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Prothrombotic status in Myeloproliferative Neoplasms: the role of JAK2V617F allele burden and platelets/leukocytes activation

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Professora Doutora Letícia Ribeiro (Departamento Hematologia CHC, EPE).

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ABBREVIATIONS

A – Adenine nucleotide

ADP – Adenosine diphosphate

AML – Acute myeloblastic leukemia

ASA – Aspirin

ASO-PCR – Allele specific oligonucleotide polymerase chain reaction

ASXL1 – Additional Sex Combs-Like 1 gene

ATP – Adenosine triphosphate

BM – Bone marrow

C – Cytosine nucleotide

CBL – Casitas B-lineage lymphoma proto-oncogene

CML – Chronic myeloid leukemia

CN – copy number

DNA – Deoxyribonucleic acid

ECLAP – European Collaboration of Low dose Aspirin

EPI – Epinephrine

EPO – Erytropoietin

ET – Essential thrombocythemia

FCM – Flow Cytometry

G – Guanine nucleotide

GM-CSF – Granulocyte-macrophage colony-stimulating factor

GP – Glycoprotein

HU – Hydroxyurea

IDH1 – Isocitrate dehydrogenase 1

IDH2 – Isocitrate dehydrogenase 2

IKZF1 – IKAROS family zinc finger 1

JAK2 – Janus 2 kinase gene

LAMP – Lysossomal membrane protein

LOH – Loss of heterozygosity

LPS – Lypopolysaccharide

mAb – Monoclonal antibodies

MDS – Myelodysplastic syndrome

MESF – Molecules of equivalent soluble fluorochrome

MFI – mean fluorescence intensity

MFI – Median fluorescence intensity

MPL – Myeloproliferative leukemia virus oncogene

MPN – Myeloproliferative neoplasm

P – Phosphate

PCR – Polymerase chain reaction

Ph – Philadelphia

PIP2 – Phosphatidyl inositol bi-phosphate

PIP3 – Phosphatidyl inositol tri-phosphate

PMF – Primary myelofibrosis

PMN – Polymorphonuclear leukocytes

PSGL-1 – P-selectin glycoprotein ligand 1

PV – Polycythemia vera

RQ-PCR – Real time quantitative polymerase chain reaction

SEM – standard error median

SPD – Storage pool deficiency

SSCP – strand conformation polymorphism

T - Thymine nucleotide

TET – TET oncogene family member 2

TF – Tissue factor

TPO – Trombopoietin

TRAP6 – Thrombin receptor agonist peptide

UPD – Uniparental disomy

VWD – von Willebrand disease

VWF – von Willebrand factor

WHO – World Health Organization

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RESUMO

Introdução: As neoplasias mieloproliferativas (MPN) são doenças da "stem-cell" hematopoiética e que estão associadas à ocorrência de eventos trombohemorragicos. Diferentes estudos sugerem que doentes com Policitémia Vera (PV) e Trombocitémia Essencial (ET) apresentam um estado protrombótico e que este poderá estar relacionado não só com a activação constitutiva da via JAK/STAT mas também com a carga alélica da mutação JAK2V617F.

Objectivo: Investigar a presença de marcadores de activação hemostática e a relação destes com a carga alélica JAK2V617F e trombose.

Métodos: Foram estudados 28 PV, 47 ET e 48 controlos saudáveis. Os doentes estão clinicamente estáveis e sob tratamento com Hidroxiureia; tempo de seguimento de 78 meses nas PV e de 84 meses nas ET. Sete doentes com PV e 16 com ET apresentaram história de trombose ao diagnóstico. Após consentimento informado, os doentes suspenderam a aspirina nos 10 dias prévios ao estudo. Por citometria de fluxo (FACSCalibur, BD) avaliou-se: expressão de P-selectina (CD62P) e granulofisina (CD63) plaquetar, basal e após estímulo com agonistas; capacidade de captação e libertação de mepacrina nas plaquetas; agregados plaqueta-leucócito; expressão de CD11b nos leucócitos e de factor tecidular (TF) nos monócitos, basal e após estímulo com LPS. A pesquisa da mutação JAK2V617F foi efectuada por PCR alelo específica e a quantificação por PCR em Tempo Real (JAK2 MutaQuant, Ipsogen). O screening de mutações no exão 10 do gene MPL, foi efectuado por SSCP e as mutações identificadas por sequenciação (ABI 310 Genetic Analyzer, AB).

Resultados: A mutação JAK2V617F foi encontrada em 28 PV (100%) e 28 ET (60%); 2 doentes com ET, apresentam mutações no exão 10 do gene MPL: W515L e R524C. Os doentes apresentam um aumento significativo de expressão basal de CD62P e de CD63 e de resposta ao ácido araquidónico; em todos os doentes a resposta ao TRAP6 está significativamente diminuída; 77% das PV e 50% das ET apresentam um fenótipo

de “storage pool disease”. Todos os doentes apresentam um aumento significativo de expressão basal de CD11b nos leucócitos e de TF nos monócitos. Os agregados plaqueta-leucócito estão significativamente aumentados em todos os doentes, sendo que os agregados plaqueta-neutrófilo (PMN) estão significativamente aumentados nas ET vs PV. Os doentes com carga alélica JAK2V617F>50% apresentam um aumento significativo na expressão de CD11b nos leucócitos e de agregados plaqueta-PMN, em comparação com os doentes com carga alélica <50%; as PV com carga alélica >50% apresentam um aumento estatisticamente significativo de TF nos monócitos. Nas ET, foi encontrada uma correlação estatisticamente significativa entre trombose e a presença de mutações JAK2V617F ou MPL, e entre trombose e carga alélica>50%. Esta associação não foi encontrada nas PV. A relação entre carga alélica e alterações plaquetares é difícil de estabelecer, uma vez que não foram encontradas alterações estatisticamente significativas.

Discussão: Os dados apresentados mostram, com significado estatístico, que os doentes com PV e ET apresentam plaquetas e leucócitos activados e um aumento de agregados plaqueta-leucócito em circulação. Os doentes com carga alélica>50% apresentam marcadores de activação significativamente aumentados em comparação com os doentes com carga alélica <50%, consistente com a influência da carga alélica e perda de heterozigotia para a mutação JAK2V617F na activação celular. Nas ET a presença da mutação e a carga alélica >50% está significativamente associada a eventos trombóticos ao diagnóstico.

Conclusão: Uma vez que as tromboses representam, nos doentes com MPN, uma das principais causas de co-morbilidade, foram investigados marcadores de activação da hemostase e a sua relação com a carga alélica JAK2V617F. Os resultados apresentados ilustram diferentes mecanismos que favorecem a trombose nas PV e ET, nomeadamente, activação basal das plaquetas, monócitos e PMN, aumento de FT nos monócitos e de aumento de agregados plaqueta-leucócito em circulação.

ABSTRACT

Introduction: Myeloproliferative neoplasms (MPN) are stem cell-derived proliferative diseases associated with thrombohemorragic diathesis. Different studies suggested that Polycythemia Vera (PV) and Essential Thrombocythemia (ET) patients have a baseline protrombotic status, which could be related to constitutive JAK2/STAT signalization in correlation with JAK2V617F mutation allele burden.

Objective: Investigate the baseline hemostatic activation markers and their correlation with the JAK2V617F allele burden and thrombosis.

Methods: 28 PV and 47 ET patients and a control group of 48 healthy volunteers were studied. All the patients are under hydroxyurea treatment and remain clinically stable with follow up periods of 78 months for PV and 84 months for ET. Seven PV and 16 ET patients have a history of thrombosis at diagnosis. With patients' written informed consent, aspirin was withdrawn for 10 days prior the studies. Using flow cytometry (FACSCalibur, BD), we evaluated: platelet P-selectin (CD62P) and granulophysin (CD63), at baseline and after stimulation with agonists; platelet uptake and release of mepacrine; platelet-leukocyte aggregates, leukocyte CD11b and monocyte Tissue factor (TF) at baseline and after stimulation with LPS. JAK2V617F allele was detected by Allele specific PCR and quantified by Allele specific Real Time PCR (JAK2 MutaQuant, Ipsogen). MPL exon 10 mutations were screened by SSCP and identified by direct sequencing (ABI 310 Genetic Analyzer, Applied Biosystems).

Results: JAK2V617F mutation was found in 28 PV patients (100%) and in 28 ET patients (60%). In two other ET patients we found MPL gene exon 10 mutations: W515L and R524C. All patients have, at baseline, a significant increased expression of CD62P and CD63, and a significant increase response to arachidonic acid and diminished levels of CD62P and CD63 following TRAP6 activation. A phenotype of storage pool disease was found in 77% of PV and 50% of ET patients. At baseline all patients have activated leukocytes with a statistically significant increase in CD11b

expression and monocyte-TF. The latter was significantly elevated in PV vs ET patients. Circulating platelet-leukocytes aggregates were significant higher in all patients; in the ET, platelet-PMN aggregates were significantly increased vs PV patients. Patients with JAK2V617F>50%, have a statistically significant increase in leukocytes CD11b expression and in platelet PMN-aggregates, in comparison with those with JAK2V617F<50%;PV patients with allele burden >50% have a statistically significant increase in monocytes-TF. In ET patients we found a statistically significant correlation between thrombosis and JAK2 or MPL mutations, furthermore, ET patients with JAK2V617F allele burden >50% have statistically higher incidence of thrombosis. No such correlation in PV patients. Regarding to platelets activation, it’s not evident such effect since no significantly differences were found.

Discussion: These data shown, with statistically significance that PV and ET patients have circulating activated platelets and leukocytes, and increased number of platelet-leukocyte aggregates. Consistent with the influence of allele burden and acquisition of loss of heterozygosity for the JAK2V617F mutation in leukocyte activation, all patients with allele burden >50% presented significantly increased activation markers comparing to patients with allele burden >50%. In ET patients the presence of JAK2V617F mutation and allele burden>50% was significantly associated with a previous history of thrombosis.

Conclusion: As thrombosis is one of the main co-morbilities in MPN patients, we investigated the baseline hemostatic activation markers and their correlation with the JAK2V617F allele burden. These data clearly illustrate several mechanisms favoring thrombosis in PV and ET patients, namely, the baseline activation of platelets, monocytes and PMNs leukocytes, increased monocyte-TF and platelet-leukocytes aggregates.

CHAPTER 1 – INTRODUCTION

1.1. MYELOPROLIFERATIVE NEOPLASMS

Myeloproliferative neoplasms (MPN) constitute a group of hematopoietic malignancies that feature enhanced proliferation and survival of one or more myeloid lineage cells (i.e. granulocytic, erythroid, megakaryocytic and mast cell (Swerdlow *et al.*, 2008). In 1951, William Damesked highlighted the phenotypic similarities among chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). In 1960, CML became the first cancer to be associated with a specific cytogenetic marker, the Philadelphia chromosome (Ph), which was shown to harbor a reciprocal chromosomal translocation, t(9;22)(q34,q11) and led to the identification of disease-causing mutation (*BCR-ABL*) (Tefferi and Gilliand, 2007). Accordingly, the classic MPN were sub-classified into Ph-positive (CML) and Ph-negative chronic myeloproliferative neoplasms including PV, ET and PMF. Collectively, MPN are stem cell-derived clonal proliferative diseases that share a common stem cell-derived clonal heritage and their phenotypic diversity is attributed to different configurations of abnormal signal transduction, resulting from a spectrum of mutations affecting protein tyrosine-kinases or related molecules (Tefferi and Vardiman, 2008). Despite an insidious onset each MPN has the potential to undergo to a stepwise progression that terminates in marrow failure due to myelofibrosis, ineffective haematopoiesis or transformation to acute blast phase (Swerdlow *et al.*, 2008).

