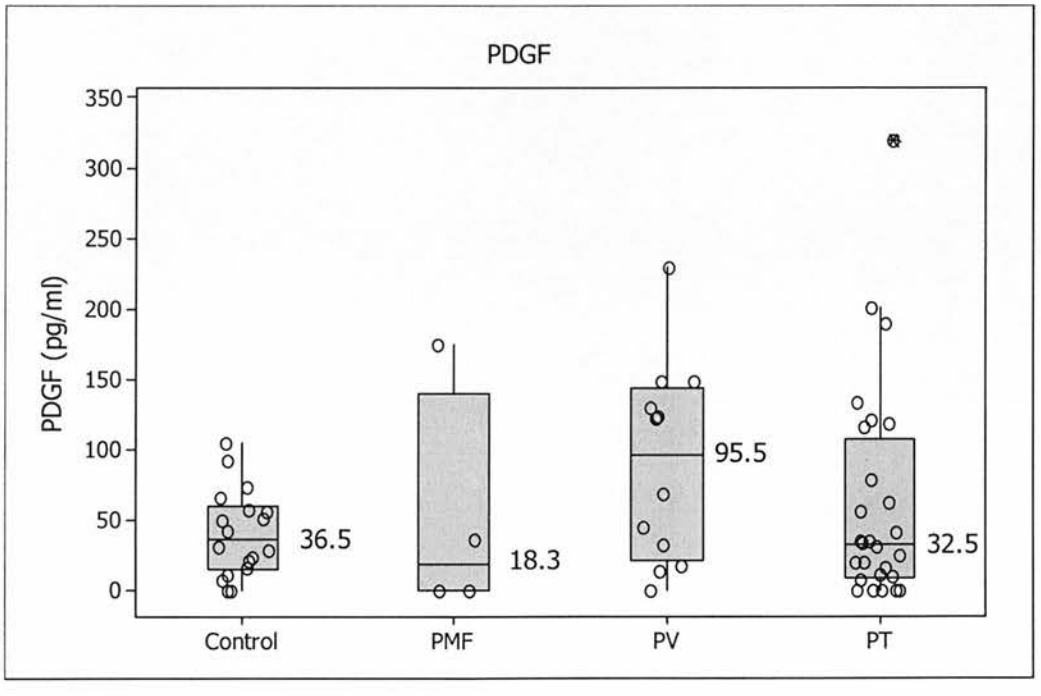


Figure 6.4 (d)

Figure 6.4 BTG, PF4, TGFβ and PDGF in patients and controls

Plasma levels of (a) BTG (b) PF4 (c) TGFβ and (d) PDGF in control subjects, PMF, PV and PT patients. Circles denote individual values and * denotes outlier results. Median values are shown.

6.3.4.5 Plasma levels of platelet α-granule contents and haematological parameters

There were no significant associations between haemoglobin or leucocyte count and BTG, PF4, TGFβ and PDGF values. Across the study cohort as a whole there were modest but significant correlations between platelet count and BTG (r =0.38, p=0.001), PF4 (r =0.36, p=0.002) and TGFβ (r =0.34, p=0.004). There was no significant correlation between PDGF and platelet count.

The higher BTG, PF4 and TGFβ in the patient group may therefore be confounded by the higher platelet counts in this group (Table 6.2). On multiple regression analysis the presence or absence of disease was the only significant predictor for BTG and TGFβ ($p<0.001$ for both) compared to platelet count which was not significant (p=0.236 and p=0.484 respectively). However platelet count was the only significant predictor for PF4 (p=0.008 v 0.164 for disease). To further explore the influence of platelet count we analysed the ratio of plasma BTG, PF4 and TGFβ to whole blood platelet count (Figure 6.5 (a-c)). The ratios of BTG and TGFβ to platelet count, but not PF4 to platelet count, were significantly higher in patients than controls.

There were also differences in platelet count within the patient group, being highest in PT patients and lowest in those with PMF (Table 6.2). Multiple regression analysis of the patient cohort only, revealed that both specific diagnosis and platelet count were significant predictors of BTG ($p<0.001$ and p=0.024). Diagnosis was the strongest predictor of TGFβ (p=0.001 v 0.077 for platelet count) while platelet count remained the most important predictor of PF4 (p=0.005 v 0.096 for diagnosis).

The BTG:platelet ratios were significantly higher in PMF and PV patients compared to controls (p=0.002 and p=0.013) but in the PT patients were not significantly different than controls (p=0.834). Similarly, TGFβ:platelet ratios were significantly higher in PMF and PV patients compared to controls (p=0.003 and p=0.029) but levels in the PT patients were not significantly different than controls (p=0.088). PF4:platelet ratios were significantly higher in PMF patients than controls (p=0.026)

but there was no difference between PV and PT patients and controls (p=0.283 and p=0.635 respectively).

6.3.4.6 Plasma levels of platelet α-granule contents and JAK2 V617F status

Within the PT patient group, JAK2 V617F mutational status was known in 35/38 patients of whom 16 were positive for the mutation. There were no significant differences between the mutated and wild type groups for BTG, PF4, TGFβ or PDGF (Table 6.6)

6.3.4.7 Plasma levels of platelet α-granule contents and treatment

There were no significant differences in BTG, PF4, TGFβ or PDGF levels between patients currently treated with aspirin or hydroxycarbamide or not (Table 6.7)

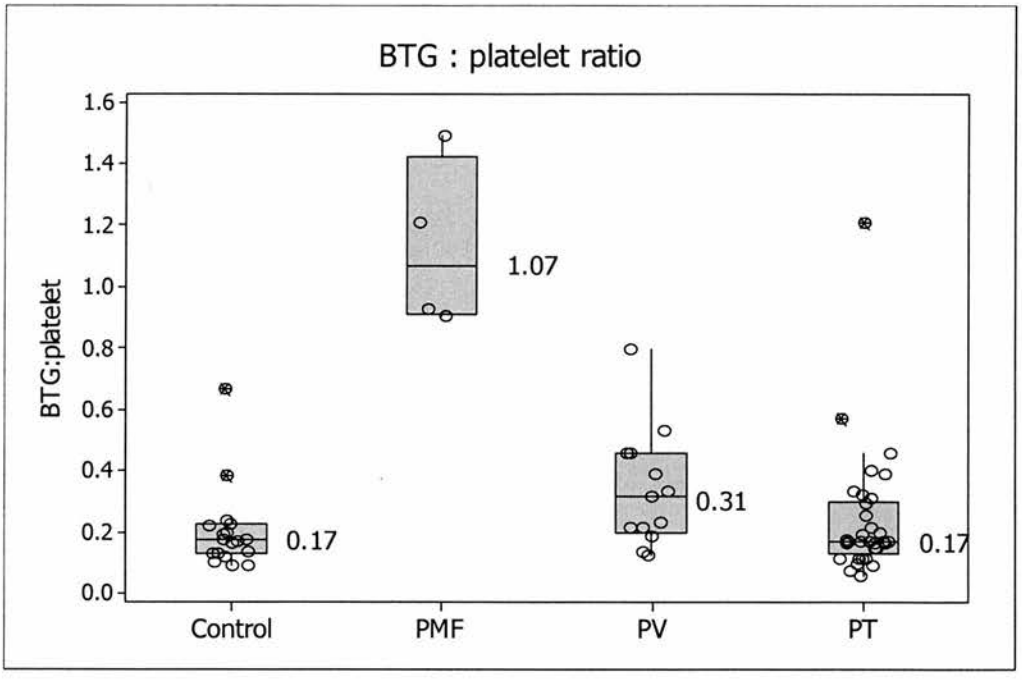


Figure 6.5(a)

