The JAK2 V617F mutation in Philadelphia chromosome negative chronic myeloproliferative disorders - Impact on clinical phenotype

Ph.D Thesis

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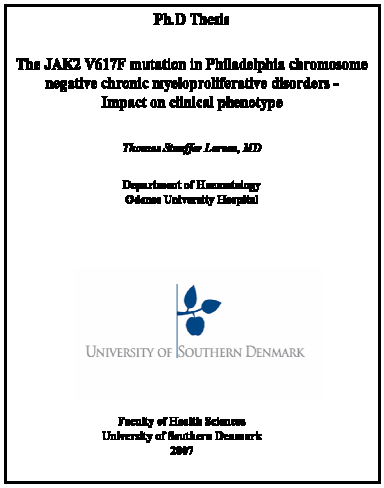
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The thesis is based on the following papers, referred to in the text by their roman numerals:

I. Larsen TS, Christensen JH, Hasselbalch HC, Pallisgaard N. The JAK2 V617F mutation involves B- and T-lymphocyte lineages in a subgroup of patients with Philadelphia-chromosome negative chronic myeloproliferative disorders. Br J Haematol 2007;136:745-51

II. Larsen TS, Pallisgaard N, Møller MB, Hasselbalch HC. Quantitative assessment of the JAK2 V617F allele burden: equivalent levels in peripheral blood and bone marrow. Leukemia 2008;22:194-5

III. Larsen TS, Pallisgaard N, Møller MB, Hasselbalch HC. The JAK2 V617F allele burden in essential thrombocythemia, polycythemia vera and primary myelofibrosis – Impact on disease phenotype. Eur J Haematol 2007;79:508-515.

IV. Larsen TS, Hasselbalch HC, Pallisgaard N, Møller MB. Bone marrow histomorphology and JAK2 mutation status in essential thrombocythemia. Acta Pathologica, Microbiologica et immunologica Scandinavica (APMIS) 2007;115:1267-73

V. Larsen TS, Pallisgaard N, Møller MB, Hasselbalch HC. High prevalence of arterial thrombosis in JAK2 mutated essential thrombocythaemia: Independence of the JAK2 V617F allele burden. Hematology 2008 (In press).

Preface

This Ph.D study was carried out at the Department of Hematology and the Department of Clinical Pathology, Odense University Hospital, Odense, Denmark in the period from December 1 2004 to December 1 2007.

I am deeply indebted to Hans Carl Hasselbalch, my principal supervisor, colleague and friend for his outstanding inspiration, guidance and support with respect to the present project, but also in general with respect to my development as a physician and scientist. I am convinced that our inspiring exchange of ideas will live on in time to come.

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Lastly, Tone and my two girls Laura and Dicte deserve my deepest gratitude for their loyal support and important role in bringing efforts in perspective.

Odense March 1 2008

Thomas Stauffer Larsen

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Abbreviations

ABL	**Abelson (gene)**
Add	**additive (chromosomal)**
AML	**Acute myeloid leukemia**
APC	**Allophycocyanin (fluorochrome)**
BM	**Bone marrow**
CHL	**Chronic hypereosinophilic leukemia**
CML	**Chronic myeloid leukemia**
CMML	**Chronic myelomonocytic leukemia**
CMPD	**Chronic myeloproliferative disorder**
CNL	**Chronic neutrophil leukemia**
CT	**Cycle threshold**
del	**deletion (chromosomal)**
der	**derivative chromosome (chromosomal)**
dNTP	**deoxyribonucleotide phosphate**
dup	**duplication (chromosomal)**
EEC	**Endogenous erythroid colonies**
EPO	**Erythropoietin**
ET	**Essential thrombocythemia**
FACS	**Fluorescence Activated Cell Sorting**
FISH	**Fluorescence in Situ Hybridyzation**
FITC	**fluorescein isothiocyanate (fluorochrome)**
G-CSF	**Granulocyte Colony Stimulating Factor**
GM-CSF	**Granulocyte Monocyte Colony Stimulating Factor**
GUS	**Beta-glucoronidase**
JAK2	**Janus Kinase 2**
Hb	**Hemoglobin**
HCT	**Hematocrit**
HEL	**Human erythrocytic leukemia (cell line)**
IE	**Idiopathic erythrocytosis**
IGF-1	**Insulin-like growth factor 1**
IL-3	**Interleukin-3**
IMF	**Idiopathic myelofibrosis**
Inv	**inversion (chromosomal)**
LOH	**Loss of heterozygozity**
MDS	**Myelodysplastic syndrome**
MPL	**Thrombopoietin receptor**
PB	**Peripheral blood**
PCR	**Polymerase-chain-reaction**
PE	**Phycoerythrin (fluorochrome)**
PE-Cy7	**Phycoerythrin-Cy7 (fluorochrome)**
PMF	**Primary myelofibrosis**
PRV1	**Polycythemia Vera Rubra gene 1**
PV	**Polycythemia vera**
qPCR	**Quantitative Polymerase-chain-reaction**
SCF	**Stem cell factor**
SE	**Secondary erythrocytosis**
SOCS	**Suppressor of Cytokine Signalling**
STAT	**Signal Transducers and Activators of Transcription**
t	**Translocation (chromosomal)**
TIA	**Transient ischemic attack**
TPO	**Thrombopoietin**
WBC	**White blood cells**
WHO	**World Health Organisation**