In early 2005, the identification of a gain-of-function mutation in the Janus kinase 2 gene, named JAK2V617F, opened a new era in the understanding of Ph-negative neoplasms. Since then, the discovery of mutations in MPL and in JAK2 exon 12 was also reported. The presence of any of these molecular abnormalities, that point a clonal myeloproliferations, stands as a major diagnosis criteria in the revised 2008 classification of myeloid neoplasms of the World Health Organization (WHO).

The three main Ph-negative neoplasms are Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) – here we will focus only on PV and ET.

1.1.1. Polycythemia Vera

PV is characterized by increased red blood cell production independent of mechanisms that normally regulate erythropoiesis. Virtually all patients carry the somatic gain-of-function mutation of the Janus 2 kinase gene (JAK2), JAK2V617F, or another functionally similar JAK2 mutation that causes the proliferation not only of the erythroid lineage but of the granulocytes and megakaryocytes as well, i.e., panmyelosis. The natural progression of PV includes a low incidence of evolution to a myelodysplastic/preleukaemic phase and/or to acute leukaemia (AML) (Swerdlow *et al.*, 2008). The reported annual incidence of PV increases with advanced age and varies from 0.7 to 2.6 per 100000 inhabitants in Europe and North America. Most reports indicate a slight male predominance, with M:F ratio ranging from 1-2:1. The median age at diagnosis is 60 years; patients younger than 20 years are rarely reported. Median survival times >10 years are commonly reported. Most patients die from thrombosis or hemorrhage, but up to 20% succumb to myelodysplasia or acute myeloid leukemia. Among patients who have not been treated with cytotoxic agents the incidence of MDS and acute leukemic transformation is only 2-3%, but it increases to 10% or more following certain types of chemotherapy. The predictive risk factors for thrombosis or hemorrhage are not well defined.

1.1.2. Essential Thrombocythemia

ET is a myeloproliferative neoplasm that involves primarily the megakaryocytic lineage. It is characterized by sustained thrombocytosis ≥ $450x10^9$/L in the peripheral blood, increased numbers of large, mature magakaryocytes in the bone marrow (BM), and clinically by episodes of thrombosis and/or haemorrhage. No specific molecular genetic or cytogenetic abnormalities are known associated with ET. Approximately 40-50% of the ET patients carry the JAK2V617F or a similar functional mutation (Swerdlow *et al.*, 2008). These mutations are present in almost all the PV and in some MF patients. A MPL gain-of-function mutation, MPL W515K/L, has been reported in 1% of ET cases. The true incidence of ET is estimated to be 0.6-2.5 per 100000 persons per year. Most cases occur in patients with 50-60 years, with no major sex tendency. However, another peak in frequency, particularly in women, occurs at about 30 years of age. It can also be seen in children, albeit infrequently. ET is an indolent disorder characterized by long symptom free intervals, interrupted by occasional life-threatening thromboembolic or hemorrhagic episodes. Median survives of 10-15 years are reported. Because ET usually occurs late in middle age, many patients life expectancy is nearly normal (Swerdlow *et al.*, 2008). Transformation to acute myeloid leukemia or MDS occurs in <5% of patients and, when it does occur, it is likely related to previous cytotoxic therapy. After many years a few patients may develop BM fibrosis associated with myeloid metaplasia, although such progression in uncommon.

1.2. CLINICAL FEATURES OF MPN

1.2.1. Bleeding

The reported incidence of bleeding in PV and ET at initial presentation varied from 1.7-20% and 3.6-37%, respectively. Retrospective studies estimated the overall risk of bleeding in ET in 0.33%/ patient-year (Elliott and Tefferi, 2004).

Bleeding manifestations in both PV and ET are predominantly mucocutaneous, with particular involvement of the gastrointestinal and genitourinary tracts. Bruising, epistaxis, and superficial mucosal hemorrhages are the most common. This type of bleeding pattern is consistent with platelets defects (quantitative or qualitative) or von Willebrand Disease (VWD) (Elliott and Tefferi, 2004). However the lack of correlation between platelet function abnormalities and clinical bleeding suggest alternate mechanisms might be involved. The risk of spontaneous hemorrhage may be increased when platelets counts are greater than 2000×10^9/L. Aspirin is an important contributing factor to the overall hemorrhage in MPN (Rao, 2007).

1.2.2. Thrombosis

PV and ET patients have an increased risk for both arterial and venous thromboses, associated with microcirculatory disturbances (Landolfi *et al.*, 2006). The manifestations are particularly common at diagnosis and can occur during the latent phase of the disease. The precise incidence is hard to ascertain; at presentation it has been reported as 12-39% in PV and 11-25% in ET. The overall risk of thrombosis for ET was estimated in 6.6% patient-year (Elliott and Tefferi, 2004; Rao, 2007). In two large trials thrombotic events occurred in 34 and 41% of the PV patients (Rao, 2007).

1.2.2.1. Microcirculatory disturbances

Microcirculatory disturbances are the most typical thrombotic manifestations, which are associated with erythromelalgia, visual and hearing symptoms, Raynaud phenomenon and untreatable headhache. Erythromelalgia never occurs in secondary thrombocytosis, suggesting that qualitative platelet abnormalities have a major implication in the pathogenesis of this phenomenon (Landolfi *et al.*, 2006); histopathological studies demonstrate platelet-rich arteriolar microthrombi with endothelial inflammation and intimal proliferation (Rao, 2007). Other clinical manifestations of microcirculatory disturbances are transient neurological and ocular ischaemias (Landolfi *et al.*, 2008).

1.2.2.2. Arterial thromboses

Arterial thromboses dominate venous events, in both PV and ET, and are quite common at diagnosis with an apparent reduction during follow-up (Landolfi *et al.*, 2008). Thrombotic occlusions of large arteries most commonly involve cerebrovascular accidents (stroke and transient ischemic attacks), myocardial infarction and peripheral arterial occlusion (Elliott and Tefferi, 2004; Landolfi *et al.*, 2008). A European Collaboration of Low dose Aspirin in PV (ECLAP) study reported a high prevalence of stroke at diagnosis and a tendency to thrombophilia 5-6 years previous to diagnosis (Landolfi *et al.*, 2008).

1.2.2.3. Venous thromboses

Deep vein thrombosis and superficial phlebitis are frequent in PV and ET and generally involves the lower extremities (Landolfi *et al.*, 2006). Among PV, venous thromboses represent approximately one-third of the total events. Thromboses at unusual sites, such as renal, mesenteric, portal, splenic hepatic and subclavian veins and intracranial sinuses, have been reported, and should lead to a careful search of a latent myeloproliferative neoplasm (Landolfi *et al.*, 2006). In patients with hepatic vein thrombosis the prevalence of a latent MPN has been estimated to range between 40 and 60% (Landolfi *et al.*, 2008).

1.2.3. Risk factors for bleeding and thrombosis

ET patients with high platelet counts have more hemorrhagic than thrombotic complications. Also, aspirin use has been repeatedly reported as the possible cause of major gastrointestinal bleeding. A history of previous bleeding is an important predictor of major bleeding during follow-up (Landolfi *et al.*, 2006).

It is widely accepted that age and a previous thrombosis are predictive risk factors for recurrent thrombosis, in both PV and ET (DeStefano *et al.*, 2008; Harrison, 2005). Regarding the conventional risk factors for cardiovascular disease, such as arterial hypertension, diabetes, smoking, and hypercholesterolemia, conflicting results are found in the literature (Barbui *et al.*, 2009; Passamonti *et al.*, 2008). Hereditary thrombophilic states, such as congenital deficiencies of natural anticoagulants (antithombin, protein C and protein S) and genetic mutations (factor V Leiden and prothrombin G20210A) may play a role in the pathogenesis of venous thromboses. Elevated homocysteine levels and the presence of antiphopholipid antibodies can increment the risk for both venous and arterial thromboses (Landolfi *et al.*, 2006).

More recently, disease-related risk factors have been considered, including leukocytosis at diagnosis, presence of JAK2V617F mutation, JAK2V617F allele burden and the role of platelet and neutrophil activation.

1.3. Platelets in MPN

1.3.1. Platelets normal function

Platelets are anuclear cellular fragments derived from BM megakaryocytes. They contain numerous cytoplasmic structures important to hemostasis; in addition to mitochondria, microtubules, microfilaments and lysosomes, platelets have two major intracellular types of granules: the α-granules and dense granules, which are found only in megakaryocytes and platelets. The α-granules contain platelet thrombospondin, fibrinogen, fibronectin, platelet factor 4, von Willebrand factor (VWF), platelet derived growth factor, β-thromboglobulin and anticoagulant factors V and VIII. The dense granules contain ADP, ATP, serotonin and lysosomal membrane proteins such as CD63 (LAMP-3) and LAMP-2. Lysosomes in platelets, like those in other cells contain acid hidrolases, cathepsins, and lysosomal membrane proteins (LAMP-1, LAMP-2 and CD63) (Reed, 2007). When platelets are stimulated, both the α and dense granules are released through the open canalicular system (Triplett, 2000).

Platelets play an important role in the hemostasis maintenance, representing the first line of defense in the prevention of hemorrhage. In primary hemostasis platelets interact with elements of the damage vessel wall, leading to the initial formation of a “platelet plug”. This interaction involves a series of events that includes platelet adhesion to components of the subendothelium, activation and shape change, release of platelet granular contents (α- and dense granules) with subsequent formation of fibrin-stabilized platelet aggregates, and clot retraction (Figure 1).

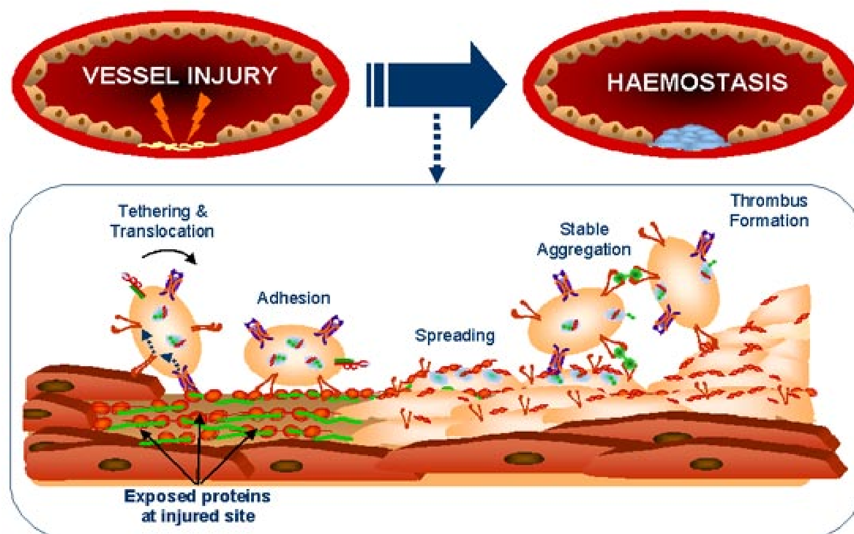

Figure 1 – Stages in platelet thrombus formation (adapted from www.med.monash.edu.au). After vessel injury, platelets adhere to endothelium, spread and aggregate leading to thrombus formation.