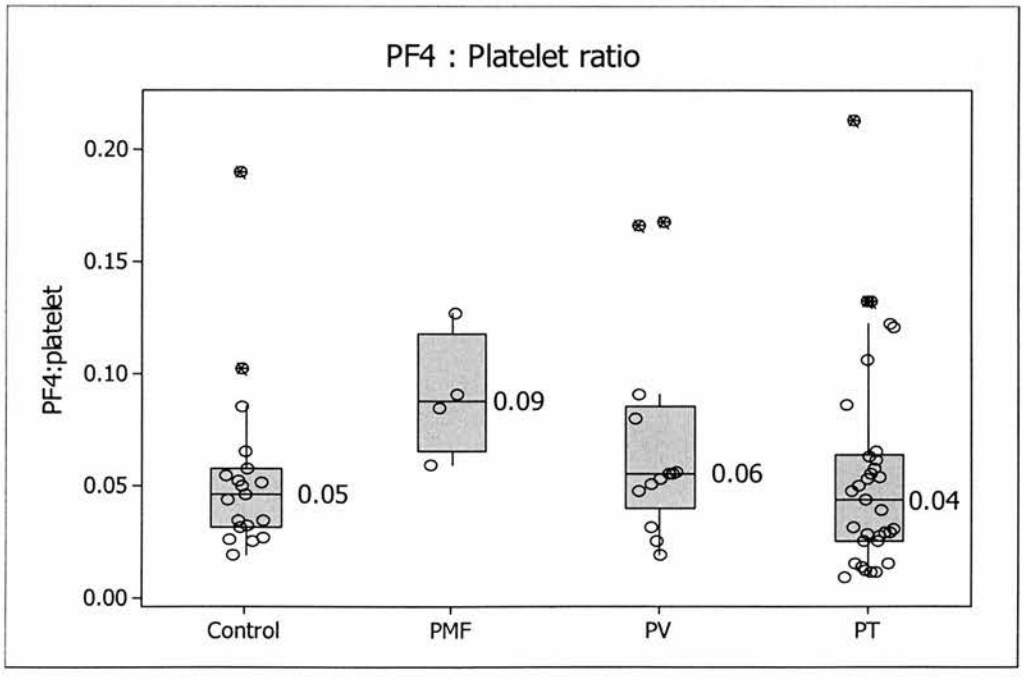


Figure 6.5(b)

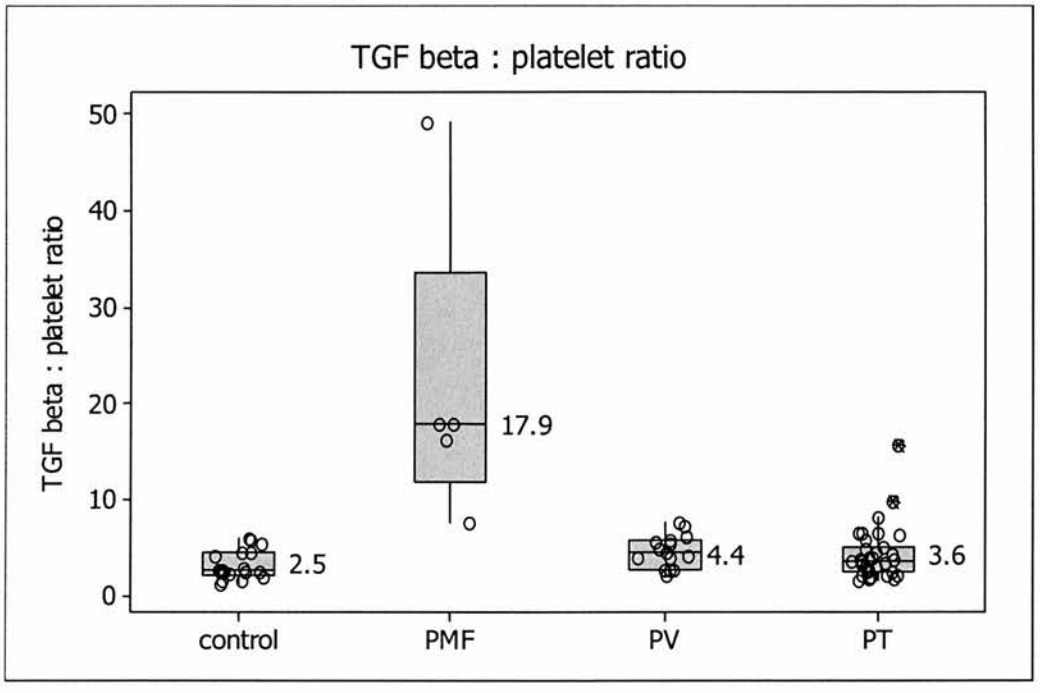


Figure 6.5 (c)

Figure 6.5 Ratio of plasma BTG, PF4 and TGFβ to whole blood platelet count

Ratio of plasma (a) BTG (b) PF4)and (c) TGFβ to whole blood platelet count in control subjects, PMF, PV and PT patients. Circles denote individual values and * denotes outlier results. Median values are shown.

	JAK2 wild type n = 19	JAK2 V617F N = 16	P value
BTG (IU/ml)	76.0 (60.8, 101.5)	61.0 (39.5, 100.5)	0.183
PF4 (IU/ml)	11.0 (7.5, 20.0)	18.0 (9.5, 31.5)	0.143
TGFβ (pg/ml)	1189 (848, 2069)	1516 (1130, 2059)	0.228
PDGF (pg/ml)	28.0 (8.5, 56.8)	35.0 (0.5, 133.0)	0.620

Table 6.6 BTG, PF4, TGFβ and PDGF in PT patients by JAK2 mutation status

Comparison of BTG, PF4, TGFβ and PDGF results in PT patients positive or negative for the JAK2 V617F mutation. Median and (IQR) values are shown. There were no significant differences between the two groups of patients.

	Hydroxycarbamide n = 46	No HC n = 6	P value
BTG (IU/ml)	81.5 (59.8, 140.8)	93.0 (63.8, 188.8)	0.637
PF4 (IU/ml)	21.0 (11.0, 38.8)	6.0 (5.8, 35.5)	0.103
TGFβ (pg/ml)	1483 (1071, 2314)	1872 (1468, 3426)	0.132
PDGF (pg/ml)	35.0 (14.5, 121.8)	0.5 (0.5, 97.6)	0.114
	Aspirin n = 40	No aspirin n = 13	P value
BTG (IU/ml)	74.0 (57.0, 131.0)	109.0 (73.8, 151.5)	0.102
PF4 (IU/ml)	22.0 (11.0, 44.0)	14.0 (8.3, 26.8)	0.299
TGFβ (pg/ml)	1416 (1068, 2336)	1521 (1346, 2455)	0.310
PDGF (pg/ml)	35.0 (18.5, 128.3)	11.0 (0.5, 128.3)	0.124

Table 6.7 BTG, PF4, TGFβ and PDGF in MPD patients by treatment

Comparison of BTG, PF4, TGFβ and PDGF results in all MPD patients according to treatment with or without hydroxycarbamide (HC) or aspirin. Median and IQR values are shown. There were no significant differences according to treatment.

6.3.5 BTG and TGFβ and relation to fibrosis in MPD patients

Bone marrow histopathology reports were available for 52/62 patients. All five patients with a diagnosis of PMF had Grade 3 or 4 fibrosis, (category C). Three PV patients were reported to have Grade 3 fibrosis, none of whom have developed clinical MF. One patient originally diagnosed with PT progressed to clinical MF around the time of the study. One patient initially presented with a platelet count $>2000x10^9$/L but defaulted from follow-up. He re-presented three months later with a progressive pancytopenia, splenomegaly and Grade 4 fibrosis, at which time he participated in the study. Without treatment, his pancytopenia and splenomegaly have subsequently resolved with a recurrence of thrombocytosis and his most recent trephine histology showed Grade 2 reticulin.