Chapter 1. Historical Overview

In the late nineteenth century the first cases of polycythemia vera (PV) and myelofibrosis (PMF) were described (1;2). Decades later (1934) essential thombocythemia (ET) was described (3). In 1951, based on observations of a considerable overlap in clinical and laboratory features of these conditions, William Dameshek introduced the concept of the chronic myeloproliferative disorders as a group of closely related disease entities featured by excessive proliferation and accumulation of myeloid cells from one or more lineages, sometimes accompanied by myeloid metaplasia (4). In the nineteen-seventies crucial experiments demonstrated the capacity of erythroid progenitors of in vitro proliferation in the absence of erythropoietin (EPO) in patients with PV – a phenomenon referred to as endogenous erythroid colonies (EEC) (5). Clonality studies revealed the origin of these diseases in a multipotent hematopoietic stem cell (6;7). In parallel with the advances in our knowledge on molecular biology through the nineteen-eighties and nineteen-nineties, it became evident that deregulated tyrosine kinase activity caused mainly by gene fusion products, was a key molecular pathogenetic mechanism causing chronic myeloid diseases as chronic myeloid leukemia (CML), systemic mastocytosis (SM), chronic myelomonocytic leukemia (CMML) and chronic hypereosinophilic leukemia (CHL) (8). Whereas CML - characterized by the BCR-ABL fusion protein, as a result of the chromosomal translocation t(9:22) - has become a true model of a molecularly defined disease, chromosomal aberrations in the other chronic myeloproliferative disorders are more infrequent and the spectrum is more heterogeneous (9;10). However, the loss of heterozygosity of the short arm of chromosome 9 (9p LOH) was recognized as a rather frequent chromosomal aberration in patients with PV (11). The recognition of 9p LOH together with the observation that EPO independent colony formation was dependent on molecular signaling through the JAK-STAT signaling pathway (12;13) provided important clues to the understanding of the molecular foundation for the three classical chronic myeloproliferative disorders – PV, ET and IMF. In spring 2005, four independent research groups identified a somatic point mutation in the gene coding for the *Janus Kinase 2 (JAK2)* tyrosine kinase. This mutation causes growth factor independent autonomous proliferation of hematopoietic precursors and was found in the majority of patients with PV and approximately half of the patients with ET and IMF (14-17).

Chapter 2. Background – Classification, Epidemiology, Clinical Phenotype and Treatment

2.1 Classification of the chronic myeloproliferative disorders

The WHO classification system from 2001 groups together as chronic myeloproliferative diseases the three classical myeloproliferative disorders (ET, PV and IMF), CML, CMML, CHL, chronic neutrophil leukemia (CNL) and a group of unclassifiable diseases (18). Because CML was molecularly characterized by the t(9;22) resulting in the BCR-ABL fusion gene and the constitutively activated protein tyrosine-kinase, and due to the lack of characteristic molecular markers, ET, PV and IMF have traditionally been referred to as the Philadelphia chromosome negative chronic myeloproliferative disorders (Ph-negative CMPD). The Ph-negative CMPD are clonal hemotopoietic stem cell disorders, characterized by autonomous proliferation of one or more myeloid cell lineages (19;20). Although ET, PV and IMF are interpreted as three distinct disease entities, considerable overlapping in clinical and laboratory features has been recognized for decades, which may impede the diagnostic accuracy. Moreover, the distinction from reactive or secondary erythro- and thrombocytosis are sometimes difficult. Accordingly, Ph-negative CMPD have historically been defined by a set of clinical, histopathologic and biochemical criteria in order to i) exclude reactive conditions with myeloproliferative features and ii) standardize diagnosis in order to enrole patients in clinical trials. Traditionally these diagnostic criteria have been of doubtful value in daily clinical practice.

PV is characterized by an increased hematocrit (HCT), often accompanied by elevated white blood cell (WBC) and platelet counts. The bone marrow is hypercellular with increased numbers of erythroid, megakaryocyte and granulocyte precursor cells, abnormal megakaryocyte morphology, often increased reticulin fiber content and a decrease in the amount of iron. Patients presenting with an isolated red cell mass and bone marrow features of erythroid hyperplasia, but without other morphological distinct features of PV and no evidence of secondary erythrocytosis (SE) is referred to as idiopathic erythrocytosis (IE) (21). ET is characterized by an isolated elevation of platelets in peripheral blood together with enlarged, mainly matured megakaryocytes in a normo- to slightly hypercellular bone marrow. In most series however, some patients are also featured by slight anemia and leucocytosis (22;23). These patients may be more correctly classified as *prefibrotic IMF* (24). Thus, with the introduction of the WHO criteria the bone marrow