Platelets promote hemostasis by four interconnected mechanisms: i) *adhesion* to damage vascular surface promoted by VWF, a large multimeric protein secreted into the extracellular matrix from endothelial cells, that binds to platelet surface glycoprotein (GP) Ib/IX/V. Platelets can also adhere to vascular wall-associated fibrin or fibrinogen via GPIIb-IIIa which is expressed when platelets are activated; ii) *releasing* compounds, of both α- and dense granules, into the surrounding milieu. The granule membranes contain many integral glycoproteins on their inner leaflet, such as P-selectin (CD62P) and gp53 (CD63) which become expressed on the outer platelet membrane. The ADP released from dense granules leads to a fibrinogen receptor conformational change, and then the GPIIb/IIIa receptor that initiates the next step; iii) the process of *platelet aggregation,* whereby the GPIIb/IIIa receptor of one platelet is bound to the same receptor on adjacent platelet via a fibrinogen bridge. The platelet release reaction and aggregation lead to the recruitment of many other platelets to the vessel wall with the formation of a hemostatic platelet plug, and iv) providing a *procoagulant* surface for activated coagulation proteins complexes on their phospholipid membranes. Platelet

membrane phospholipids undergo a rearrangement during activation, with phosphatidylserine exposure, providing a binding site for phospholipid-dependent coagulation complexes that activate both factor X and prothrombin (Kottke-Marchant and Corcoran, 2002).

Platelet activation results from exposure of the platelet to damage endothelium or underlying components of the vessel wall. Other biological compounds are involved in platelet activation, including thrombin, epinephrine, ADP, and thromboxane A_2 (Triplett, 2000).

1.3.2. Platelets abnormalities in MPN

Owing to the particular features of a thrombohemorrhagic diathesis in MPN, most experimental *in vitro* studies have attempted to demonstrate and characterize possible platelet defects. A large number of morphological, functional and biochemical abnormalities have been identified including qualitative and quantitative platelet defects; however, their clinical significance remains elusive. Platelets abnormalities likely result from an abnormal clone of stem cells, however some alterations may be secondary to *in vivo* enhanced platelet activation. These abnormalities possibly contribute to the morbidity and mortality of these disorders, but the precise mechanisms are poorly understood (Rao, 2007). Platelet function is most commonly assessed by platelet aggregation studies; unfortunately, although platelet aggregation studies are frequently abnormal (demonstrating either or both hypo- and hyperfunction), a disappointing lack of clinical correlation with haemostatic complications (either bleeding or thrombosis) has been the rule (Elliott and Tefferi, 2004; Shafer, 1984). Nevertheless, aggregation measurements have provided some interesting findings, such as the reduced platelet response to some aggregating agents that include decreased primary and secondary aggregation patterns to either or both

epinephrine and ADP and decreased response to collagen, with generally normal responses to arachidonic acid (Shafer, 1984). The occurrence of bleeding and thrombotic events in the same patient creates further complexity in the interpretation of the laboratory findings.

1.3.2.1 Platelet receptors

The hemostatic response of an individual's platelets is influenced by the quantity and quality of receptors expressed on the platelet surface. In MPN, receptor abnormalities have been reported in GPIb, GPIIb/IIIa, GPIV, GPVI adrenergic receptors and in the trombopoietin receptor MPL (Harrison, 2005). A study by Jensen *at al* (2000) demonstrated an increased expression of GPIV in association with a thrombotic history.

1.3.2.2 Platelet activation

Enhanced platelet activation is common reported in MPN. Increased expression of P-selectin, thrombospondin and the activated receptor GpIIb/IIIa have been variably correlated with thrombosis (Harrison, 2005). Platelet activation includes formation of platelet microparticles that are associated with procoagulant activity. Currently, the pathogenesis of platelet activation in MPN is unknown, although some studies revealed that a proportion of patients have a deficiency of lipoxygenase, which could increase availability of endoperoxides to produce tromboxane A2 (Shafer, 1984). Alternative explanations for increased platelet activation include an effect of JAK2-activating mutation, interaction of the abnormal hematocrit and activated white cells. Activated

platelets interact with other blood components, both cellular and circulating, and have the capacity to provoke endothelial activation/damage (Harrison, 2005).

1.3.2.3 Acquired storage pool deficiency

Deficiency of the platelet dense granule pool of ATP and ADP (storage pool deficiency, SPD) is associated with impaired platelet function and bleeding diathesis. Acquired dense granule SPD is a common find in MPN patients (Kottke-Marchant and Corcoran, 2002). Decreased numbers of platelets dense-granules have been found by both electron microscopy and by fluorescent mepacrine labeling (Wall *et al.*, 1985). Diminished intracellular and releasable platelet adenine nucleotides or diminished serotonin uptake and release by platelets, in association with aggregation abnormalities, are consistent with storage pool disease.

Acquired dense granule SPD in myeloproliferative neoplasms appears to result of *in vivo* activation and release of platelet dense granule contents or due to the production of abnormal platelets by bone marrow (Kottke-Marchant and Corcoran, 2002; Shafer, 1984).

1.3.2.4 Thrombocytosis

Platelets counts have not been significantly correlated with thrombotic risk in either PV or ET (Austin and Lambert, 2008). Paradoxically, marked thrombocytosis might be responsible for hemorrhagic rather than thrombotic manifestations in ET patients; this is partially attributed to an acquired von Willebrand syndrome, as result of increased clearance of the large von Willebrand factor multimers from plasma. Microcirculatory disturbances are more frequent in patients with thrombocytosis than in

those with normal platelet counts. Platelets counts reduction lead to a substantial amelioration of both hemorrhagic and microcirculatory disturbances. The antithrombotic efficacy of chemotherapy in high-risk ET patients is clearly demonstrated and can be attributed not only to platelets reduction but also to mechanisms related to inhibition of proliferation of all myeloid cell lines, with a possible effect on mechanisms regulating platelets and leukocytes activation (Harrison, 2005; Landolfi *et al.*, 2006).

1.4. LEUKOCYTES IN MPN

Increased white blood cells count – leukocytosis - is a risk factor for thrombosis in patients with PV and ET. However, also qualitative abnormalities of leukocytes, particularly the polymorphonuclear leukocytes (PMNs), can occur and contribute to the hemostatic system activation, thus favoring a hypercoagulable state.

Activated neutrophils can affect the hemostatic system mainly by: 1) production of reactive oxygen species; 2) release of storage pools granules, such as CD11b/CD18, CD66 and β1 integrins, in the circulation; and 3) active interactions with other vascular cells, as platelets, monocytes, and endothelial cells.

The neutrophils and platelets interaction is coordinated through an adhesion cascade of events in which platelet P-selectin binds to P-selectin glycoprotein 1 (PSGL-1) on neutrophils. Subsequent adhesion is mediated by the neutrophil surface β-2 integrin CD11b/CD18 (Mac-1) to either platelet GPIb or to fibrinogen bound to platelet GPIIb/IIIa. Mac-1 is constitutively expressed on the neutrophil surface and is additionally stored in secondary granules, which are mobilized to the cell surface via exocytosis (Falanga *et al.*, 2005). *In vitro*, interaction between the activated platelets, neutrophils, and monocytes increases the procoagulant activity.

The interplay between activated neutrophils and activated platelets generates neutrophil/platelet mixed aggregates. Increased levels of circulating mixed aggregates

have been found in pathologic conditions associated with a susceptibility to thrombosis (unstable angina, cardiac infarction, venous stasis ulceration, stoke) and more recently to myeloproliferative neoplasms (PV and ET) (Falanga *et al.*, 2005; McEver, 2007). Different studies have attributed this phenomenon to platelet activation, however, PMN from MPN patients also express high levels of the β2 integrin CD11b, which is a prominent site for platelet activation (Figure 2). In a study of Fallanga *et al.* (2005), circulating PMN/platelets aggregates were measured simultaneously to the levels of activated PMN and activated platelets and results suggested a role for the activated PMN in the formation of high percentage of circulating mixed aggregates; this was further supported by the evidence that, *in vitro* induced PMN activation resulted in a significant increase of PMN-platelet aggregate formation. In ET patients receiving aspirin, the increment in CD11b and PMN/platelet aggregates was significantly lower compared with non-aspirin treated ET subjects.

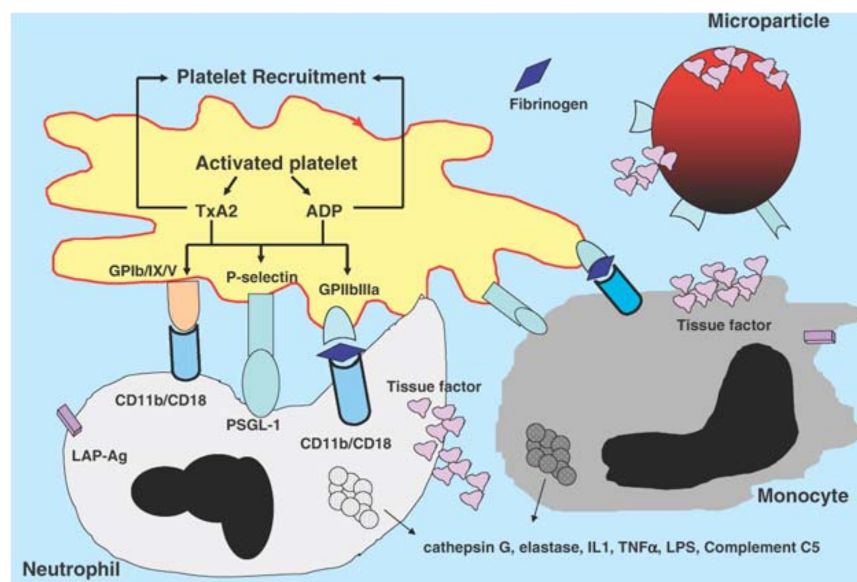

Figure 2 - Mechanisms promoting interaction of platelets, neutrophils and monocytes and the production of prothrombotic substances (Landolfi *et al*, 2008)**.** LAP, leukocyte alkaline phosphatase; TNF, tumor necrosis factor.

1.5. Molecular mechanisms of MPN

1.5.1. The JAK2 mutations

The Janus kinase (JAK) family proteins are cytoplasmic tyrosine kinases that participate in cytokine receptor superfamily signaling, which transduces signals downstream of type I and II cytosine receptors via signal transducers and activators of transcription (STAT). Four JAK proteins have been identified: JAK1, JAK2, JAK3 and the tyrosine kinase 2 (Tyk2). On the basis of homology, JAKs share seven homology domains (JH), denoted as JH1-JH7 (Figure 3).

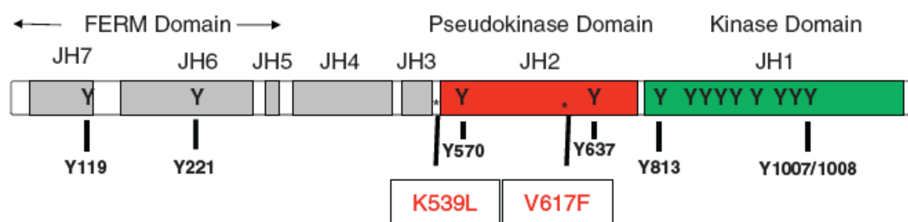

Figure 3 – Schematic illustration of JAK2 and the different homology (JH) domains (adapter from Kota J *et al.* 2008). The V617F mutation occurs in the pseudokinase domain rendering the kinase domain constitutively active. Exon 12 mutations, such as K539L, occur in the linker region between the JH3 and JH2 domains. Tyrosine residues that can be phosphorilated are depicted by their single letter.

From de C to the N terminus, JH1 represents the kinase domain, JH2 the pseudokinase domain, JH3 and JH4 contain the SH2-like domain and linker regions, whereas JH5-JH7 contain a FERM domain. The FERM domain of JAKs is responsible for appending JAKs to cytokine receptors (Kota *et al.*, 2008). JAKs N terminus is required for binding to receptors, chaperoning and stabilizing them at the surface, whereas the kinase domain is absolutely crucial for signaling. The pseudokinase

domain precedes the kinase domain and, because of sequence differences at key residues required for catalysis, it cannot transfer phosphate and thus is catalytically inactive. Nevertheless, the pseudokinase domain is structurally required for the response of JAKs to cytokine receptor activation and for inhibiting the basal activity of the kinase domain.