Platelet count, BTG, PF4, TGFβ and PDGF levels according to classification of fibrosis are shown in Table 6.8. There was no significant difference in platelet count or any of the plasma α-granule contents between those patients for whom reports were or were not available. Patients with more severe fibrosis (C) had significantly higher BTG and TGFβ levels compared to the group with milder fibrosis (A/B) although the ranges were very wide; there was no significant difference in PF4 or PDGF levels. The mean platelet count was lower in patients with more severe fibrosis due to the inclusion of thrombocytopenic PMF patients, but the difference was not statistically significant.

	Unknown n = 9	A/B n = 38	C n = 7	A/B v C P value
BTG (IU/ml)	78.0 (48.0, 92.0)	74.0 (57.0, 136.5)	115.5 (103.0, 378.0)	**0.036**
PF4 (IU/ml)	22.00 (8.5, 32.5)	17.00 (10.8, 35.0)	25.0 (6.0, 38.0)	0.730
TGFβ (pg/ml)	1846 (1178, 2301)	1307 (1017, 2159)	2750 (1521, 3499)	**0.007**
PDGF (pg/ml)	30.0 (17.0, 68.0)	43.0 (8.5, 132.3)	27.0 (0.5, 135.0)	0.5889
Platelets x 10^9/L	390.0 (336.3, 449.3)	389.0 (325.0, 449.5)	379.0 (85.0, 417.0)	0.184

Table 6.8 BTG, PF4, TGFβ and PDGF and degree of marrow fibrosis

Comparison of BTG, PF4, TGFβ and PDGF results in the MPD patients only according to grading of bone marrow fibrosis described on most recent trephine biopsy histopathology report. Fibrosis was categorised A-C as described in Table 6.1. A/B represents milder reticulin fibrosis (Bauermeister Grade 0-2) compared to C, more severe fibrosis (Bauermeister Grade 3-4). Median and IQR values are shown. BTG and TGFβ were significantly higher in patients with more severe fibrosis. Statistically significant differences are highlighted in bold.

6.3.6 Assessment of bone marrow fibrosis

Bone marrow histopathology reports were available for 52/62 patients and 5/8 control subjects with secondary polycythaemia or reactive thrombocytosis. Archived bone marrow trephine slides were available and suitable for evaluation for 33/62 patients and 4/8 secondary controls. Table 6.9 (below) shows the numbers graded in each of the categories of reticulin fibrosis.

	Missing	A	B	C
Histopathology report	13	28	19	10
Researcher	34	20	8	8
Pathologist	33	7	6	24

Table 6.9 Grading of bone marrow trephines for fibrosis

Of the 35 bone marrow trephines where the histopathology report was available and the trephine slides were evaluated. Concurrence of grading between the report and either the researcher or the pathologist is tabulated below (Table 6.10).

	A	**B**	**A/B**	**C**
Report/Researcher (35)	12/16	4/13	23/29	2/6
Report/Pathologist (36)	4/17	1/13	11/30	5/6

Table 6.10 Concurrence of grading for bone marrow fibrosis

6.4 Discussion

We observed elevated plasma levels of platelet α-granule proteins in patients with MPD compared to both healthy controls and those with secondary thrombocytosis or polycythaemia. This included elevated plasma levels of the profibrotic cytokine TGFβ, which was highest in those patients with more marked bone marrow fibrosis. In some of the patient groups, differences in the plasma levels of the platelet α-granule proteins could be attributed to the higher platelet counts. However a disease effect independent of platelet count was also observed, especially in the PMF and PV patients. Our results also cast uncertainty on the reliability of using the BTG:PF4 ratio to recognise *in vitro* platelet activation and reinforce the problems of inter-observer variability in the assessment of bone marrow histology.

Sampling and use of the BTG:PF4 ratio

We aimed to measure circulating levels of platelet α-granule proteins in treated MPD. Plasma samples were chosen to try to assess the constitutive release of α-granule contents *in vivo*. Other studies have measured serum levels, which may be more reflective of the intraplatelet content which is released following *ex vivo* platelet degranulation. Previously published data has suggested that due to the different clearance kinetics of BTG and PF4 *in vivo*, a reduced BTG:PF4 ratio (less than 2-3) may indicate *in vitro* activation and conversely that higher ratios would exclude this (Kaplan & Owen, 1981).

Examination of the 56 duplicate sets of results in the current study revealed inconsistencies in the relationship of BTG:PF4 ratios to the absolute values. In half of the pairs where one ratio was less than 2.5, the associated BTG and TGFβ results were both higher, which would be consistent with the theory that a reduced ratio may indicate *in vitro* platelet granule release. However in the remainder, the BTG and TGFβ results from both occasions were similar despite differences in the PF4 level and consequently the BTG:PF4 ratio. Conversely, in 32 of the 56 paired results both

had a BTG:PF4 ratio greater than 2.5, but in 13 of these there was at least a two-fold difference in the BTG or TGFβ between the samples.

These observations question the usefulness of the BTG:PF4 ratio for determining whether *in vitro* activation has occurred in a single sample. In addition the previous studies of BTG:PF4 ratios did not consider the effect of thrombocytosis specifically but did suggest that the BTG:PF4 ratio varies inversely with the BTG level. Lower ratios might therefore be expected in view of the elevated BTG levels seen in the MPD patients.

The observed discrepancies in the absolute results and the BTG:PF4 ratios between episodes may reflect a number of factors. The patients and controls were all tested in a clinic setting which introduces methodological variables known to influence platelet activation; phlebotomy was performed by a number of different individuals and it is therefore likely that technique was variable. Individual patient variation due to external influences such as concomitant illness or drug changes may also have resulted in true variation in the plasma levels of the proteins.

The use of duplicate samples and selection of those with the lower BTG level was intended to help exclude those samples in which activation and α-granule release may have occurred *in vitro*. This may however have attenuated the observed differences between patients and controls, if some of the higher values did in fact reflect biological variation as opposed to *in vitro* activation.

Assessment of Fibrosis

The bone marrow reports available for the patients span a period of 25 years from 1981 to 2006 and were reported in a non-standardised way by a number of different histopathologists. Clearly this introduces bias in both the assessment made of the degree of fibrosis and our interpretation of the report. To try to appreciate the degree of inter-observer variability in assessment and reporting, the trephines were

reassessed by the researcher and an independent histopathologist and comparison was made with the original report.

Initially the reports of the researcher and histopathologists were divided into normal, intermediate or marked fibrosis (Grades grade A – C) based on the Bauermeister grading system and the reports were similarly interpreted. Using this system there was concurrence of all three assessments for only 6/35 reports. Comparing the reports for the researcher and the histopathologist, a significant degree of inter-observer variation was apparent even when the trephines were assessed by the same grading system. There also appeared to be systematic bias towards reporting either a lower or higher degree of fibrosis and inter-observer variability was most marked at the intermediate grades of fibrosis. Combining groups A and B, who have no fibrosis or a non-pathological degree of fibrosis, compared to group C, resulted in improved concurrence with the original report of 25/35 and 16/36 for the researcher and histopathologist respectively. Our findings reflect the recognised difficulties in the use of trephine histology in the diagnosis of MPD, particularly outside the research setting or specialist centres (Kuter *et al*, 2007). We did not reassess megakaryocyte morphology which is used in the WHO criteria to distinguish pre-fibrotic and early myelofibrosis from PT but which is also recognised to be difficult to assess reproducibly (Campbell & Green, 2005).

BTG, PF4 and TGFβ - clinical and haematological associations

In keeping with previous studies our results show that plasma levels of the platelet α-granule proteins BTG, PF4 and TGFβ are elevated in MPD patients compared to both healthy controls. Furthermore, levels were elevated compared to patients with either secondary polycythaemia or thrombocytosis, which has not been previously reported. Of the MPD patients, those with PMF had the highest BTG, PF4 and TGFβ levels but this was only statistically significant for TGFβ compared to PV and PT patients and for BTG compared to PT patients. The lack of statistical

significance for some of these comparisons is likely to reflect the wide range in results and the small number of PMF patients in the study.