morphological criteria were extended and the

concept of prefibrotic IMF was introduced (18). This new concept describes a condition with bone marrow hypercellularity, neutrophil proliferation, megakaryocyte proliferation and atypia and minimal or absent reticulin fibrosis. Together with these bone marrow characteristics, patients with prefibrotic IMF often but not always present with a normal hemoglobin (Hb) or slight anemia, mild to moderate leukocytosis and moderate to marked thrombocytosis as the most prominent features. Prefibrotic IMF should be interpreted as a pre-phase (cellular phase) of the classical IMF phenotype with marked fibrosis ranging from pronounced reticulin fibrosis to extensive collagen fibrosis with or without osteosclerosis, together with prominent megakaryocyte proliferation, leuko-erythroblastic blood smear and splenomegaly. The key to identification of the prefibrotic IMF is the abnormal, atypical megakaryocytic morphology and topography, typically in a hypercellular bone marrow, compared to a more restricted proliferation of enlarged mature megakaryocytes in classical or "true" ET. In the literature idiopathic myelofibrosis is also referred to as chronic idiopathic myelofibrosis (CIMF), agnogenic myeloid metaplasia (AMM) and myelofibrosis with myeloid metaplasia (MMM). Recently the International Working Group for Myelofibrosis Research Treatment (IWG-MRT) agreed on standardization of the nomenclature and the term primary myelofibrosis (PMF) was accepted as the preferred nomenclature when referring to the above mentioned conditions (25).

2.2 Epidemiology of the chronic myeloproliferative disorders

Epidemiological studies on chronic myeloproliferative disorders conducted during the last five decades have reported variable annual incidence rates. In PV, the entity studied most extensively, the annual incidence rate per 100.000 inhabitants varies considerably but most studies have reported it in the range of 1.9-2.6 (26-29). As regards ET and IMF the same variability is observed with annual incidence rates ranging from 0.6-2.5 (28-31) and 0.3-1.5 (28-30) per 100.000 inhabitants, respectively. These discrepancies may reflect geographic, demographic and ethnic variability. Additionally, and presumeably a more important contributor to discrepancy are the differences in diagnostic procedures and criteria used for classification. The median age at diagnosis is 69-74 years in PV (28;29) , 67-72 years in ET (28-31) and 67-76 years in PMF (28-30). There is a male predominance in PV and IMF (27), whereas ET is most common in females (31). Although life expectancy exceeds 15 years in both ET and PV most studies have reported a reduced life expectancy in PV (27;32), whereas data on ET regarding this matter is conflicting (31;32). However, the diseases follow a chronic clinical course characterized by considerable morbidity due to a high risk of thrombohemorrhagic
complications. Moreover, fibrotic and leukemic transformation occurs as part of the natural evolution of these disorders (33;34). Treatment

with certain myelosuppressive agents increases this risk, especially of leukemic transformation considerably (35-38). A higher propensity of fibrotic transformation ranging from 5-33 % is observed in PV (32;37;39-43), being higher in patients treated only with phlebotomy, compared to 2-7% in ET (44-51) after 10 to 15 years of follow up. The great majority of studies addressing the risk of fibrotic and leukemic transformation have included both patients treated with phlebotomy alone and patients treated with different myelosuppressive agents. Mixed study populations, variable follow-up time as well as a general reluctance to perform sequential bone marrow biopsies compromise firm conclusions on this issue. Studies have pointed to the possibility that a well controlled platelet count reduces myelofibrotic transformation (42), However, the latest large scale randomized study (PT1) demonstrated an increased risk of myelofibrotic transformation in ET patients treated with anagrelide compared to hydroxyurea, although platelet counts were equally controlled, suggesting a potential role of other myeloid cells (granulocytes and monocytes) in driving the myelofibrotic process (22).

The prognosis in PMF is very heterogenous ranging from less than 1 year to more than 15 years, but is in most studies reported with a median of 4-5 years (52-55). Robust parameters predicting prognosis have been difficult to identify. However the Dupriez (Lille) score using simple clinical and biochemical parameters subdivides patients into three risk groups with distinct survival expectancies (54).

2.3 Clinical phenotypes of the chronic myeloproliferative disorders

Symptoms and clinical characteristics of CMPD patients are caused by increased cell proliferation and turn-over of one or more myeloid cell lineages, and if pronounced, giving rise to hypermetabolic symptoms such as weight loss, fever and night sweats, which is most prominent in proliferative PMF. However the dominant symptoms and major cause of morbidity and mortality in PV and ET are due to quantitative and qualitative changes of clonally derived myeloid cells leading to vascular disturbances such as microvascular transient occlusions and major thrombosis in both the venous and arterial systems. The excessive myeloid proliferation may also cause sequestration of myeloid cells in various organs (myeloid metaplasia), most often causing splenomegaly. Although many clinical and biochemical characteristics are shared by the three CMPD's some manifestations are unique or more pronounced in one of the three distinct disease entities.