JAKs are crucial for normal haemopoiesis; The JAK/STAT pathway is the crucial step in signaling for numerous cytokines, including erythropoietin (EPO), thrombopoietin (TPO), granulocyte-macrophage colony-stimulating factor (GM-CSF), growth hormone, interleukin-3 and interleukin-5.

The type I cytokine receptors lack intrinsic tyrosine kinase activity. When a ligand binds a receptor, a conformational change in the receptor brings two JAK2 proteins close enough together to allow them to phosphorylate each other (Figure 4). Phosphorylated JAK2 acts as an activated tyrosine kinase, phosphorylating the cytoplasmic domains of type I cytokine receptors, which become a docking site of STAT proteins. Following activation, Jak kinases are able to phosphorylate another family of proteins known as the STAT proteins (*s*ignal transducers and *a*ctivators of transcription). Phosphorylated STAT proteins are then able to enter the nucleus and bind to DNA acting as transcription factors, and leading to a cellular response. Activated Jaks can also phosphorylate the protein Shc, which, in turn, associates with the adaptor protein Grb2. The latter connects to the nucleotide exchange factor Sos, which controls the GTPase activity of a small membrane-bound protein termed ‘Ras’. Activated Ras in the GTP-bound state can then induce signaling via a number of phosphorylation cascades, including the MEK–MAP kinase pathway and the phosphatidylinositol 3-kinase (PI-3 kinase) pathway, which, in turn, lead to gene induction and a cellular response (Smith and Fan, 2008).

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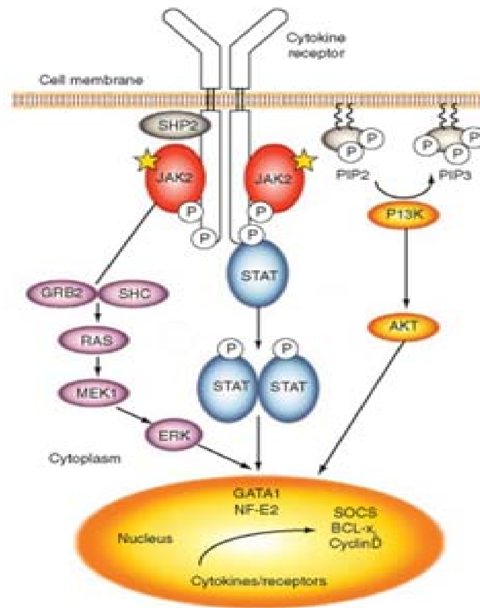

Figure 4 – JAK2V617F signaling in MPN (Delhommeau F *et al*., 2006). In the presence of a homodimeric receptor (like EPOR), the two JAK2V617F proteins bound to the intracellular domain of the receptor transphosphorylate its tyrosine residues. In turn, STAT5, PI3K, and RAS signaling pathways are activated, leading to downstream modulation of transcription and protein levels for cell cycle, proliferation and apoptosis-related factors. P,phosphate; PIP2 and PIP3, phosphatidyl inositol bi- and triphosphate.

Most cytokine receptors can associate more than one JAK kinase, but it has been shown that Jak2 deficient myeloid progenitors fails to respond to EPO, TPO, or GM-CSF, and that Jak2 deficiency results in an absence of definitive erythopoiesis. These data suggest that JAK2 is the predominant kinase involved in myeloid cell proliferation and differentiation.

1.5.1.1. JAK2V617F mutation

In 2005, while studying the mechanisms responsible for the Epo-independent growth characteristic of PV progenitors, and the acquired uniparental disomy (UPD) of chromosome 9p24, several groups reported the presence of a mutated form of the JAK2 protein (Kilpivaara and Levine, 2008). They found a G→ T transversion at nucleotide 1849, in exon 14, resulting in the substitution of valine to phenylalanine at codon 617 - JAK2V617F (Kralovics *et al.*, 2005). The JAK2V617F mutation occurs in the pseudokinase domain (JH2) of JAK2 gene, and is found in the majority of patients with Ph-negative MPN (PV, ET, PMF). The precise structure of JH2 domain has not been solved, however, based on the homology to JH1 domain, it has been suggested that JH2 domain negatively regulates JH1 kinase activity, likewise to the autoinhibitory role of juxtamembrane domains in receptor tyrosine kinases. Thus, it is expected that JAK2V617F mutation disrupts the inhibitory effect on JAK2 kinase activity (see Figure 2) (Kota *et al.*, 2008). The V617F amino acid change results in a gain-of-function of JAK2, which, in an autonomous growth factor-independent manner, activates downstream signaling pathways (Figure 3). This mutation is not present in the germ line, consistent that JAK2V617F is acquired as a somatic disease allele in the hematopoietic compartment (Levine and Gilliand, 2008). The JAK2 is the predominant kinase involved in myeloid cell proliferation and differentiation, therefore, the V617F gain-of-function mutation in JAK2 is observed in a spectrum of myeloid malignancies and JAK2V617F positive cells are hypersensitive to cytokine stimulation.

The identification of JAK2V617F provided an important insight into the pathogenesis of PV, ET and MF. However, the same JAK2V617F is present in 95% of PV in about 50% of ET and MF, raising questions how a single mutation is commonly associated with apparently distinct phenotypes.

1.5.1.2. Exon 12 JAK2 mutations

Since 2007, different somatic missense, deletion and insertion mutations in JAK2 exon 12 have been identified in JAK2V617F-negative PV involving amino acid residues F537-E543 (Butcher *et al.*, 2007). This region of seven highly conserver amino acids lies between the Scr homolog 2 (SH2)-like motif and the JH2 domain of JAK2. In vitro studies demonstrated that mutations is this region make hematopoietic cells' growth cytokine independent, and expression of JAK2 exon 12 *in vivo* causes polycythemia and leukocytosis, as is observed for JAK2V617F. Studies have found these alleles in PV but not in ET or MF.

1.5.2. The MPL mutations

MPL (myeloproliferative leukemia virus oncogene) belongs to the hematopoietin receptor superfamily. MPL is the key growth and survival factor for megakaryocytes and is located on chromosome 1p34, includes 12 exons and encodes for the thrombopoietin receptor (Tefferi, 2008). MPL somatic mutations were first described in 2006 among patients with JAK2V617F-negative PMF and induce PMF-like disease with thrombocytosis in mice. MPL mutations are rare and their occurrence is largely limited to patients with MPN, namely ET and PMF. MPLW515L, the most frequent MPN-associated MPL mutation, results from a G to T transition at nucleotide 1544 (exon 10), resulting in a tryptophan to leucine substitution at codon 515. MPLW515K and MPLS505N were described in ET and PMF, with mutational frequencies ranging from 3 to 15%. MPLS505N has been found in familial thrombocytosis, associated with an MPN phenotype, including splenomegaly, myelofibrosis and increased risk to thrombosis (Kilpivaara and Levine, 2008).

Like JAK2 mutations, MPL515 mutations are stem cell-derived events that involve both myeloid and lymphoid progenitors (Tefferi, 2010). MPL mutant-induced oncogenesis also results in constitutive JAK-STAT activation. Some patients with ET or PMF display multiple MPL mutations and others a low allele burden JAK2V617F clone together with a higher allele burden MPL. Homozygosity for MPL mutations is also ascribed to acquired UPD (Tefferi, 2010).

1.5.3. One mutation and different phenotypes

How does a single mutation contribute to the pathogenesis of three clinical distinct clinical disorders, PV, ET and MF?

The answer to this question remains unclear, but clinical, biological and pathological data have lead to three potential hypotheses that although explanatory, are not mutually exclusive.

The gene dosage hypothesis, postulates a correlation between disease phenotype and the proportion of JAK2V617F mutant alleles introducing the concept of allele burden, i.e, ratio between mutant and wild type JAK2 in hematopoietic cells (Francesco and Elisa, 2009). Studies describing JAK2V617F identified subsets of patients homozygous for the JAK2V617F allele, consistent with the loss of heterozygosity (LOH) at the JAK2 locus. Unlike classical LOH observed with tumor suppressor genes, in which one allele is inactivated by mutation and the second by deletion, LOH and the resultant JAK2V617F homozygosity is copy neutral – result of acquired uniparental disomy (UPD) at chromosome 9p24 after mitotic recombination (Kilpivaara and Levine, 2008). Conceivably, duplication of mutant allele is expected to result in a higher level of JAK2/STAT activation than in cells harboring one mutant and one wild-type allele, possibly because of the loss of competition between normal and mutated allele and/or impaired interaction of mutant JAK2 with cellular regulators such

as the suppressor of cytokine signaling-3 (SOCS3) (Vannucchi and Guglielmelli, 2008). Patients' genetic data is consistent with the notion that JAK2V617F gene dosage influences MPN phenotype, as homozygous JAK2V617F mutant erythroid colonies are observed in most patients with PV, but are rarely observed in ET. Thus, a high level of JAK2 signaling favors an erythroid phenotype and a low JAK2 state favors a megakaryocyte phenotype.

A second hypothesis advocates the existence of a pre-JAK2 phase in which additional somatic mutations or inherited predisposing alleles establish clonal hematopoiesis before the acquisition of JAK2V617F. Blast cells of AML developed in patients with a preceding JAK2V617F-positive MPN were often JAK2V617F negative, indicating that they might derive from the transformation of a pre-JAK2V617F mutated hematopoietic stem cell that was originally at the basis of the MPN itself (Vannucchi and Guglielmelli, 2008).

Finally, host genetic factors may contribute to phenotypic diversity among patients with MPN. The possibility of independently emerging multiple abnormal clones has recently been raised and challenges the prevailing concept that considers an ancestral abnormal clone that gives to mutually exclusive subclones. Recently, a number of stem cell-derived mutations involving JAK2 (exon 14 and 12), MPL (exon 10), TET (across several exons), ASXL1 (exon 12), CBL (exons 8 and 9), IDH1 (exon 4), IDH2 (exon 4) and IKZF1 (deletion of several exons) have been described in chronic or blast-phase MPN (Tefferi, 2010).

CHAPTER 2 – OBJECTIVES

2.1. MAIN OBJECTIVE

As thrombosis is one of the main co-morbilities in MPN patients, we investigated the baseline hemostatic activation markers and their correlation with the JAK2V617F allele burden.

2.1.1. Specific objectives

- ✓ quantify JAK2V617F allele burden, using Real Time Quantitative-PCR;
- ✓ evaluate baseline activation markers accessing by flow cytometry:
    - platelet activation and response to agonists;
    - platelet leukocyte-aggregates;
    - monocytes and PMNs leukocytes CD11b expression;
    - monocyte-TF;
- ✓ correlate the allele burden and activation parameters;
- ✓ correlate the JAK2V617F allele burden and thrombosis.

CHAPTER 3 – MATERIAL AND METHODS

3.1. PATIENTS

Seventy-four patients with PV and ET were enrolled into the study after giving written informed consent. The patients were diagnosed according to WHO 2008 classification and included 28 patients with PV and 47 patients with ET. All the patients are under hydroxyurea treatment and remain clinically stable with follow up periods of 78 months for PV and 84 months for ET. Forty-eight healthy subjects without history of thrombohemorrhagic events acted as the control group.

3.2. BLOOD SAMPLES AND REAGENTS

Peripheral blood samples were obtained in trisodium citrate tubes (vacutainer system) and the first 3 ml of blood were discarded. Platelet studies were initiated within one hour after blood collection and leukocyte studies were accomplished within a maximum of 4 hours after sample collection. Monoclonal antibodies (mAbs) used were all purchased from Becton Dickinson (BD*, USA*). Platelets were activated using Thrombin Receptor Activator Peptide 6 (TRAP6), ADP, Arachidonic Acid sodium salt and Epinephrine bitartrate salt (Sigma Chemical, St Louis, USA). Quinacrine (*Sigma Chemical, UK*) was used as fluorescent to detect platelets dense granules. For leukocyte activation studies was used lipopolysaccharide (LPS) from E. coli (Sigma Chemical, USA).