TGFβ has been clearly associated with marrow fibrosis in PMF and by extrapolation is also implicated in myelofibrosis complicating the other MPD (Chagraoui *et al*, 2002;Dong & Blobe, 2006;Rameshwar *et al*, 1998;Yanagida *et al*, 1997). A lesser degree of reticulin fibrosis may also be present at diagnosis in PV and PT or may develop during the course of the disease. In the ten patients (five PMF, three PV and two PT) who were reported to have bone marrow fibrosis equivalent to Grade 3 or higher, we observed higher BTG and TGFβ levels compared to those with fibrosis equivalent to Grade 2 or less. However many patients within the lower grade fibrosis group (which may be considered to be within normal limits) had BTG and TGFβ levels above the median level of those with higher grade fibrosis and overall had significantly higher BTG and TGFβ levels compared to controls.

Where available the most recent bone marrow report was used; post-diagnosis bone marrow assessments were likely to have been performed for a clinical indication such as suspicion of progression. However, for the majority of patients only a diagnostic marrow had been performed. It is therefore possible that the degree of fibrosis at the time of the study, although subclinical might have progressed since diagnosis thus underestimating the association with BTG and TGFβ levels. Repeat bone marrow examinations would be required to explore this possibility.

As the factors that we have measured are platelet and megakaryocyte derived we might expect them to be higher in the patient group due to their higher platelet counts. Consistent with this, moderate but significant correlations were observed between the whole blood platelet count and BTG, PF4 and TGFβ. Multivariate analysis suggested that the presence or absence of disease was the most important factor determining BTG and TGFβ but that platelet count was more significant for PF4. In keeping with this, the ratios of BTG and TGFβ to platelet count were higher in patients than controls but PF4 to platelet ratios were similar.

Platelet counts were also variable between the patient groups, being lowest in PMF and highest in PT. Multivariate analysis within the patient group only suggested that both disease type and platelet count were significant determinants of BTG but that diagnosis was the most important predictor of TGFβ and that platelet count remained the more significant determinant of PF4. Investigation of the factor to platelet ratios within the patient subgroups showed that BTG and TGFβ ratios were elevated in PMF and PV patients but in PT patients were similar to controls.

Pathophysiology of increased plasma BTG, PF4 and TGFβ

The above observations suggest two separate mechanisms whereby levels of platelet α-granule proteins are elevated in MPD. The first is that the platelet mass itself does, as would be expected, influence the plasma levels of platelet α-granule proteins and that this may be particularly relevant in PT patients. Uncontrolled thrombocytosis might therefore be expected to increase the risk of fibrosis in an individual via the release of higher quantities of the profibrotic TGFβ. This might explain the increased rates of fibrotic progression seen in follow-up from the PVSG-01 trial in PV patients treated with phlebotomy alone compared to a cytoreductive agent (Najean *et al*, 1994). Against this theory is the apparently increased rate of fibrotic progression observed in the anagrelide arm of the high risk PT-1 trial despite equivalent control of the platelet counts (Harrison *et al*, 2005). We found no difference in platelet α-granule levels in relation to treatment with hydroxycarbamide but the majority of patients who were not treated with hydroxycarbamide either did not have a thrombocytosis (in the case of PV patients), or received an alternative cytoreductive.

Secondly, our observations suggest that in MPD patients, particularly those with PMF, there is also a qualitative difference in platelet release of BTG and TGFβ. One mechanism for this could be that increased platelet activation in MPD patients may promote platelet degranulation with release of α-granule contents. We observed no difference in BTG and TGFβ levels in relation to treatment with aspirin but increased

platelet activation has been observed in MPD despite aspirin therapy. Other postulated mechanisms of α-granule release include neutrophil-platelet interactions (Schmitt *et al*, 2000) or intracellular NFkB activation by dysregulated Tpo signalling as can be caused by the JAK2 V617F mutation (Rameshwar *et al*, 2000;Khwaja, 2006;Komura *et al*, 2005). We found no association between BTG and TGFβ levels and the presence or absence of the JAK2 V617F mutation. Neither was there an association with leucocyte count, however we did not measure PLA which are reported to be elevated in patients with PT and PV (Falanga *et al*, 2005;Jensen *et al*, 2001).

As these postulated mechanisms of increased α-granule protein release are common to the MPD they do not explain the observation that BTG and TGFβ levels were higher in patients with clinical PMF or higher grade fibrosis than in the other patient groups (independent of platelet count). This suggests either that these mechanisms may be more active in some patients, or that additional sources are important, for example megakaryocytes. Another possibility is that non-platelet or megakaryocyte sources of TGFβ are significant but this would not explain the correlation of TGFβ and BTG.

We also observed that PF4 levels were more dependent on platelet count than disease status and that the BTG to PF4 ratio was significantly higher in PMF patients. This might be more in keeping with specific changes in the production and/or release of TGFβ (and BTG) as opposed to non-specifically increased release of all platelet α-granule proteins. However the more rapid clearance of PF4 from plasma (compared to TGFβ and BTG), resulting in relatively lower levels of this protein, may have influenced any observed associations.

PDGF levels

In contrast to the findings of previous studies, PDGF levels were not significantly different between the patient and control groups (Gersuk *et al*, 1989;Lev *et al*, 2002). No correlation was seen between PDGF levels and those of the other α-granule proteins, although similar to TGFβ and BTG, there was a weak correlation with the platelet count. Again, this might suggest that the elevated levels of BTG and TGFβ are not the result of a general increase in the release of platelet α-granule proteins. However any interpretation should be made with caution in view of the high number of ELISA results falling below zero on the standard curve. The cause of this problem was unclear but as it did not arise with the other measurements, it suggests a problem with the assay rather than the samples. It is also possible that the quality of the frozen samples may have deteriorated due to the delay in analysis of PDGF because of assay supply problems.

Summary

Our observations of elevated plasma levels of α-granule proteins, including TGFβ, in MPD patients compared to both healthy controls and to patients with reactive thrombocytosis or secondary polycythaemia, concur with and extend the findings of previous studies. Furthermore, we observed that the highest levels were in those patients with greater degrees of bone marrow fibrosis, which supports the pivotal role of TGFβ in fibrosis. The strong correlation of TGFβ with BTG confirms that platelet α-granules are an important source of TGFβ in these patients. As expected the plasma levels of the α-granule proteins correlated with platelet count and this appeared to be a significant factor contributing to the elevated levels in PT patients. However in the patients with PMF and PV plasma levels were disproportionately elevated compared to the platelet count, suggesting a specific mechanism of increased α-granule release in this group of patients. Elucidation of such a mechanism may help to direct treatment towards reducing lowering the risk of fibrotic transformation.

CHAPTER 7

GENERAL DISCUSSION

The main objective of this work was to set up an assay to measure plasma microparticles and to apply this to a population of patients with MPD in whom we might expect microparticles to be elevated considering their prothrombotic risk. We also wished to identify any association of microparticle levels with clinical and laboratory characteristics and to compare this novel measurement to established markers of platelet and vascular abnormalities. In addition we investigated the role of platelets in the fibrotic complications of MPD.

Microparticle assay

We have established a method for the measurement of plasma microparticles by flow cytometry. This method includes the quantitation of microparticles in cell free samples thus permitting more reliable comparisons between samples analysed at different times. The assay was optimised for the effects of preanalytical variables, the most important of which was identified as the centrifugation protocol; slower centrifugation resulted in greater platelet contamination of the samples and increased numbers of PMP. Previously published studies have used a variety of centrifugation protocols in their sample preparation and this is therefore one factor limiting the comparison of results between studies. A more important implication may be the effect of this preanalytical variable on the investigation of microparticle coagulation activity; coagulation assays dependent on phospholipid activity may be significantly influenced by residual platelet phospholipid in the samples. This is likely to be a particular problem for stored samples that have been freeze-thawed as this process will increase platelet membrane phospholipid exposure.