Polycythemia Vera

PV is characterized by trilinear myeloid hyperplasia and proliferation. The hallmark is erythrocytosis, reflected by an increased Hb concentration as well as an elevated hematocrit (HCT). In PV, whole blood viscosity increases dramatically when the HCT exceeds 45 %. The

elevated HCT has been shown to be a significant risk factor associated with an increased risk of arterial and venous thrombosis, which is the dominant morbidity in patients with PV. Lowering the HCT by phlebotomy markedly reduces the thromboembolic risk (56). Together with the increased red cell mass (RCM) some degree of thrombocytosis and leukocytosis are often apparent. Although platelet counts are sometimes massively increased > 1500 $x10^9$/liter, a modest elevation (450-1000 $x10^9$/liter) is most often observed. Whereas the platelet level itself has not proved to be an independent risk factor for thrombosis, structural and functional platelet abnormalities are correlated to thrombotic risk (57-59). Qualitative platelet changes may result in hyperactive circulating platelets. The in vivo platelet activation in part caused by high shear stress with increased platelet-platelet and platelet-endothelium interactions results in a secretion of granular products causing formation of loose vWF-mediated aggregates, which mediate transient plugging in the microcirculation (60-62). In addition, leukocytosis and leukocyte activation may play a key role in the pathogenesis of thrombosis. A slightly increased leukocyte count is frequent, and a significant correlation has been demonstrated between granulocyte cell surface activation markers and abnormally increased clotting tendency in vitro in PV and ET (63). It is also evident that a substantial proportion of patients with ET and PV have increased numbers of in vivo circulating monocyte-granulocyte-platelet aggregates (64;65). Most recent data suggests a direct correlation between an elevated leukocyte count and increased risk of thrombosis (23;66;67), being in accordance with the well established anti-thrombotic effect of hydroxyurea treatment in ET (68) as compared to anagrelide (22). Plugging of micro-aggregates of activated clonal myeloid cells in the microcirculation in concert with the release of local endothelial vasoactive cytokines may account for the classical clinical manifestations of acroparesthesias, erythromelalgia and transient neurological symptoms such as migraine-like headaches, amaurosis fugax and transient ischemic attacks (TIA), which in addition to constitutional symptoms characterize the clinical phenotype in PV. The TIA's are most often categorized as a major thrombotic event together with cerebral infarction, myocardial infarction and peripheral arterial occlusions. Also lower extremity deep vein thrombosis and pulmonary embolism are major thrombotic complications reported with increasing frequency in CMPD patients. Of importance, and unique is the unusual high frequency of splanchnic vein thrombosis such as the Budd Chiari syndrome recorded in patients with CMPD, being higher in PV than in ET (69). In general arterial thromboses are more prevalent than venous events (70). In patients with PV the prevalence rates of major thrombosis at time of diagnosis is 34-39%, whereas the corresponding figures at follow-up is 8-19 % (43;71;72).

After approximately 10 years disease the clinical phenotype changes in about 10-15 % of PV patients, being featured by the appearance or enhancement of constitutional symptoms, a decline in phlebotomy requirements in concert

with rising leucocyte counts and an increasing spleen size due to myeloid metaplasia. The total red cell mass is often still massively increased in spite of a falsely normal or low Hb. This phenomenon is partly caused by pooling of red blood cells in the spleen and hemodilution consequent to an increased plasma volume. This phenotype has traditionally been referred to as the "spent phase" of PV (73). A disease state with pancytosis, splenomegaly and bone marrow fibrosis can remain unaltered for many years, and is known as a 'transitional myeloproliferative disorder' (39). Patients diagnosed in this transitional disease state may be misclassified as PMF when the presenting features are slight anemia, huge splenomegaly together with a fibrotic bone marrow. In these patients the total red cell mass is nevertheless increased and the venous Hb concentration and HCT misleadingly low or sub-normal because of hemodilution.

Essential Thrombocythemia

Essential thrombocythemia is characterized by thrombocytosis, a normal Hb concentration or slight anemia and normal or slightly elevated WBC counts. In a number of patients the spleen is moderately enlarged. When also presenting with slight anemia, leuko- and thrombocytosis many of these patients are most likely those with the aforementioned - but controversial state - of prefibrotic myelofibrosis. The range of the platelet count is exceedingly broad, from just above the upper normal limits to massively increased numbers, exceeding 2000 x 10^9/l. Like in PV symptoms are caused by formation of microaggregates of activated platelets and leukocytes, resulting in microvascular disturbances and accordingly an increased risk of major thrombosis. A history of previous thrombosis as well as thrombosis during follow up is reported in 10-30% of patients with ET (23;31;46;67;74-78). Microvascular disturbances are reported at similar frequencies 13–34 % (31;46;67;74-76;78). As in PV, age over 60 and a history of thrombosis is the most well established risk factors predicting a high risk of future thrombotic events (23;46;67). Data on the impact of cardiovascular risk factors (i.e smoking, hypertension, hyperlipidemia, diabetes) on thrombotic risk are limited and difficult to interpret (79). Data on platelet and WBC levels show consistent similarities with PV (57-59;63-65;80). High platelet levels above 1500 x 10^9/l are significantly associated with an increased risk of mucocutaneous bleeding. This paradox, which founded the nomenclature of the past: 'Hemorrhagic thrombocythemia' is presumably reflecting an acquired von Willebrand factor (vWF) coagulation defect (81-85). This hemorrhagic state is reversed and a thrombogenic condition is reemerging as platelet levels are reduced below 1000-1500 x 10^9/l. Constitutional symptoms and splenomegaly are sometimes present, but not as frequent as in PV.