3.3. ROUTINE HEMATOLOGICAL ASSAYS

White blood cell differential count, hematocrit, hemoglobin, red blood cell and platelet counts were determined by automated methods using a Cell Dyn 4000 (Abbott).

3.4. MOLECULAR STUDIES

3.4.1. DNA extraction

DNA was extracted from whole blood using JETQUICK Blood & Cell Culture Spin Kit (Genomed) protocol according to manufacture instructions. Briefly, whole blood cells were lysed by a combination of proteolytic enzyme (Proteinase K), detergent and a chaotropic salt. The lysate was directly applied in high specified silica membrane; several washes were performed to remove contaminants, and the purified DNA was eluted in 100 µL of water. DNA samples were stored at 4ºC or -20ºC.

3.4.2. DNA quantification

DNA was quantified by spectofotometry with a NanoDrop1000 (Thermo Scientific). Samples were diluted in sterile water at a final concentration of 5 ng/µL for RQ-PCR assays.

3.4.3. Allele-specific polymerase chain reaction for JAK2V617F mutation

Allele-specific polymerase chain reaction (ASO-PCR) exploits the fact that oligonucleotides primers must be perfectly annealed at their 3’ ends for a DNA polymerase to extend these primers during PCR. In this technique, primers are designed to match with a specific nucleotide and to increase the specificity of primer binding, a mismatch at the third nucleotide from the 3’ end is included to maximize discrimination of the wild-type and mutant alleles.

The ASO-PCR for the JAK2V617F mutation enables simultaneous amplification of the mutant and normal alleles plus a DNA control band with two pairs of primers in a single PCR tube. The primers used were the follow:

Primer F0: 5’ TCCTCAGAACGTTGATGGCAG 3’
Primer R0: 5’ ATTGCTTTCCTTTTTCACAAGAT 3’
Primer F wild-type: 5’ GCATTTGGTTTTAAATTATGGAGTAT*A*T**G** 3’
Primer R mutant: 5’ GTTTTACTTACTCTCGTCTCCACA*A*A**A** 3’

Note: mismatch nucleotide are pointed in italic; specific wild type and mutant nucleotide in bold.

For the ASO-PCR, 2 µL of DNA were added to a final reaction volume of 20 µL, containing Quiagen Multiplex PCR Kit (Quiagen), primers F0, R0, F wild-type and R mutant, and water. Amplification was performed in a thermocycler (Biometra), with an initial enzymatic activation of 95ºC, 10 minutes, followed by 30 cycles of amplification with denaturation at 95ºC, 30 seconds, annealing at 58ºC, 30 seconds, and extension at 72ºC during 60 seconds.

3.4.3.1. Gel electrophoresis of amplification products

PCR products were submitted to electrophoresis in agarose gel 2% (Ultrapure™ agarose, Invitrogen). Gel stain was performed using Sybr® Safe DNA

(Molecular Probes, Invitrogen) and a 100 bp DNA ladder (DNA ladder 100bp, Invitrogen) was used as reference for amplification products. Amplification products were visualized in a UV transiluminator and after photographed (Vilber Lourmat, UV Kodac EDAS 290).

3.4.4. Real-Time quantitative PCR for JAK2V617F mutation

Real-time quantitative PCR (RQ-PCR) permits accurate quantification of PCR products during the exponential phase of the PCR amplification process. The method employed here uses a hydrolysis probe, FAM-TAMRA, which consists of an oligonucleotide labeled with a 5' reporter dye (FAM) and a downstream 3' quencher dye (TAMRA). Hydrolysis probes exploit the 5'-nuclease activity of the *Taq* polymerase; when the probe is intact, the proximity of the reporter dye to the quencher results in the suppression of the reporter fluorescence. If the probe hybridizes to the target, DNA polymerase cleaves the probe between the reporter and the quencher and probe fragments are then displaced from the target. The increase in fluorescence is directly proportional to the target copy number amount in the sample at the beginning of the amplification. The number of PCR cycles necessary to detect a signal above the threshold is Cycle threshold (Ct) and is directly proportional to the amount of target present at the beginning of the reaction. When using standards with a known number of molecules, a standard curve can be established and the precise amount of target present in the sample determined.

The quantitative allele specific PCR technology employed here is based on the use of specific forward primers, for the wild-type and the V617F allele respectively. All samples with JAK2V617F mutation (ASO-PCR) were quantified using

JAK2MutaQuant™ Kit (Ipsogen) according to manufacture instructions, using a Real-Time PCR 7300 from Applied Biosystems.

JAK2V617F allele burden was calculated using the formula: JAK2V617F % = [CN V617F/ (CN V617F + CN WT)]*100, where CN is the copy number.

3.4.5. Reference to MPL mutations screening

Samples negative for JAK2V617F, were screened for MPL exon 10 mutations using single strand conformation polymorphism (SSCP). In the presence of an altered mobility compared with control, samples were sequenced by Sanger method in an ABI 310 Genetic Analyzer (Applied Biosystems). Note: MPL mutation screening was performed by other element of the group.

3.5. FLOW CYTOMETRY

Flow cytometry (FCM) is a technique that simultaneously measures and analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. Briefly, single cells in a suspension are labeled with a fluorescent conjugated monoclonal antibody (mAb) and then passed through a flow chamber through the focused beam of a laser. After the laser light activates the fluorophore at the excitation wavelength, detectors process the emitted fluorescence and light scattering properties of each cell. The intensity of the emitted light is directly proportional to the antigen density.

3.5.1. Platelet baseline and activation studies

A whole blood FCM assay was used to evaluate platelets P-selectin (CD62P) and CD63 (dense granules). For activation assays AA, TRAP6, EPI and ADP were added at final concentration of 0.1 mM, 5 µM and 25 µM, 20 µM and 5 µM, respectively. Briefly, whole blood was diluted in saline (1:10) and anti-CD42b-FITC and anti-CD62P-PE or anti-CD63-PE mAbs and were added to the polypropylene tube. Samples were left undisturbed for 15 minutes in the dark, at room temperature, and resuspended in saline before analysis in a FACSCalibur. Platelets were identified by their characteristic side-scatter and FITC-conjugated anti-CD42b positivity. Ten-thousand events were collected from each sample, and data acquisition and processing were performed with Cell-Quest software (BD). Results for CD62P and CD63 were expressed as percentage.

3.5.2. Platelet mepacrine uptake and release test

Platelets have been shown to selectively take up mepacrine (Quinacrine) into the dense granules of platelets. The uptake of mepacrine is useful because it emits a green fluorescence when excited at the appropriate wavelength of light (Wall *et al.*, 1985).

For mepacrine uptake are release assay, citrated whole blood was diluted 1:10 with saline and incubated for 20 minutes, in the dark at room temperature, with mepacrine 40 µM (uptake test); for release test, TRAP6 30 µM was added to the tube. Samples were resuspended in saline and analyzed by FCM. Ten-thousand events were collected and platelets were identified by their forward/side-scatter logarithmic mode, and green fluorescence (FL1) associated to mepacrine was quantitated. A positive result was recorded when mean fluorescent index (MFI) ratio between

mepacrine uptake and release was greater than 1.5. For each analysis a normal control sample was included.

3.5.3. Evaluation of Platelet-leukocyte aggregates

Whole blood samples (50 µl) were incubated with FITC-conjugated anti-CD42b, PE-conjugated anti-CD14 and PerCP-conjugated anti-CD45. After 15 minutes of incubation, samples were lysed, centrifuged, resuspended in PBS and analysed on the FACSCalibur (BD). 50000 events were collected for each sample. Platelet-polymorphonuclear leukocytes aggregates (platelet-PMN) were identified by the forward/side-scatter proprieties of PMN and positivity for CD42b and platelet-monocyte aggregates were identified by CD14 and positivity for CD42b. PMN and monocyte aggregates were expressed as the percentage of PMN and monocytes, respectively, with bound platelets.

3.5.4. CD11b and Tissue factor (CD142) expression in PMN and Monocytes at baseline and after LPS stimulation

Whole blood samples (50 µL) were incubated with FITC-conjugated anti-CD14, PE-conjugated anti-CD11b or anti-CD142 PE and PerCP-conjugated anti-CD45. After 15 minutes of incubation at room temperature, samples were lysed, centrifuged and ressuspended in PBS, before analysis in the FACSCalibur (BD). For the activation assays, we used a method adapted from Amirkhosravi *et al* (1996). Briefly, 1 mL of citrated whole blood was incubated at 37ºC with LPS (10 µg/ml) for 1 hour, and subsequently processed as per other samples.

PMN and monocytes were identified by their forward and side-scatter properties and monocytes were identified by gating the CD14 positive cells; 50000 events were collected from each sample. Since CD11b is constitutively expressed by PMN and monocytes, and the number increases upon activation, therefore the results are expressed as mean fluorescence intensity (MFI units), representing the mean level of marker's expression/cell; CD142, tissue factor, is a glycoprotein synthesized by monocytes, and is expressed as percentage of positive cells because is not constitutively expressed, and is expected that upon activation the number of positive cells increases. To circumvent the day to day variations in MFI values, we convert MFI values to molecules of equivalent soluble fluorochrome (MESF) units using standardized fluorescent beads (Quantum™ PE Medium Level, BangsLabs, USA).

3.6. STATISTICAL ANALYSIS

t-test was performed to assess the significance of differences between the mean values of continuous variables among the groups. Chi-squared (χ^2) was used to determine significance between nominal variables. Differences were considered significant at a p-value<0.05. StatView 5 was used for statistical analysis.

CHAPTER 4 – RESULTS

4.1. CHARACTERISTICS OF THE PATIENTS

A total of 75 adult patients, 47 ET and 28 PV, entered in this study. At the time of the study all patients were receiving cytoreductive therapy with hydroxyurea (HU). All patients signed a written informed consent to suspend aspirin 10 days prior flow cytometry studies. Patients and controls hematological parameters are summarized in Table 1. Platelets are significantly increased in ET patients ($p<0.01$). Regarding to thrombotic manifestations, 16 ET and 7 PV patients have a positive history of thrombosis at diagnosis.

Table 1 – Characteristics of study subjects.

	Controls	ET	PV
Subjects (n)	48	47	28
Male/Female	15/33	25/22	11/17
Age (years)	30 (20-64)	65 (29-96)	75 (52-92)
Hemoglobin (g/L)	13.9 (11.9-17.4)	13.9 (9.6-16.5)	14.0 (10.6-14)
Hematocrit (%)	41.2 (35.6-51.9)	40.8 (27.6-49.9)	41.5 (29-54)
Red blood cells ($x10^{12}$/L)	4.5 (4.0-5.7)	3.9 (2.5-5.7)	3.9 (2.5-8.1)
Leukocytes ($x10^{9}$/L)	6.8 (4.4-10.1)	6.3 (4-11.1)	7.1 (2.1-15)
Platelets ($x10^{9}$/L)	271.5 (127-524)	457.0* (110-794)	288.0 (51-288)
Thrombotic antecedents (n)	0	7	16

All values are expressed as median (range).

*p<0.01 versus controls and PV

4.2. MOLECULAR STUDIES

4.2.1. Allele-specific polymerase chain reaction for JAK2V617F mutation

The JAK2V617F point mutation was detected by ASO-PCR in 28 PV (100%) patients and in 28 ET (60%) patients. All ET patients with no detectable JAK2V617F mutation were screened for MPL exon 10 mutations.