When the current assay was applied in practice to the measurement of microparticles in MPD patients, a number of problems were identified. We frequently encountered high levels of positivity in the negative isotype controls for the CD31 monoclonal antibody that was used in the identification PMP and EMP. This had a more significant effect of the measurement of EMP due to their relatively lower numbers and so the investigation of the use of other endothelial markers would be helpful.

The numbers of LMP that we identified in both patients and controls was proportionately somewhat lower than has been reported in previously published studies (Daniel *et al*, 2006;Berckmans *et al*, 2001). We used a CD45 monoclonal antibody that identified leucocytes in samples of whole blood however the use of an *in vitro* model of LMP generation might be a preferable positive control.

A further issue that we identified with the current method was that the population of small platelets was continuous with that of microparticles (by forward light scatter measurement) using a size limit of 1μm for differentiation. Although this is the widely accepted size limit for microparticle identification there is no clear evidence for its use (Biro *et al*, 2004;Dignat-George *et al*, 2004;Hugel *et al*, 2004;Jimenez *et al*, 2004;Jy *et al*, 2004;Nomura, 2004;Shet *et al*, 2004). Concomitant measurement of Annexin V binding as a measure of membrane PPS in comparison to particle size may aid differentiation of PMP and small platelets, as PMP would be expected to have proportionately greater membrane PPS exposure (Sinauridze *et al*, 2007). We also recognised a population of events of <1μm that did not stain for the cell specific antigens used. Annexin V double staining would help to identify whether this population represent microparticles or are non-significant debris, which can be a problem at the lower threshold of the detection ability of the flow cytometer.

Interest in the investigation of microparticles has greatly expanded in the last few years, even since we began this project. Data similar to ours with regard to the influence of preanalytical variables, especially centrifugation and platelet contamination, has been presented by a number of other investigators. There has also been a move away from the measurement of PMP and towards measurement of EMP and LMP, in particular monocyte microparticles. This may reflect some of the difficulties that we have described with the reliable discrimination of PMP from platelets. In addition, the use of functional assays that can assess the pathophysiological properties of microparticles, has taken prominence. There is now more emphasis on the identification of microparticles bearing tissue factor and the measurement of their coagulation potential. The use of flow cytometry with double staining for cell specific markers and functional molecules, in combination with

coagulation type assays, may be the most appropriate way to take forward our investigation of plasma microparticles in disease states. With this knowledge, we plan further refinement of the assay with regard to the choice of cell specific antibodies and double staining for tissue factor and annexin V binding. Samples have also been stored for thrombin generation analysis as a measure of microparticle coagulation potential. However we will need to determine whether the residual platelets in these frozen samples will obscure any meaningful data.

Microparticles in myeloproliferative disorders

We were able to recruit a cohort of patients with PT and PV with a demographic comparable to the overall local group of patients with MPD although the PT patients included in the study were slightly younger. The clinical and laboratory characteristics of the study cohort were largely similar to those reported in previously published studies. However we observed a lower incidence of thrombotic events both at presentation and during follow-up (Gangat *et al*, 2006;Gangat *et al*, 2007;Harrison *et al*, 2005;Marchioli *et al*, 2005;Passamonti *et al*, 2004;Wolanskyj *et al*, 2006). This is in line with recent trends towards a greater proportion of asymptomatic patients being diagnosed with PT or PV after investigation for an incidental finding of thrombocytosis or raised haematocrit on the ever-increasing workload of peripheral blood counts (Jensen *et al*, 2000b).

We observed increased numbers of PMP and EMP in the PV and PT patients compared to a healthy control group but the influence of a number of potential confounding factors should be considered. Our control population were significantly younger than the patient cohort as a whole and within the patient cohort an effect of age on PMP numbers was evident. Further, the majority of the patient cohort had either a history of cardiovascular disease or risk factors; circulating microparticles have been reported to be elevated in vascular disease in a number of previous studies (Amabile *et al*, 2005;Bernal-Mizrachi *et al*, 2003;Koga *et al*, 2005;Preston *et al*, 2003;Simak *et al*, 2006). Comparison of our patient cohort with a control group

matched for the presence of cardiovascular disease or risk factors would allow investigation of whether microparticles are elevated in patients with MPD in excess of these background factors.

It is however important to consider that the incidence of thrombotic complications in younger patients with MPD, although lower than in older patients, remains greater than that of an age-matched healthy population (Alvarez-Larran *et al*, 2007). It is therefore relevant that in our younger patients without a history of cardiovascular disease, both PMP and EMP were significantly elevated compared to controls. We cannot exclude the presence of unrecognised cardiovascular disease in these patients and certainly the low incidence of hypercholesterolemia and smoking suggests that these may be under-reported or under-diagnosed in our patient group. Further, microparticles have been reported to be predictive of sub clinical atherosclerosis in diabetic patients and this would be another possible explanation. The measurement of microparticles in a larger number of low risk MPD patients and thorough cardiovascular investigation may be enlightening in this regard.

Our observations of the relationship between microparticles and the more established markers of platelet and endothelial dysfunction were mixed. A correlation between PMP and sP-sel was noted, in keeping with PMP being a marker for platelet activation. However as sP-sel was measured in PPP prepared in the same way as for microparticle measurement we cannot exclude that in fact this "soluble" form was in fact microparticle bound. Concomitant measurement of PMP, microparticle positivity for P-sel by flow cytometry and measurement of sP-sel in PPP and microparticle free plasma would be useful to explore this. We did not measure platelet-leucocyte aggregates (PLA) that are elevated in MPD and other vascular disorders, but it is probable that some of the leucocyte positivity for platelet antigens is in fact due to PMP-leucocyte binding. This may merit further investigation, as the functional potential of such cell-bound microparticles is unknown.

Although altered endothelial dysfunction has been shown in MPD by plethysmography, previous studies of soluble endothelial markers have reported

mixed results (Bellucci *et al*, 1993;Falanga *et al*, 2000;Musolino *et al*, 2000;Neunteufl *et al*, 2001;Robertson *et al*, 2007). Despite the elevation in EMP we did not observe an elevation in sE-sel or a correlation between EMP and sE-sel. One reason for this may be that whilst we used antigens constitutively expressed by endothelial cells to measure EMP, in comparison sE-sel is expressed only on activated endothelium. Differences in the expression of a variety of endothelial antigens by EMP *in vivo* have been reported in patients with acute CVA and *in vitro* studies have suggested different EMP antigen profiles depending on the nature of the microparticle trigger (Jimenez *et al*, 2003b;Simak *et al*, 2006). This may suggest that EMP are released by non-activating stimuli in MPD; again concomitant measurement of EMP by constitutive and activation antigens would be useful in this regard. We did however find a correlation between sP-sel and EMP. This may simply reflect a general prothrombotic state in these patients but an alternative explanation is that PMP induced by activating stimuli have been shown *in vitro* to induce endothelial cell changes which may result in EMP release (Barry *et al*, 1997;Barry *et al*, 1998).

Interestingly, microparticle numbers were elevated despite the fact that the majority of the patient cohort was receiving standard therapy with venesection and/or cytoreductives. This is in keeping with the observation that although correction of the elevated haematocrit and thrombocytosis reduces thrombotic risk, it does not normalise it. Serial measurement of microparticles in new patients commencing treatment would be useful to examine the effects of cytoreduction on microparticle levels. The point data collected suggested that our patient group may have been less aggressively managed compared to current recommendations, however recent studies have reported no increased risk of thrombosis at comparable haematocrit or platelet levels (Di Nisio *et al*, 2007). The observation that PMP have proportionately higher procoagulant activity compared to platelets implies that despite relatively normal platelet counts higher circulating levels of microparticles could significantly increase thrombin generation potential (Sinauridze *et al*, 2007). It is likely that in the future, studies in other patient groups with vascular or prothrombotic disorders will address the effects of treatment modalities on microparticle levels and in view of their

procoagulant activities microparticles may themselves become a potential therapeutic target.