Primary myelofibrosis

The clinical manifestations of PMF are heterogenous, depending on disease state. Symptoms can be related to excess myeloproliferation such as splenomegaly,

hepatomegaly, extramedullary hematopoiesis of various organs (e.g pulmonary hypertension), bone pain and thrombosis. Constitutional symptoms such as fatigue, night sweats, weight loss and fever, caused by the massively increased hematopoietic cell turn-over often accompany this proliferative form of PMF. On the contrary, symptoms such as fatigue, bleeding and infections caused by cytopenia and sometimes huge splenomegaly may dominate the clinical picture in the more advanced disease phase. Moreover the broad clinical spectrum also includes asymptomatic patients. However, the classical description of PMF is the complex of a leuko-erythroblastic anemia with teardrop poikilocytosis, bone marrow fibrosis and splenomegaly. The number of circulating CD34+ cells are always increased in classical PMF (86-88). The same phenomenon is present in some patients with ET and PV, but at much lower levels (87;89). A cut-off level of 15/µl to distinguish true PMF has been proposed (90). The prognostic relevance of increasing CD34+ counts on follow-up is controversial (86;88). A diagnosis of PMF requires the exclusion of other diseases accompanied by reactive myelofibrosis, both non-malignant (e.g autoimmune diseases) and malignant (such as lymphomas, hairy-cell leukemia and metastatic carcinomas). PMF is clinically and histopathologically indistinguishable from post polycythemic/thrombocythemic myelofibrosis. In up to 25% of patients with PMF the disease will progress to acute leukemia with a dismal prognosis (54;91-93).

2.4 Treatment of the chronic myeloproliferative disorders

In ET and PV treatment objectives can be divided into three categories: i) relief of microvascular symptoms and primary or secondary prevention of major thrombosis ii) relief of constitutional symptoms, pruritus and discomfort from an enlarged spleen if apparent, iii) prevention of transformation to post ET/PV myelofibrosis and or AML. Randomized clinical trials on myelosuppressive therapy in CMPD are scarce and the available drugs have potential adverse effects of importance. This fact in combination with the observation that the median overall survival in most patients exceeds 15 years a strategy of *primum non nocere* ("First, do no harm") has traditionally been the concept of choice. Regarding PMF, the treatment objectives may differ. If symptoms are caused by excessive myeloproliferation, treatment aims at the aforementioned goals, whereas patients in whom cytopenia is the dominating feature, transfusion therapy and supportive care aiming at increasing the insufficient hematopoiesis with colony stimulating factors and erythropoietin are the main objectives. In the following a more detailed description of treatment possibilities and principles will be given for the three distinct disease entities.

Polycythemia Vera

The corner stone in the treatment of PV is to

reduce the increased red cell mass by phlebotomy. Phlebotomy alleviates symptoms caused by hyperviscosity and restores the hemostatic balance. Furthermore, iron deposits are depleted and accordingly the erythropoiesis is restricted. Phlebotomy aims at a stable hematocrit < 0.45 in males and < 0.42 in females (94;95). A hematocrit below this threshold will relieve microvascular disturbances in the large majority of patients, restore hemostatic balance and greatly reduce risk of major thrombotic events (56). All patients without clear contraindications should be treated with low dose aspirin (75-100 mg) daily to reduce the risk of major thrombosis (96-98). Aspirin treatment also benefits patients with microvascular symptoms (60). Phlebotomy requirements are very individual, ranging from a few units a year to one unit every third or fourth week. Unacceptable high phlebotomy requirements and/or thrombocytosis in the context of previous thrombosis and patient age, are the major determinants of whether myelosuppressive treatment should be initiated. Age above 60 and/or previous thrombosis warrant myelosuppressive therapy. In patients younger than 60 with no previous thrombosis the evidence of a reduction in thrombotic risk by lowering the platelet count is not clear and recommendations are diverging and controversial (99-101). Recent data suggest an important role of elevated leukocyte count (66) and leukocyte activation on thrombotic risk (64;65). However, studies on a beneficial effect of lowering leukocyte counts are lacking. Hence these observations have not yet changed therapeutic recommendations. Some patients with PV spontaneously progress over years to post-polycythemic myelofibrosis and/or acute myeloid leukemia. Data on the effect of myelosupressive agents in preventing transformation into myelofibrosis are very limited, but data from the PT1 trial indicate that hydroxyurea is more effective than anagrelide in the prevention of myelofibrosis (22). On the contrary it is well established that certain treatment modalities i.e chlorambucil, busulfan, P^{32} are leukemogenic (35-38). The leukemogenic potential of the non-alkylating agent hydroxyurea - the treatment of choice - remains controversial (102). Apart from traditional myelosuppressive drugs, alternative agents such as anagrelide and pegylated interferon-alpha are available. Anagrelide inhibits megakaryocyte maturation and proliferation and effectively lowers platelet counts. However, the drug has no effect on the overall increased myeloproliferation (103). A potent global myelosuppressive effect can be achieved with the immunomodulatory drug interferon-alpha in its native or pegylated form (104-107). Interferon-alpha has been shown to inhibit the in vitro proliferation of hematopoietic progenitors (108). Reports on restoration of polyclonal hematopoiesis (109) and reversal of cytogenetic abnormalities (104;110), as well as lowering of PRV1 gene expression (107) and JAK2 V617F allele burden (111-114) have raised special interest on interferon-alpha as an agent with the potential of clonal suppression at the molecular level. However, the drop-out rate because of intolerable adverse effects is approximately 30 % with interferon-alpha (106;107) and approaches

50 % with anagrelide (115). In most western countries the treatment of choice for patients older than (40)-60 years is hydroxyurea because of its – in general - favorable tolerability. Because of the unanswered questions in regards to the potential leukemogenicity of HU patients younger than (40)-60 years are often considered candidates for treatment with interferon-alpha or anagrelide.