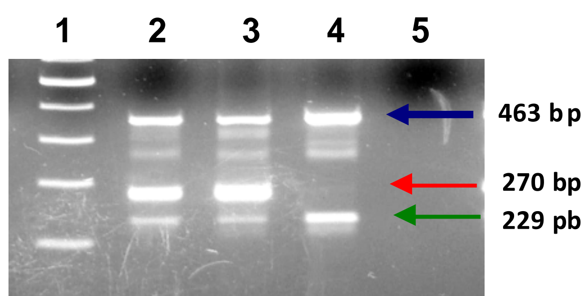

Figure 5 – Gel electrophoresis of ASO-PCR products for JAK2V617F. The 463 bp fragment corresponds to PCR internal control; the 270 bp to the V617F allele and the 229 bp to the wild-type allele.

Legend: lane 1: 100 bp DNA ladder; lane 2 and 3: samples with V617F allele and normal allele; lane 4: normal sample; lane 5: negative control.

4.2.2. MPL exon 10 mutations screening

SSCP revealed a difference in electrophoretic mobility in 2 ET patients in relation to control samples. MPL gene exon 10 direct sequencing revealed two different mutations: W515L, a G to T transition, and a non described R524C mutation, a C to T transition.

4.2.3. Quantitative Real Time PCR for JAK2V617F

JAK2V617F allele burden was evaluated by RQ-PCR in all patients that have the mutation: 28 PV and 28 ET patients. PV patients present significantly higher levels of JAK2V617F allele burden compared to ET patients (66.1% vs 32.4%, $p<0.01$) (Figure 6). In 18 PV patients JAK2V617F allele burden was >50% and in the ET group 10 patients presented JAK2V617F allele burden>50%.

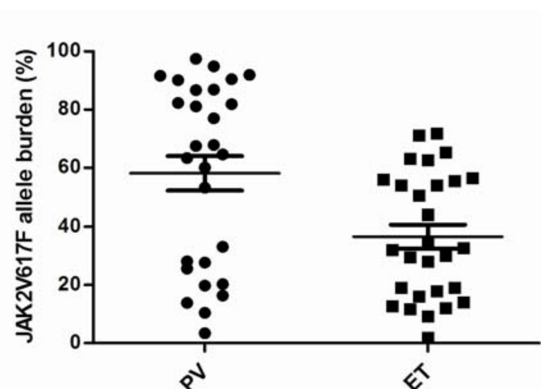

Figure 6 – JAK2V617F allele burden in PV and ET patients. The JAK2V617F allele burden found in PV patients was significantly higher than in ET. The horizontal line marks the median and the bars show the upper and lower range of values.

4.3. FLOW CYTOMETRY

4.3.1. Platelet activation studies

Platelet activation studies were performed to evaluate platelet baseline expression of CD62P and CD63, and to evaluate platelet degranulation of both alpha (CD62P) and dense granules (CD63) after stimulus with different agonists. Results are summarized in Table 2.

Table 2– Platelet activation studies.

	Controls (n=48)	ET (n=47)	PV (n=27)
% CD62P			
Baseline	2.8 (1.0-8.3)	7.1 (1.0-45.3)*	8.2 (2.3-18.3)*
AA 0.1 mM	11.8 (2.6-47)	24.4 (2.1-82.8)*	30.5 (4.3-44.2)*
TRAP6, 5 µM	84.4 (4-98.8)	66.5 (1.1-95)	59.8 (13.2-92)
TRAP6, 25 µM	97.4 (87.9-99.4)	92.1 (8.9-98.5)*	89.1 (28.2-98)*
EPI 20 µM	29.3 (9.3-52.9)	39.3 (9.9-87.8)	38.5 (5.6-59.2)
ADP 5 µM	74.4 (31.4-98)	70.6 (41.6-93.3)	76.7 (49.9-89.6)
% CD63			
Baseline	1.9 (0.8-3.7)	2.8 (0.5-32)*	3.4 (0.4-18.5)*
TRAP6, 5 µM	37 (1.4-89.2)	22.2 (1.3-76.3)*	15.6 (4-57.3)*
TRAP6, 25 µM	76.4 (49.4-96.6)	54.8 (6.2-92.9)*	49.2 (6.7-96)*

All values are expressed as median (range). *$p<0.01$ vs controls

Baseline platelet CD62P and CD63 expression were significantly elevated in patients with NMP compared with controls ($p<0.01$). Platelet response to AA was significantly increased in both PV and ET ($p<0.01$). Regarding the TRAP6 agonist, no difference was found in CD62P expression after exposure to low TRAP6 (5 µM) concentration, however, statistically significant differences were observed after exposure to a higher concentration of TRAP6 (25 µM). CD63 expression in patients' platelets was significantly diminished after TRAP6 activation, in both concentrations, comparing to controls. No difference in CD62P expression was observed between patients and controls after stimulation with Epinephrine and ADP.

4.3.2. Mepacrine uptake and release test

Platelet mepacrine uptake and release tests were negative (ratio<1.5) in 20 out of 26 PV patients (63%) and in 22 out of 44 ET patients (50%) (Table 3). In all controls the test was positive. A statistically significant reduced mepacrine uptake was verified in both groups of patients (*p*<0.01). Mepacrine release after TRAP stimulation is decreased in PV (p=ns).

Table 3 - Platelet mepacrine uptake and release test.

	Mepacrine Uptake (MFI)	Mepacrine Release (MFI)	Ratio uptake/release	Positive results (%)
Controls (n=46)	11.3 (8.5-16.3)	5.3 (3.7-9)	1.97 (1.5-3)	100%
ET (n=44)	8.2 (5.8-19.6)*	5.4 (3.1-14.9)	1.5 (1-2.9)	50%
PV (n=26)	8.5 (3.6-18.7)*	6.3 (3.3-14-3)	1.36 (1.1-1.9)	23%

MFI- median fluorescence intensity. All values are expressed as median (range). Results are considered positive when ratio uptake/release>1.5.
*P<0.01 vs controls

4.3.3. Platelet leukocyte-aggregates

Circulating platelet-monocyte (PM) and platelet-PMN aggregates, were determined as the percentage of monocytes and PMN, respectively, positive for CD42b. The results show statistically significant increased levels of PM and platelet-PMN aggregates in both ET and PV patients compared with the control group (Figure 7). Particularly, the percentage of PM-aggregates was 83±13 in ET, 80±10 in PV and 64.8±13 in controls (p<0.0001) and the percentage of platelet-PMN aggregates was 31.1±13.2 in ET and 22.5±8.9 in PV patients versus 16.1±7.2 in controls (*p*<0.01). ET

patients present a statistically significant increase in platelet-PMN aggregates comparing to PV patients (p<0.01).

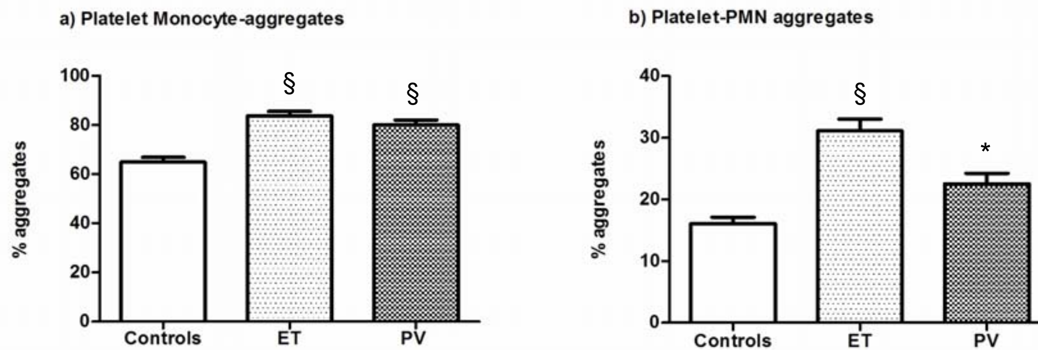

Figure 7 – a) Platelet-monocyte and b) platelet-PMN aggregates. Percentage of circulating platelet-monocyte and platelet-PMN aggregates. Results are expressed as mean ± SEM. $^{§}p$<0.0001 vs controls; *p<0.05 vs controls; p<0.01 ET vs PV.

4.3.4. CD11b expression: baseline and after LPS stimulation

Monocytes and neutrophils express CD11b antigen at membrane surface; increased CD11b levels are expected in response to an activating stimulus, as result of cell degranulation and mobilization of storage pool granules. Results are summarized in Table 4.

Table 4 – Monocyte and PMN CD11b expression in controls and patients.

	CD11b - Monocytes		CD11b - PMN	
	Baseline	*LPS*	*Baseline*	*LPS*
Controls (n=48)	138±98	342±129	70.9±41	398±156
ET (n=47)	232±127**	432±120*	90±38*	487±150*
PV (n=28)	274±153**	456±153*	102±95	499±149*

Results are expressed in MESFx10^3 units and are given as mean and standard deviation.
** p<0.0001 vs controls
*p<0.01 vs controls

Baseline monocyte-CD11b expression was statistically significant increased in patients compared to the control group (*p*<0.0001). Baseline PMN-CD11b was statistically significant increased in ET patients comparing to control subjects (*p*<0.01), and although increased in PV patients no statistically significant differences were found comparing with controls. Stimulation with LPS increased monocyte and PMN CD11b surface expression in all groups. Statistically significant differences were observed after monocytes and PMN LPS stimulation of both ET and PV patients comparing to control subjects (*p*<0.01). Whether at baseline or after LPS activation, no significantly differences were observed between ET and PV subjects.

4.3.5. Monocyte tissue factor expression: baseline and after LPS stimulation

Lipopolysaccharide (LPS) has been shown to stimulate monocytes TF expression. The percentage of monocytes bearing TF (CD142) was evaluated by whole blood flow cytometry before and after LPS stimulation. In basal conditions, PV and ET patients show a statistically significant increase of TF (*p*<0.0001) compared to

controls (PV: 15.7±17%; ET: 5.7±4.8%; CT: 2±1.6%) (Figure 8). The percentage of monocytes bearing TF, was significant increased in PV patients when compared to ET (p<0.05). After 1 hour incubation with LPS, an increased expression of TF in monocytes was observed in all groups (PV: 45.1±25%; ET: 41±19%; CT: 21.5±16%), with statistically significant differences between patients and controls (p<0.0001), and no differences between PV and ET.

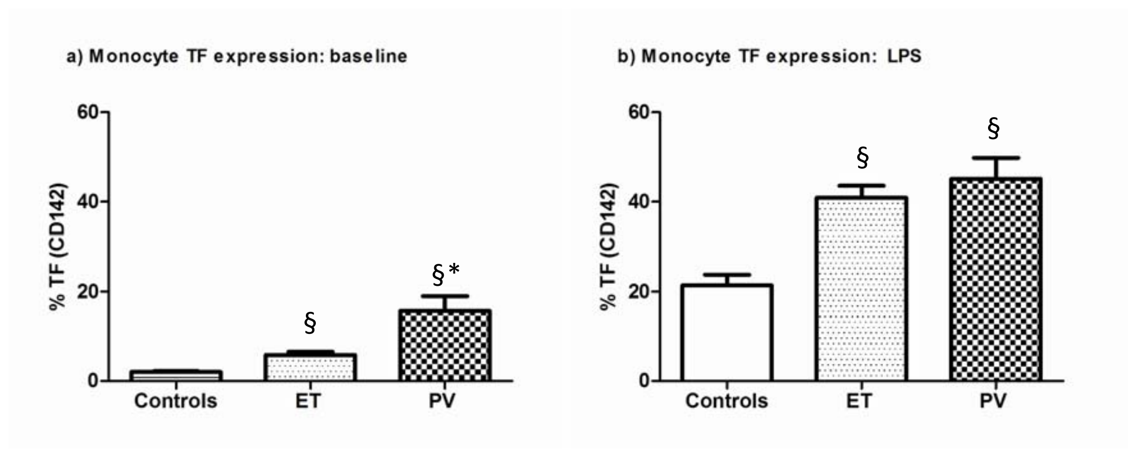

Figure 8 – Percentage of monocyte expressing tissue factor. a) Baseline conditions, b) after incubation with LPS. Results are expressed as mean ± SEM. [§]p<0.0001 vs CT; *p<0.05 PV vs ET.