The importance of leucocytes in MPD is increasingly recognised, with the observation of elevated PLA and the association between leucocyte count at diagnosis and thrombosis – an effect that is abrogated by the reduction of the leucocyte count by cytoreductive therapy (Carobbio *et al*, 2007). The absence of a significant difference in LMP was therefore perhaps surprising and possible problems with the LMP assay have already been discussed. As with the possibility of PMP binding to leucocytes, any circulating LMP may become platelet bound, a possibility that could be investigated by measurement of platelet positivity for leucocyte antigens or tissue factor.

We did not identify a direct correlation between microparticle numbers and thrombotic history, similar to many previous studies that have identified vascular abnormalities in MPD patients and to studies of microparticles in other prothrombotic disorders. At least in part, this is likely to reflect the small numbers of thrombotic events in our patient population but also it is also probable that a combination of factors contribute to the thrombotic risk. There has been much interest in the role of the JAK2 V617F mutation in thrombosis and an association with the incidence of venous thrombosis has been reported in two large studies but not by others (Campbell *et al*, 2006;Carobbio *et al*, 2007;Kittur *et al*, 2007;Wolanskyj *et al*, 2005). Two recent studies investigating vascular markers in myeloproliferative patients reported an association of the mutation with markers of platelet activation (Arellano-Rodrigo *et al*, 2006;Robertson *et al*, 2007). In contrast we did not identify a correlation of sP-sel or microparticles with the JAK2 V617F mutation status in the PT patients. However one of these studies did not differentiate PT from PV patients who comprised the majority of patients in the JAK2 positive group which may have confounded their results.

Platelets and myelofibrosis

The other direction of our investigation of the role of platelets in the complications of MPD was in relation to myelofibrosis. The importance of the profibrotic cytokine TGFβ is well established but the mechanism of its elevation in MPD patients is less clear. As TGFβ is contained within the α-granules of both platelets and megakaryocytes, we investigated whether other α-granule proteins were similarly elevated and if circulating levels were related to fibrosis. In keeping with previously published studies we found significantly elevated plasma levels of TGFβ in the MPD patients compared to controls (Rameshwar *et al*, 1998). We included a control group of patients with secondary polycythaemia or thrombocytosis, excluding thrombocytosis *per se* as the explanation for this difference. Furthermore, we observed that levels were highest in patients with PMF, however the number of these patients was small and the range of TGFβ levels was wide. Investigation of more of these patients would be desirable to confirm and validate the results. We also observed a correlation between severity of marrow fibrosis and TGFβ levels in the PV and PT patients. However the degree of fibrosis was based on the most recent trephine report, which in the majority of cases was from diagnosis. The strength of the association may therefore have been underestimated if fibrosis had progressed since diagnosis. This could also explain the observation of elevated TGFβ levels in the range of the PMF patients in a number of PV and PT patients who had no increase in fibrosis. Clearly further bone marrow assessment of these patients would be required to investigate this possibility.

We also observed an increase in the circulating levels of other α-granule proteins, with BTG and PF4 similarly elevated and BTG strongly correlated with TGFβ levels. This would support a general mechanism of increased release of platelet α-granule contents as opposed to specific release of TGFβ from platelets or another source. Comparison with PDGF levels would have been useful to support this interpretation of our results but these were difficult to analyse due to technical difficulties with the assay. We did not directly investigate the mechanism of increased release of platelet α-granule proteins but the elevated sP-sel and PMP in our other MPD patient cohort

would support platelet activation as at least one contributory mechanism. Another question is the degree to which systemic TGFβ levels influence bone marrow fibrosis, if at all or whether megakaryocyte TGFβ is more important. If local production of TGFβ is more important, then the correlation of systemic platelet α-granule protein levels with fibrosis may suggest a shared mechanism of increased release, for example due to defective granule packaging.

Platelets in PV and PT

Although our two MPD patient cohorts were separate, the clinical characteristics of the PV and PT patients were similar and certainly there was some crossover in the patients. The results of both studies underline the persistent platelet related abnormalities in MPD patients despite standard therapy. We did not observe a difference in the measured platelet parameters (PMP, sP-sel and platelet α-granule proteins) between the JAK2 positive and negative PT patients consistent with the absence of a prognostic effect of JAK2 status on clinical course and in particular thrombotic risk. However, interestingly, in both studies the influence of the peripheral platelet count on the measured platelet parameters was greater in the PT patients than the PV patients. This suggests that there may be qualitative differences in the platelets between PV and PT with perhaps greater platelet activation in the PV patients. The mechanisms responsible for these platelet abnormalities remain elusive although, as discussed, leucocytes are increasingly implicated in the vascular disturbances in MPD and may be important in platelet activation. The observed similarities in the JAK2 wild-type and mutated PT patients and the differences between the PT and PV patients support the theory that other as yet undetected molecular abnormalities are likely to be important in the pathophysiology of these disorders.

In summary, our investigation of platelet and vascular abnormalities in MPD we have combined two areas of growing interest. The discovery of the JAK2 tyrosine kinase mutation in MPD has renewed enthusiasm for investigation into the mechanisms responsible for these disorders and their thrombotic and fibrotic complications. As in previous studies, we have observed a variety of vascular and platelet abnormalities in MPD patients which are not reversed by our current therapeutic options of cytoreduction and antiplatelet agents. That differences in these abnormalities exist between the clinical groups but are not associated with JAK2 mutation status, confirms the need to identify whether other pathogenic molecular mechanisms exist. Such mechanisms may be potential therapeutic targets to reverse not only the cellular overproliferation but also the vascular abnormalities of MPD. The field of microparticles is also rapidly evolving, with a growing emphasis on investigation of their functional properties. Further optimisation of our current assay by minimisation of platelet contamination, identification of microparticle phenotype by functional molecules in addition to cell specific antigens and its use in conjunction with a coagulation assay will hopefully produce a useful addition to our current research tools and will be applicable to the investigation of a variety of vascular disorders.

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APPENDIX A - GUIDELINES FOR THE DIAGNOSIS OF POLYCYTHEMIA VERA AND PRIMARY (ESSENTIAL) THROMBOCYTHEMIA

PVSG parameters for the Diagnosis of Polycythaemia Vera (Murphy *et al*, 1986)

Categories

A1. Raised red cell mass
Male > 36ml/kg
Female >32ml/kg

A2. Normal arterial oxygen saturation>92%

A3. Splenomegaly

B1. Thrombocytosis
Platelet count >400 x 10^9/l

B2. Leucocytosis >12 x 10^9/l
No fever or infection

B3. Raised leucocyte alkaline phosphatase >100
No fever or infection
Raised serum B12 (>900pg/ml)

Diagnosis acceptable if following combinations are present: A1 + A2 + A3; A1 +A2 + any two from category B.