Because of its effectiveness and easy administration sequential therapy with busulfan is an alternative to hydroxyurea in patients older than 75 years (116).

Essential Thrombocythemia

As in PV, aspirin treatment is beneficial in lowering the risk of thrombotic events and reducing microvascular symptoms. A benefit of lowering the platelet count is evident in patients older than 60 and patients regardless of age with a history of previous thrombosis (68). A platelet count > 1500 $x10^9$/l is also an indication of myelosuppressive therapy. Because of the acquired vWF disease and hence, the risk of mucocutaneus bleedings when platelet levels exceed 1500 $x10^9$/l, aspirin treatment must be paused until platelets are reduced to < 1000 $x10^9$/l. If patients display microvascular symptoms or have significant cardiovascular risk factors, myelosuppressive therapy should be considered regardless of age, previous thrombosis and platelets < 1500 $x10^9$/l. Treatment options are similar as in PV and recommendations often the same, although anagrelide is sometimes preferred instead of Interferon-alpha taking into account the one-lineage – megakaryocytic- proliferation.

Primary Myelofibrosis

Conventionally, the treatment of proliferative PMF aims at reducing constitutional symptoms and discomfort due to a huge splenomegaly. Classical myelosuppressive agents such as melphalan and busulfan have been used, but because of the leukemogenic potential of these agents, administration is limited to elderly patients. Hydroxyurea with its unsolved leukemogenic potential is a usefull agent as is 2-chlorodeoxyadenosine. In younger patients interferon-alpha is an alternative but clinical data are scarce. If symptomatic thrombocytosis is the main problem, anagrelide can be temporarily administered. A huge splenomegaly often results in mechanical discomfort, but is also a major cause of anemia and thrombocytopenia because of pooling of red cells and platelets. Moreover, a massively enlarged spleen is associated with portal hypertension, which may result in oesophagal varices, increasing the risk of severe upper gastrointestinal bleeding. These associated features may strengthen the indication for splenectomy, although this procedure is associated with a significant morbidity of approximately 10 % (117). As an alternative in patients not candidates for a splenectomy, splenic irradiation may be considered, although often complicated by severe myelosuppression (118). Allogeneic stem cell transplantation is the only curative treatment, but because of a high transplant related mortality of approximately 25-30 % this procedure is restricted to very few patients (119).

As previously outlined the treatment of PMF is

heterogeneous and multifactorial. In a large proportion of patients the primary problem is cytopenias, most often anemia. Transfusion of red blood cells and platelets is an opportunity. In order to increase quality of life by lowering the need of transfusions EPO or darbopoietin-alpha can be administered (120;121). The responses are however heterogeneous and can to some degree be predicted by the s-EPO values (120). Alternative agents are danazol and thalidomide or newer thalidomide analogues which all have immunomodulatory and anti-angiogenic properties. Thalidomide is most often used in combination with prednisone, which may increase Hb-levels in about 40 % of patients, often with a reduction in spleen size as well (122;123).

Chapter 3. Introduction - Molecular markers

3.1 Cytogenetics

Originally CML was grouped together with PV, ET and PMF (4). Beginning with the identification of the Philadelphia chromosome in 1960 and ongoing through the 1970-80 with the description of the t(9;22) followed by characterization of the BCR-ABL fusiongene, CML became the first malignant disorder to be characterized in detail at the molecular level (124-126). However, chromosomal aberrations in the Ph-negative CMPDs are sporadic and heterogeneous lacking consistent information in regards to pathogenesis. Chromosomal abnormalities identified by standard G-band karyotyping are more frequent in PV (~30 %) than ET (~5 %). The most frequent identified aberrations are trisomy 9, trisomy 8, trisomy 1q and del20q (127). In PMF the same chromosomal aberrations are recorded but also the del13q, del5q and del7q, the latter two presumeably being associated with previous myelosuppressive therapy (127). The del 20q lesion seems to be associated with the JAK2 V617F mutation (**Suppl Paper b)** (128). A predictive prognostic value of chromosomal aberrations has not been demonstrated in ET and PV, contrasting their prognostic impact in PMF (52). Data exist that indicate a possible association of the der(9;18) lesion with a more proliferative PV phenotype, resembling transitional PV in conversion to IMF **(Suppl Paper a)** (129).

Before the identification of the JAK2 V617F mutation it was demonstrated using more sensitive methods like FISH that aberrations on chromosome 9p are relatively common (130-133), and the loss of heterozygozity on chromosome 9p (9pLOH) was described in 2002 as a frequent stem cell defect in PV (11). This observation among others laid the foundation for the identification of the JAK2 V617F mutation three years later.