4.4. INFLUENCE OF JAK2V617F ALLELE BURDEN

In order to evaluate the influence of JAK2V617F allele burden in all the studies performed above, ET patients were divided in three groups: ET no mutation (JAK2 wild-type) (n=17), ET JAK2V617F<50% (n=18) and ET JAK2V617F>50% (n=10). The two patients who presented MPL mutation were excluded to this analysis. PV patients were divided in two groups: PV JAK2V617F<50% (n=10) and PV JAK2V617F>50% (n=18).

4.4.1 Platelet studies

PV and ET patients with JAK2V617F allele burden >50%, when compared to the others groups, have a diminished expression of both CD62P and CD63 after stimulation with the different agonists (Table 5). PV patients with mutant allele burden >50% have a significant reduced expression of CD63 after TRAP6 25 μM and a significant reduced expression of CD62P after ADP when comparing with PV JAK2V617F<50%. Mepacrine test results are not significantly different between groups.

Table 5 – Platelet activation and mepacrine test according to patients' mutant allele burden.

	ET			PV	
	No mutation (n=17)	**V617F<50%** (n=18)	**V617F>50%** (n=10)	**V617F<50%** (n=10)	**V617F>50%** (n=18)
% CD62P					
Baseline	10.1 ± 10	12.5 ± 12	8.6 ± 9.5	8.5 ± 3.1	7.7 ± 3.7
AA 0.1mM	26 ± 19	30 ± 19	27.8 ± 14	29.6 ± 11	30.5 ± 8.8
TRAP6, 5μM	61.3 ± 30	62.8 ± 25.9	35.2 ± 22	50.6 ± 26.5	56.3 ± 19.8
TRAP6, 25μM	83.3 ± 22.8	91.3 ± 5.7	57.3 ± 25.7	84.4 ± 18.2	80.1 ± 17.5
EPI 20μM	38.3 ± 20.2	41.6 ± 18.1	32.9 ± 13.9	44.7 ± 16.2	32.6 ± 14.4
ADP 5μM	69.8 ± 17	72.2 ± 11.4	67.7 ± 10.4	81.2± 10	71.8 ± 10.3*
% CD63					
Baseline	3.1 ± 2.5	5.9 ± 8.3	3.6 ± 2	5.6 ± 4.3	5.3 ± 4.6
TRAP6, 5μM	25 ± 20.9	29.4 ± 18.5	21.9 ± 13.4	21.4 ± 17.1	21.3 ± 13.4
TRAP6, 25μM	49.6 ± 22.2	58.6 ± 13	42.2 ± 19.6	52.1 ± 24.8	43.4 ± 23.9*
Mepacrine positive test	1 (17%) (n=6)	5 (28%) (n=18)	8 (50%) (n=16)	9 (53%) (n=17)	4 (40%) (n=10)

All values are expressed as mean ± standard deviation; *p<0.05: PV<50% versus PV>50%

4.4.2. Platelet-leukocyte aggregates

The percentage of platelet-PMN aggregates was found higher in patients with JAK2V617F>50% (ET: 39.7% ±12, PV: 25.6±9%), and statistically significant different from ET patients with no mutation and from ET and PV patients JAK2V617F<50% (Figure 9). No differences were found on the percentage of platelet-monocyte aggregates, regarding allele burden.

a) Percentage of platelet-PMN aggregates **b)** Percentage of platelet-monocyte aggregates

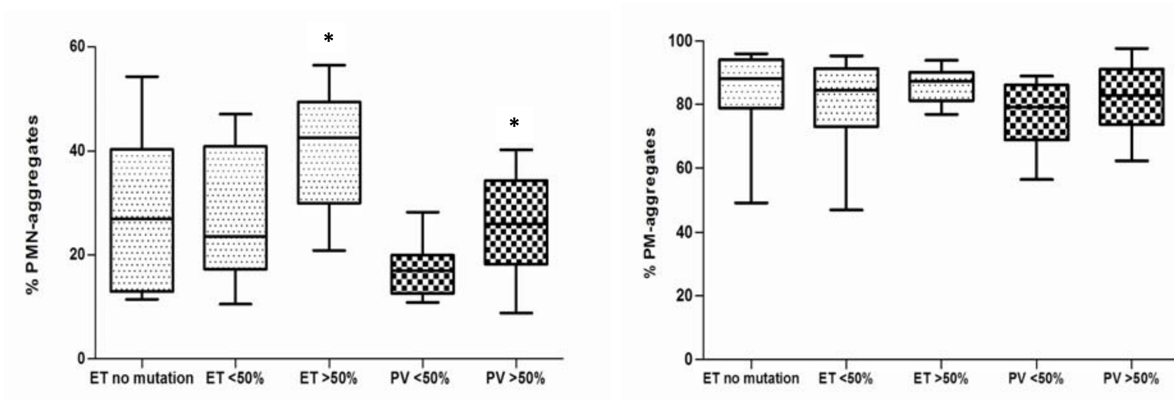

Figure 9 – a) Percentage of plateler-PMN aggregates and b) percentage of PM-aggregates in patients with no mutation, JAK2V617F allele burden <50% and >50%. *p<0.05 when comparing: ET>50% vs ET <50%; ET>50% vs ET no mutation; PV<50% vs PV>50%; ET and PV groups. Boxes represent the maximum and minimum values and the horizontal line the mean; the bars show the upper and lower range of values.

4.4.3. CD11b expression in monocytes and PMN

ET and PV patients with JAK2V617F allele burden >50% have increased monocyte and PMN CD11b expression, at baseline levels and after activation with LPS (Figure 10).

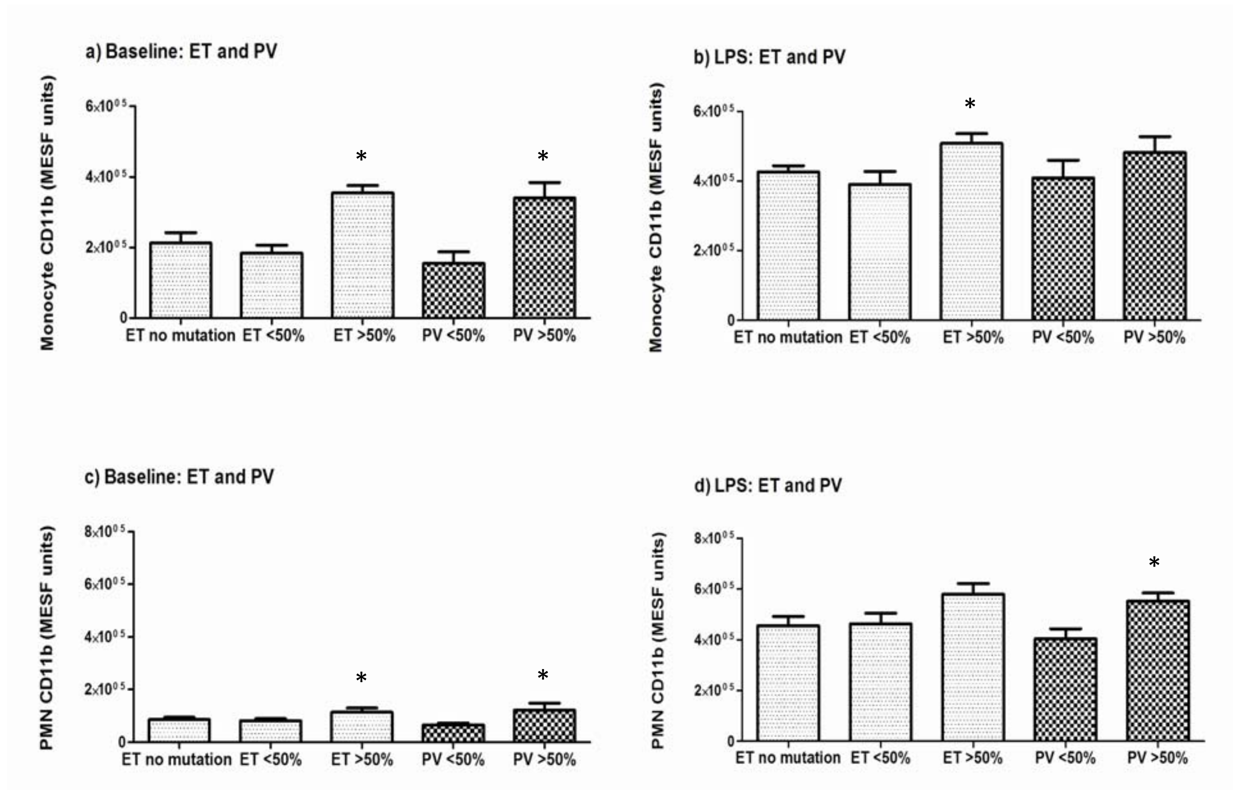

Figure 10 – Monocyte and PMN CD11b expression in patients with ET and PV according to allele mutation burden. a) at baseline level, *p<0.01: ET >50% vs ET no mutation and ET <50%; PV>50% vs PV<50%; b) after LPS activation, *p<0.01: ET >50% vs ET no mutation and ET <50%; c) at baseline level, *p<0.01: ET>50% vs ET<50% and no mutation, PV>50% vs PV<50%; d) after LPS activation, *p<0.01: PV >50% vs PV <50%.

4.4.4. Tissue factor expression in monocytes

Patients with JAK2V617F allele burden >50% present an increased percentage of TF at monocyte surface, both at baseline and after LPS activation (Figure 11). PV patients with allele burden >50% have a statistically significant increase on TF at baseline compared with PV patients with allele burden <50% (22.4±18 versus 3.8 ±2.6, *p*<0.01). ET patients with allele burden >50% have higher baseline TF (7.3 ±3.5), comparing to ET patients with no mutation (5.1 ±5.9) and to the allele burden <50% (5.3 ±4.3), although differences are not statistically significant. After LPS stimulus, an increased monocytes TF expression occurs in both PV and ET samples, with statistically significant differences observed in PV patients with mutant allele burden >50% (*p*<0.01).

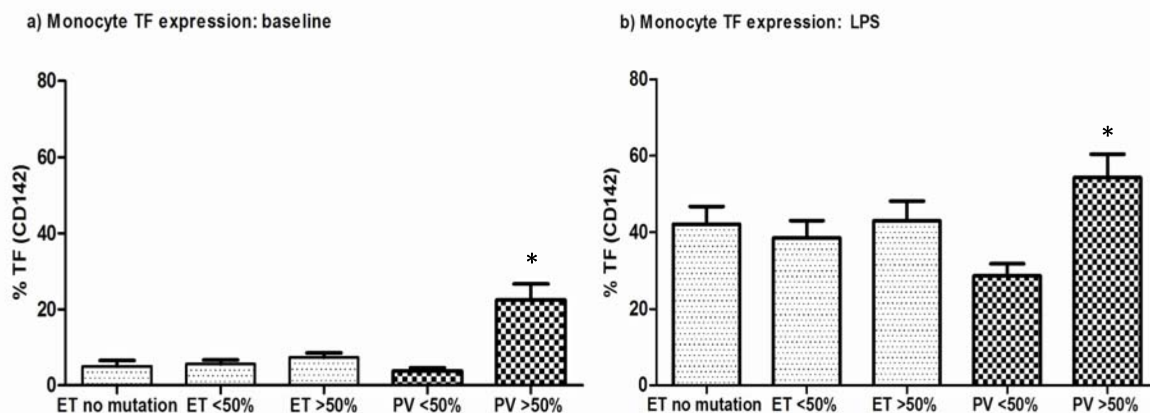

Figure 11 – Percentage of monocyte expressing TF. a) Baseline conditions, b) after incubation with LPS. Results are expressed as mean ± SEM. **p*<0.05 PV<50% vs PV>50%.