British Committee for Standards in Haematology (BCSH) Guidelines for the diagnosis of polycythaemia vera (McMullin *et al*, 2005)

Major
A1: Raised red cell mass (>25% above mean normal predicted value*) or Hct ≥0·60 males; ≥0·56 females
A2: Absence of cause for secondary erythrocytosis (consider possibility of dual pathology)
A3: Palpable splenomegaly†
A4: Clonality marker, i.e. acquired abnormal marrow karyotype
Minor
B1: Thrombocytosis (platelet count >400 × 10^9/l)
B2: Neutrophil leucocytosis (neutrophil count >10 × 10^9/l in non-smokers; >12·5 × 10^9/l in smokers)
B3: Splenomegaly (demonstrated on isotope/ultrasound scanning)†,‡
B4: Characteristic BFU-E growth or reduced serum erythropoietin§
A1 + A2 + A3 or A4 establishes PV. A1 + A2 + any 2B criteria establishes PV.
RCM – mean normal predicted value: for males =(1486 × S) − 825 ml; for females = (1·06 × age) + (822 × S*) ml (*S, surface area).
†Without evidence of a secondary cause such as portal hypertension.
‡Splenomegaly can be calculated from the ultrasound result
§Serum erythropoietin level varies depending on the assay used

Amendment to the BCSH guideline for diagnosis and investigation of polycythaemia /erythrocytosis (McMullin *et al*, 2007)

***JAK2*-positive polycythaemia vera**	
A1	High haematocrit (>0.52 in men, >0.48 in women) OR raised red cell mass (>25% above predicted)*
A2	Mutation in *JAK2*
Diagnosis requires both criteria to be present	
***JAK2*-negative polycythaemia vera**	
A1	Raised red cell mass (>25% above predicted) OR haematocrit $\geq$0.60 in men, $\geq$0.56 in women.
A2	Absence of mutation in *JAK2*
A3	No cause of secondary erythrocytosis
A4	Palpable splenomegaly
A5	Presence of an acquired genetic abnormality (excluding *BCR-ABL*) in the haematopoietic cells
B1	Thrombocytosis (platelet count >450 × 10^9/l)
B2	Neutrophil leucocytosis (neutrophil count > 10 × 10^9/l in non-smokers; >12.5 × 10^9/l in smokers)
B3	Radiological evidence of splenomegaly
B4	Endogenous erythroid colonies or low serum erythropoietin
Diagnosis requires A1 + A2 + A3 + either another A or two B criteria	
*Dual pathology (co-existent secondary erythrocytosis or relative erythrocytosis) may rarely be present in patients with a *JAK2*-positive myeloproliferative disorder. In this situation, it would be prudent to reduce the haematocrit to the same target as for polycythaemia vera.	

2001 WHO criteria for polycythemia vera (Michiels *et al*, 2006)

A-criteria

1. Elevated red cell mass > 25% above mean normal predicted value, or hemoglobin > 18.5 g/dL in men, 16.5 g/dL in women, or > 99th percentile of method-specific reference range for age, sex, altitude of residence
2. No cause of secondary erythrocytosis, including:
   a. Absence of familial erythrocytosis
   b. No elevation of erythropoietin caused by:
      i. Hypoxia (arterial $pO_2 \leq 92\%$)
      ii. High oxygen affinity hemoglobin
      iii. Truncated erythropoietin receptor
      iv. Inappropriate erythropoietin production by tumor
3. Splenomegaly
4. Clonal genetic abnormality other than Philadelphia chromosome or *BCR-ABL* fusion gene in marrow cells
5. Endogenous erythroid colony formation

B-criteria

1. Thrombocytosis > 400 x 10^9/L
2. Leukocytosis > 12 x 10^9/L
3. Bone marrow biopsy showing panmyelosis with prominent erythroid and megakaryocytic proliferation
4. Low serum erythropoietin levels

Diagnosis requires the presence of the first 2 A-criteria together with either any 1 other A-criterion or 2 B-criteria.

2008 Proposed revised WHO criteria for polycythemia vera (Tefferi *et al*, 2007)

Major criteria

1. Hemoglobin > 18.5 g/dL in men, 16.5 g/dL in women or other evidence of increased red cell volume*
2. Presence of *JAK2*617V>F or other functionally similar mutation such as *JAK2* exon 12 mutation

Minor criteria

1. Bone marrow biopsy showing hypercellularity for age with trilineage growth (panmyelosis) with prominent erythroid, granulocytic, and megakaryocytic proliferation
2. Serum erythropoietin level below the reference range for normal
3. Endogenous erythroid colony formation in vitro

Diagnosis requires the presence of both major criteria and 1 minor criterion or the presence of the first major criterion together with 2 minor criteria.

* Hemoglobin or hematocrit greater than 99th percentile of method-specific reference range for age, sex, altitude of residence or hemoglobin greater than 17 g/dL in men, 15 g/dL in women if associated with a documented and sustained increase of at least 2 g/dL from an individual's baseline value that can not be attributed to correction of iron deficiency, or elevated red cell mass greater than 25% above mean normal predicted value

PVSG Diagnostic criteria for essential thrombocythaemia (Murphy *et al*, 1986)

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1. Platelet count > 600,000/μl
2. Haemoglobin <13g/dl or normal red cell mass
3. Stainable iron in marrow or failure of iron trial (<1g/dl rise in haemoglobin after 1 month of iron therapy
4. No Philadelphia chromosome
5. Collagen fibrosis of marrow
    a. Absent: or
    b. <1/3 of biopsy area without both splenomegaly and leukoerythroblastic reacton
6. No known cause for reactive thrombocytosis.

2001 World Health Organization criteria for essential thrombocythemia
(Michiels *et al*, 2006)

Positive criteria

1. Sustained platelet count ≥600 x 10^9/L
2. Bone marrow biopsy specimen showing proliferation mainly of the megakaryocytic lineage with increased numbers of enlarged, mature megakaryocytes

Criteria of exclusion

1. No evidence of polycythemia vera
   a. Normal red cell mass or hemoglobin < 18.5 g/dL in men, 16.5 g/dL in women
   b. Stainable iron in marrow, normal serum ferritin, or normal MCV
   c. If the former condition is not met, failure of iron trial to increase red cell mass or hemoglobin levels to the PV range
2. No evidence of chronic myeloid leukemia: no Philadelphia chromosome and no *BCR-ABL* fusion gene
3. No evidence of chronic idiopathic myelofibrosis
   a. Collagen fibrosis absent
   b. Reticulin fibrosis minimal or absent
4. No evidence of myelodysplastic syndrome
   a. No del(5q), t(3;3)(q21;q26), inv(3)(q21q26)
   b. No significant granulocytic dysplasia, few, if any, micromegakaryocytes
5. No evidence that thrombocytosis is reactive caused by
   a. Underlying inflammation or infection
   b. Underlying neoplasm
   c. Prior splenectomy

2008 Proposed revised WHO criteria for essential thrombocythemia
(Tefferi *et al*, 2007)

Diagnosis requires meeting all 4 criteria.

1. Sustained platelet count ≥450 x 10^9/L*
2. Bone marrow biopsy specimen showing proliferation mainly of the megakaryocytic lineage with increased numbers of enlarged, mature megakaryocytes; no significant increase or left-shift of neutrophil granulopoiesis or erythropoiesis
3. Not meeting WHO criteria for PV,† PMF,‡ CML,§ MDS,¶ or other myeloid neoplasm
4. Demonstration of *JAK2*617V>F or other clonal marker, or in the absence of a clonal marker, no evidence for reactive thrombocytosis‖

* During the work-up period.

†Requires the failure of iron replacement therapy to increase hemoglobin level to the PV range in the presence of decreased serum ferritin. Exclusion of PV is based on hemoglobin and hematocrit levels, and red cell mass measurement is not required.

‡Requires the absence of relevant reticulin fibrosis, collagen fibrosis, peripheral blood leukoerythroblastosis, or markedly hypercellular marrow for age accompanied by megakaryocyte morphology that is typical for PMF– small to large with an aberrant nuclear/cytoplasmic ratio and hyperchromatic, bulbous or irregularly folded nuclei and dense clustering.

§Requires the absence of *BCR-ABL*.

¶ Requires absence of dyserythropoiesis and dysgranulopoiesis.

‖ Causes of reactive thrombocytosis include iron deficiency, splenectomy, surgery, infection, inflammation, connective tissue disease, metastatic cancer, and lymphoproliferative disorders. However, the presence of a condition associated with reactive thrombocytosis does not exclude the possibility of ET if the first three criteria are met.