3.2 Erythropoietin Independent Erythroid Colonies (EEC)

Growth and proliferation capacities of hematopoietic progenitor cells have been studied for decades. Progenitors are hypersensitive to several known growth factors, including EPO, TPO, IGF-1 IL-3, SCF and GM-CSF in serum-free cultures from a large proportion of CMPD patients (134-136). When cultured in serum, but in the absence of EPO the hematopoietic progenitors form burst forming units of erythroid (BFU-E) – a phenomenon referred to as EEC's (5). This phenomenon can be observed in virtually all patients with PV and a large proportion of patients with ET and PMF (137). Progenitors from patients with reactive and familial erythrocytosis do not form EEC's in the absence of EPO

(138;139). The capability of EEC formation was accepted as a minor criterion in the WHO diagnostic criteria (18). However, the EEC assay, although consistent and concise has not been widely introduced in the diagnostic setup outside large laboratory units. The observation of deregulated growth of hematopoietic progenitors stimulated investigations of altered receptor cytokine signaling. Growth factor receptor signaling is dependent on tyrosine kinases through STAT proteins (12;140).

3.3 Erythropoietin (EPO)

The plasma concentration of EPO is subnormal in patients with PV at diagnosis as compared to normal controls and cases of secondary erythrocytosis (SE) and relative polycythemia (141-144). This subnormal level of EPO seems to persist after phlebotomy treatment (143), although a sligt increase is observed in some patients. A subgroup of ET patients also displays subnormal EPO levels (145;146). Measurements of plasma EPO concentrations can be used in the diagnostic work-up in patients suspected of a CMPD, and a subnormal plasma EPO concentration is a minor criterion in the WHO classification.

3.4 Deregulated genes and proteins

In effort to identify pathogenetic mechanisms and diagnostic or prognostic markers, research within the last 10 years has revealed several candidate genes and proteins with altered expression levels. Their potential role as disease or phenotype modifying genes remains to be established. The plasma membrane expression of the trombopoietin (TPO) receptor c-MPL is decreased in megakaryocytes and platelets in patients with CMPD (147;148). Despite the findings of correlations between MPL expression and other markers such as *PRV-1* and a close relationship with JAK-STAT signaling molecules is obvious, since these are downstream targets of TPO-MPL receptor signaling, decreased MPL expression is not of diagnostic or prognostic value (149;150). However, recently a pointmutation MPLW515K/L in the trombopoietin receptor was identified in a subset of patients with JAK2 V617F negative ET (1%) and PMF (5%) (151;152).

In parallel with the increased proliferation of myeloid cells in CMPD these mature myeloid cells also accumulate because of decreased apoptosis. The gene *Bcl-x,* which acts as an antiapoptotic agent has been shown to be upregulated (153). This dysregulation is a consequence of its regulation by STAT5, being excessively phosphorylated consequent to the mutant JAK2 tyrosine kinase (14;154).

The transcription factor NF-E2 has also been found to be upregulated in PV (155;156). Data suggest an important role in driving erythropoiesis (157), and ectopic expression can redirect monocytic cells towards erythroid and megakaryocyte differentiation (158). Hypothetically NF-E2 may be a key determinant of lineage commitment, and when overexpressed in PV important for the excessive erythroid and in part megakaryocyte proliferation (155).

The Polycythemia Rubra Vera Gene 1 (PRV1)

was first described in 2000. A significant upregulation was found in patients with PV compared to patients with other hematological malignancies, secondary erythrocytosis and healthy controls (159). A subgroup of patients with ET and PMF also proved to have significant *PRV1* overexpression (137;159-161) and ET patients with elevated *PRV1* expression were demonstrated to have a higher propensity of transforming into PV (162). *PRV-1* overexpression may be associated with increased risk of thrombosis (162-164) in both ET and PV. However, more recent data indicate, that *PRV1* overexpression is not limited to the Ph-negative CMPD, but can be observed not only in other malignant hematologic diseases, but also in reactive erythro- thrombo- and leukocytosis (161;165). Elevated *PRV1* expression is induced in healthy controls treated with G-CSF and is also observed in inflammatory conditions, such as severe sepsis (156). No evidence of structural genetic rearrangements or epigenetic aberrations of the *PRV1* gene locus responsible for the deregulation have been identified (166). The pathophysiological role of the PRV1-protein (CD177), a transmembrane receptor of the uPAR receptor family remains to be elucidated (167). *PRV1* overexpression most likely reflects a non-specific neutrophil activation (156;165). However, as *PRV1* expression correlates with the capacity of EEC formation (160;168;169) and the presence of the JAK2 V617F mutation (169-173) a clear, although not specific association, with clonal CMPD is evident. Sequential analysis of *PRV1* expression levels has indicated a possible role as a marker of disease activity (107;174).