4.5. THROMBOSIS AND JAK2V617F MUTATION

Our data show that ET patients with the JAK2V617F mutation have a statistically significant higher incidence of thrombosis (*p*<0.01, χ^2=5.3) and that patients

with JAK2V617F allele burden >50% have the highest incidence of thrombosis, with statistical significance ($p<0.01$, $\chi^2=9$). In PV patients, no association was found between allele burden and previous thrombosis (Table 6).

Table 6 – Allele burden and thrombosis at diagnosis.

	ET (n= 47)		PV (n=28)	
	No thrombosis (n=31)	**Thrombosis (n=16)**	**No thrombosis (n=21)**	**Thrombosis (n=7)**
JAK2V617F allele burden (%)	16.1± 22.4	35.1±25*	58.7± 30.9	56.4± 35
JAK2V617F<50% (n)	15	6	7	3
JAK2V617F>50% (n)	3	7*	14	4
No mutation (n)	13	2	-	-

Values are expressed as mean± standard deviation. *p<0.01 vs No thrombosis

CHAPTER 5 – DISCUSSION

This study evaluates a group of 79 subjects with myeloproliferative neoplasms, 28 with polycythemia vera and 47 with essential thrombocythemia. All the patients are under hydroxyurea treatment and remain clinically stable with follow up periods of 78 months for PV and 84 months for ET. Among them, 7 PV and 16 ET patients have a positive history of thrombosis at diagnosis. By using allele-specific PCR the JAK2V617F mutation was found in 28 PV patients (100%) and in 28 ET patients (60%). Two ET patients, JAK2V617F negative, carry a mutation in MPL gene exon 10: W515L and R524C. Both JAK2V617F and MPL mutations are somatic acquired mutations that have been shown to induce constitutive JAK/STAT activation. Several studies identified patients that carry the JAK2V617F allele in homozygosity, consistent with the lost of heterozygosity for the JAK2 locus. This is expected to result in a higher level of JAK/STAT activation, possibly due to the lack of competition between normal and mutated allele. The same is true for MPL mutations, although less common. Using quantitative real time-PCR to distinguish patients "heterozygotic" (<50%) from those "homozygotic" (>50%) for the JAK2V617F, we found 18/28 PV (64.3%) and 10/28 ET (37.5%) patients with a JAK2V617F allele burden >50%. The mutant allele burden was statistically significant greater in PV when compared to ET patients. This observation is in line with previous reports (Antonioli *et al.*, 2008; Vannucchi *et al.*, 2008), and in agreement to the gene dosage hypothesis which suggests that homozygosity favors the erythroid phenotype.

In addition, other authors found significant correlations between the JAK2V617F mutation and the occurrence of complications in MPN patients. In our patients' group we found that ET patients with JAK2 or MPL mutations have a statistically significant occurrence of thrombotic events at diagnose. Other studies (Vannucchi *et al.*, 2008) described a significant association between thrombosis and homozygosity in ET patients, which is sustained after multivariate analysis, and no significant difference in thrombotic events between heterozygous and homozygous PV patients. In line with these observations, our data show, in ET patients, a statistically significant correlation

between JAK2V617F allele burden >50% and thrombosis. We found no association regarding allele burden and thrombosis in PV patients.

To investigate if JAK2V617F allele burden influenced platelet and leukocyte activation, we tested different cellular activation markers and found an increased membrane expression of both CD62P (P-selectin) and CD63 (granulophysin) in non-stimulated platelets, indicating platelet activation. Similar observations were reported by (Arellano-Rodrigo et al., 2006; Jensen et al., 2000). When performing stimulation assays with different agonists, we observed statistically significant increase response to arachidonic acid and diminished expression of CD62P and CD63 following TRAP6 activation, comparing to controls. The increased response to arachidonic acid could be explained by an increased and sustained thromboxane A_2 (TXA_2) generation, that has been reported in 40% of MPN patients, in correlation with lipoxygenase deficiency (Shafer, 1984). As it has been suggested, but not demonstrated by other authors, we observed an acquired storage pool disease in 20/26 (77%) PV and 22/44 (50%) ET patients. This phenotype may be a consequence of a reduced number of platelets dense granules or due to continuous activation conjugated with abnormal receptor mediated granule secretion, consistent with a low response to TRAP6 agonist.

As referred above, both PV and ET patients have circulating activated platelets, expressing P-selectin, thus may adhere to PMN and monocytes via the PSGL-1, with subsequent activation of β2 integrin CD11b/CD18 and generation of mixed aggregates. Increased circulating platelet-leukocyte aggregates have been previously demonstrated in several pathological conditions associated with thrombosis propensity (Jensen et al., 2001; McEver, 2007). Accordingly, when comparing to controls, we found a statistically significant increase of circulating platelet-monocytes and platelet-PMN aggregates in ET, confirming previous findings (Falanga *et al.*, 2007; Vilmow et al., 2003) and in PV patients, which have not been described previously. Furthermore, we found that ET patients have a statistically significant increase of platelet-PMN aggregates, but not in platelet-monocytes, when comparing to PV.

Circulating platelet-leukocyte aggregates may be triggered not only by P-selectin expression at platelets surface, but also by activated leukocytes. Whether the presence of activated leukocytes promotes platelets activation and how this interaction is translated into hemostatic activation is unclear. During cellular activation, leukocytes undergo phenotypic modifications with changes in expression of adhesion molecules on the cellular surface. The CD11b integrin (Mac-1) is responsible for the firm attachment of leukocytes to endothelium and platelets and is currently accepted as a marker of activation. Furthermore, the cooperation between activated leukocytes and platelets is suggested to be involved in tissue factor generation and activation of extrinsic coagulation system (TF binding to factor VII/VIIa) (Vilmow *et al.*, 2003). In normal conditions, 80–90% of monocytes TF is latent or encrypted, having little or no procoagulant activity. TF expression is up-regulated by a number of pathophysiological agonists, as well as following P-selectin binding to PSGL-1 on monocytes (Bouchard and Tracy, 2002).

When performing *in vitro* activation assays with LPS, an endotoxin that induces inflammatory response, we observed a significantly increase on CD11b and TF expression in ET and PV patients and in controls, confirming that cellular activation is related to CD11b and TF expression increments. Furthermore, we demonstrate that ET and PV patients have baseline activated monocytes and PMNs leukocytes in circulation, as they have statistically significant increase in CD11b and monocyte-TF when comparing to control subjects, and that TF expression is higher in PV than in ET patients, with statistically significant differences. These observations in ET patients are in line with Falanga *et al* (2005) and Arellano-Rodrigo *et al* (2006) published results; they did not study PV patients. Our data strongly suggest that increased monocyte TF expression observed in ET and PV patients is a consequence of platelets and leukocytes activation.

Our observation of a consistent increase on platelets and leukocytes activation, in PV and ET patients, is expected to have a direct correlation to the intensity of

constitutive JAK/STAT pathway activation. To access this hypothesis we studied the influence of allele burden in platelet activation and we found that PV and ET patients with allele burden >50% have a low response to agonists, comparing to patients with allele burden <50%, but not statistically different. However, in the PV homozygous JAK2V617F patients the CD62P expression, in response to ADP, and CD63 expression, after TRAP6 stimulation, are significantly decreased. The low CD63 expression may be a consequence of storage pool disease, which also is more frequently observed in our PV group of patients.

On the other hand, we found a correlation between increased leukocytes activation parameters in PV and ET patients and mutant JAK2V617F allele burden: patients with allele burden >50% present a statistically significant increase in circulating platelets-leukocytes aggregates and statistically significant differences in monocytes and PMN CD11b for ET and PV, comparing to allele burden <50%. Finally, we found that patients with allele burden >50% also have increased expression of TF at monocyte surface, with significantly differences in PV patients.

The data presented here strongly suggest that homozygosity for the JAK2V617F mutation increases leukocyte activation, in line with Oku S. *et al* (2010) suggestion that JAK2V617F signaling stimulates the mobilization of neutrophil secretory vesicles (CD11b, leukocyte alkaline phosphatase) through specifically activation of STAT3-depedent signaling pathway. We can also conclude that platelet activation and abnormal platelet function are not direct consequences of JAK2V671F allele burden.

Recently, a retrospectively study (Antonioli *et al.*, 2010) found that allele burden remains stable over a median follow-up time of 34 and 23 months in PV an ET patients, respectively, independently of whether patients were or not under hydroxyurea, and that the reduction of JAK2V617F allele burden is confined to subsets of patients. Since we have longer follow up periods and our patients are being treated with hydroxyurea,

it would be interesting to evaluate the allele burden at the time of diagnose, its association with thrombosis, and the hetero to homozygous progression over time.

CHAPTER 6 – CONCLUSION

We studied a group of patients with myeloproliferative neoplasms - 28 with PV and 47 with ET. All PV patients and 28 ET (60%) patients presented the JAK2V617F mutation. Two other ET patients have MPL exon 10 mutations. JAK2V617F allele burden >50% was more frequently found among PV patients, with statistically significance when compared to ET patients. Seven PV and 16 ET patients have a positive history of thrombosis at diagnosis.

As thrombosis is one of the main co-morbilities in MPN patients, we investigated the baseline hemostatic activation markers and their correlation with the JAK2V617F allele burden.

All patients have, at baseline, a significant increased membrane expression of both CD62P (P-selectin) and CD63 (granulophysin), indicating platelet activation, and abnormal platelet functional expressed by a significant increase response to arachidonic acid and diminished levels of CD62P and CD63 following TRAP6 activation.

At baseline all patients have activated monocytes and PMNs leukocytes, in circulation as showed by a statistically significant increase in CD11b expression, and monocyte-TF, the latter being significantly elevated in PV vs ET patients, probably reflecting a higher activation status in leukocytes.

In all patients circulating platelet-leukocytes aggregates are significant higher, likely resulting from platelets and leukocytes activation status. The platelet-PMN aggregates were significantly increased in ET than PV patients, in line with a higher percentage of thrombosis among ET patients.

These data clearly illustrate several mechanisms favoring thrombosis in PV and ET patients, namely, the baseline activation of platelets, monocytes and PMNs leukocytes, increased monocyte-TF and platelet-leukocytes aggregates.

This activated status probably results from the constitutive JAK/STAT signalization, as result of the acquired JAK2V617F mutation. Corroborating this

hypothesis in ET patients we found a statistically significant correlation between thrombosis and JAK2 or MPL mutations, and those with JAK2V617F allele burden >50% have statistically higher incidence of thrombosis. However, as other authors, we found no such correlation in PV patients.

Consistent with the influence of allele burden and acquisition of LOH for the JAK2V617F mutation and leukocyte activation we found, in all patients with JAK2V617F>50%, a statistically significant increase in leukocytes CD11b expression and in platelet PMN-aggregates in comparison with those with JAK2V617F<50%, and a statistically significant increase in monocytes TF in PV patients with allele burden >50%. Regarding to platelets activation, it’s not evident such effect since no significantly differences were found.

It would be interesting to evaluate the allele burden at the time of diagnose, its association with thrombosis, and the hetero to homozygous progression over time.

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