APPENDIX B - PATIENT INFORMATION SHEETS

Patient Information Sheet

Microparticles in Myeloproliferative Disorders.

You are being invited to take part in a research project. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information before deciding whether to take part. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of the study?

Patients with myeloproliferative disorders have either too many red blood cells (polycythaemia) or too many platelets (primary thrombocythaemia). Platelets are the blood cells which help to stop bleeding. Patients with either polycythaemia or primary thrombocythaemia have an increased risk of blood clots. The reasons for this are not completely understood. Research into other conditions with an increased risk of blood clots has found high levels of microparticles in patients' blood. Microparticles are small pieces of the surface of blood cells or the blood vessel lining cells, which can contribute to blood clotting.

We wish to measure the levels of microparticles in patients with too many red blood cells or platelets compared to people without blood disorders. We will also look at the role of microparticles in blood clotting.

Why have I been chosen?

You have been chosen as a patient who is attending the haematology clinic for management of one of these blood disorders. We hope to look at around 80 patients.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and asked to sign a consent form. You are still free to withdraw at any time and without giving a reason, and your blood samples and the information collected

would be destroyed. If you decide not to take part, or to withdraw, this will not affect your care.

What will happen to me if I take part?

We will contact you to arrange for you to attend the clinical research unit, which is in the hospital, at your next routine clinic appointment. You will have your blood taken as usual but at the same time 3 extra tubes of blood (about 1 tablespoon) will be taken for the research tests. Then you will go along to clinic as usual and your clinic blood results will be sent there. Only one research visit is required. We will also look at your medical notes as part of the study.

What are the possible disadvantages and risks of taking part?

The risks are the same as those of having your usual blood sample taken, possibly some discomfort or bruising at the site where the blood is drawn.

What are the possible benefits of taking part?

There are no direct benefits to you but we hope that the information we get might help us to understand why people with high red blood cells or platelets get blood clotting problems.

What if there is a problem?

Any complaint about the way you have been dealt with during this study will be addressed. The detailed information on this is given in Part 2.

Will my taking part in this study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

Contact for further information:

Dr Susan Lynch, Clinical Lecturer
Department of Haematology, Royal Infirmary Edinburgh. Tel: 0131 242 6814 or 242 6096

This completes Part 1 of the information sheet. If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making your decision.

Part 2

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (Dr Susan Lynch 0131 242 6814). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against Lothian NHS Trust and Edinburgh University but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. The results of your routine clinic blood tests will be stored on the hospital computer system. All other information obtained from your samples will be stored securely within the department of haematology under the care of Professor Ludlam. Reseachers will have access to identifiable data. This may also be looked at by representatives from regulatory authorites or authorised people from NHS Lothian to check that the study is being carried out correctly. With your consent, your own GP will be informed of your participation in the study.

What will happen to any samples I give?

The samples you give will be processed within the Clinical Research Facility and the Department of Haematology. Study researchers will have access to these samples which will be identified by a unique study number. The research samples will be stored securely within the department of haematology for use in this study for up to 5 years. At the end of the study, or if you decide to withdraw, the samples will be destroyed.

What will happen to the results of the research study?

When the results of the study are available it is planned to present these at academic meetings and publish them in medical journals. You will not be identified in any publication. In addition a short report will be provided for the participants.

Who is organising and funding the research?

The study is organised by NHS Lothian and Edinburgh University and is funded by an award from the Royal College of Pathologists/Jean Shanks Foundation.

Who has reviewed the study?

This study was given a favourable ethical opinion for conduct in the NHS by the Lothian Research Ethics Committee 01.

Thank you for taking the time to read this information sheet.

Patient Information Sheet

Platelet alpha-granule contents in myeloproliferative disorders

You are being invited to take part in a research project. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information before deciding whether to take part.

Thank you for taking the time to read this

What is the purpose of the study?

Patients with myeloproliferative disorders such as myelofibrosis, primary thrombocythaemia (high platelets) and polycythaemia (high red blood cells) have different amounts of fibrosis or scarring in their bone marrow. Previous research has shown that this might be related to the abnormal release of substances from the bone marrow cells which produce platelets. These substances are also found in the platelets in our blood.

We wish to measure the blood levels of these substances to see how they differ between patients with different blood disorders. We will also look to see whether they are related to the amount of fibrosis in the bone marrow.

Why have I been chosen?

You have been chosen as a patient attending the Haematology Clinic for investigation or treatment of high red blood cell or platelet counts, or myelofibrosis.

Do I have to take part?

It is up to you to decided whether or not to take part. If you decide to take part you will be asked to sign a consent form. If you take part you are still free to withdraw at any time and without giving a reason and your blood samples would be destroyed. If you decide not to take part or to withdraw this will not affect your care.

What will happen to me if I take part?

When you next attend the haematology clinic you will be asked to sign a consent form, a copy of which is enclosed, and you will be given a copy to keep along with this leaflet. This also asks for your consent to look at your medical notes as part of the study. Then you will have your blood taken as usual but at the same time 2 extra tubes of blood (about 1 tablespoon) will be taken for this research. These extra samples will be taken on one further occasion, the next time that you attend haematology clinic. These samples will be stored in the Haematology Department for use in this study for up to 5 years. At the end of the study, or if you decide to withdraw, the samples will be destroyed.

Contact for further information:

Dr Susan Lynch,
Department of Haematology,
Royal Infirmary Edinburgh.

or

Dr M Turner, Consultant Haematologist
Blood Transfusion Service
Royal Infirmary Edinburgh.

Version 2.0, 6/6/05

APPENDIX C – FLOW CYTOMETER INSTRUMENT SETTINGS

Microparticle collection

Detectors / Amps:

Parameter	Detector	Voltage	Amp gain	Mode
P1	FSc	E00	2.0	Log
P2	SSc	396	1.0	Log
P3	FL1	591	1.0	Log
P4	FL2	578	1.0	Log

Threshold:

Primary parameter: FSc
Value: 10
Secondary parameter: None

Compensation:

FL1 – 2.4% FL2
FL2 – 18.1% FL1

Trucount bead collection

Detectors / Amps:

Parameter	Detector	Voltage	Amp gain	Mode
P1	FSc	E00	2.0	Log
P2	SSc	396	1.0	Log
P3	FL1	505	1.0	Log
P4	FL2	505	1.0	Log

Threshold:

Primary parameter: FL1
Value: 181
Secondary parameter: None

Compensation:

FL1 – 2.4% FL2
FL2 – 18.1% FL1

APPENDIX D – PRESENTATIONS ARISING FROM THIS THESIS

S F Lynch, D Stirling, C A Ludlam. Microparticles (platelet, endothelial and leucocyte) are elevated in patients with myeloproliferative disorders.

> This work was presented as a poster at the 49th American Society of Haematology Annual Meeting, December 2007

S F Lynch, D Stirling, C A Ludlam. Platelet and endothelial microparticles are elevated in patients with myeloproliferative disorders.

> This work was presented at the British Society for Haemostasis and Thrombosis Annual Scientific Meeting, September 2007

SF Lynch, D Stirling, CA Ludlam. Effect of preanalytical variables on microparticle numbers.

> This work was presented as a poster at the XXIst Congress of the International Society on Thrombosis and Haemostasis, July 2007

SF Lynch, P Dawson, D Stirling, CA Ludlam. Platelet derived growth factors and bone marrow fibrosis in myeloproliferative disorders.

SF Lynch, D Stirling, P Hayes, CA Ludlam. Hyaluronic acid and liver function in myeloproliferative disorders

> This work was presented as a poster at the British Society of Haematology Annual Scientific Meeting, May 2007

SF Lynch, K Shaw, D Stirling, CA Ludlam Prevalence of the JAK2 tyrosine kinase mutation in patients with an established diagnosis of myeloproliferative disease and to aid diagnosis.

> This work was presented as a poster at the British Society of Haematology Annual Scientific Meeting, April 2006