3.5 JAK-STAT signaling

Type 1 hematopoietic cytokine receptors (EPO, TPO, GM-CSF and several interleukin receptors) lack cytoplasmatic tyrosine kinase activity and are dependent on phosphorylation of Janus Kinase (JAK) proteins and subsequently downstream activation of **S**ignal **T**ransducers and **A**ctivators of **T**ranscription (STAT) proteins (175). The JAK family comprises four different members JAK1, JAK2, JAK3 and TYK2. These tyrosine kinases consist of the JAK homology 1 (JH1) domain with kinase activity, the JH2 domain (pseudokinase), which is catalytically inactive and have autoregulatory function and the JH3-JH7 which contains the protein interaction FERM domain, required for targeting of the membrane receptors (176). Binding of the FERM domain to a transmembrane receptor leads to a series of conformational changes in the JAK protein, necessary for the receptor ligand to induce phosphorylation and activation of the JAK tyrosine kinase allowing further downstream signaling through mainly STAT proteins (176;177). As the name implies, these proteins serves as signal transducers and activators of transcription, and accordingly they regulate expression of target genes. Several growth factors (EPO, TPO, G-CSF, GM-CSF, SCF, IL-3 IGF-1) essential for normal hematopoiesis all use JAK2 for signaling, mainly through STAT3 and STAT5 (14;176;178-180). JAK-STAT signaling is controlled by several regulatory mechanisms, of which the **S**uppressors **O**f **C**ytokine **S**ignaling

(SOCS) proteins play an essential role and are the most well described. The SOCS proteins act as negative regulators of signaling through binding to phosphotyrosine residues of either activated JAK's or cytokine receptors (181). SOCS 1 induction by interferon-alpha is known to suppress megakaryopoiesis (182), and within recent years deregulated activity of both SOCS1 and SOCS3 at the epigenetic and genetic levels has been described and may contribute to the pathogenesis in CMPD (183-186).

3.6 Biology of the JAK2 V617F mutation

In early spring 2005 four independent research groups simultaneously published the identification of a novel acquired somatic point mutation in the *Janus Kinase 2 Gene (JAK2)*. A nucleic acid conversion of guanine to thymine results in a substitution of the highly conserved amino acid residue, valine to phenyalanine in codon 617 (V617F) of the autoregulatory pseudokinase domain *JH2* (14-17). Based upon information from previous published protein structure models (187) it is proposed that the phenylalanine residue, which has a more bulky structure compared to valine compromises the interaction of the activation loop of the kinase domain *JH1* with the autoregulatory *JH2* domain, resulting in a maintenance of the protein tyrosine kinase in its active state (177). The JAK2 V617F mutation confers EPO independent in vitro growth of erythroid colonies (EEC's) (14;16;17), which can be significantly suppressed by a small interfering RNA (siRNA) reagent reducing JAK2 expression (14). When the mutant JAK2 V617F tyrosine kinase is expressed in cell lines autophosphorylation and activation as well as autonomous growth factor independent proliferation is observed (14;15;17). Cells homozygous for the JAK2 V617F mutant have a proliferative advantage compared to wild type cells and only co-expression of the EPO receptor and the JAK2 V617F mutant but not the wildtype results in a downstream activation of the target molecule STAT5 (14). Transfection of the JAK2 V617F mutated gene into recipient mice causes development of a PV like phenotype, which tends to terminate in myelofibrosis (14;188;189).

Although evidence proving the origin of the CMPD in a pluripotent stem cell has been available for years (7;20), several publications have described the JAK2 V617F mutation as a genetic event restricted to myeloid cells (14;16;190). However, evidence from the '*pre-JAK2 era'* of clonal lymphoid cells exists (11;191-193), and using more sensitive techniques for identification of the JAK2 V617F mutation, several groups have demonstrated the presence of the JAK2 V617F mutation in lymphoid cells, and thereby proving the origin of the mutation in a progenitor with both myeloid and lymphoid differentiation potential **(Paper I)** (194-197). The JAK2 V617F mutation can be detected in hematopoietic progenitors as well as mature hematopoietic cells. Individual cells can either be heterozygous or homozygous for the JAK2 V617F mutation and the homozygous state is a result of

mitotic recombination (15), which by microsatelite mapping was first demonstrated as loss of heterozygosity of chromosome 9p (9pLOH) (11). Cell numbers homozygous for the JAK2 V617F mutation increases with time (198), suggesting a proliferative and survival advantage of mutant progenitor cells. Homozygosity in hematopoietic precursors is often found in PV, whereas it is a rare event in ET (198). Regarding the question of whether the JAK2 V617F mutation is the disease initiating event, suggestions of cooperating genetic events, which might precede The JAK2 V617F mutation are apparent. The chromosomal aberration del20q in granulocytes was in a few patients found to outnumber granulocytes harboring the JAK2 V617F mutation (199). In line with this observation, patients with JAK2 V617F mutated ET and PV have clonal granulocytes not harboring the JAK2 V617F mutation **(suppl. Paper b)** (128). Furthermore, patients with preceding JAK2 V617F mutated MPD transformed to AML, have leukemic blasts, in which the JAK2 V617F mutation cannot be detected **(suppl. Paper b)** (128;200). Finally, in familial CMPD the JAK2 V617F mutation is acquired, suggesting a predisposing genetic event (201).

3.7 Clinical aspects of the JAK2 V617F mutation

In the original publications on the JAK2 V617F mutation, the frequency of the mutation detected in patients with PV varied in the range of 65-89 %, when DNA sequencing with a sensitivity around 10-25 % was used (14;15;17), whereas Baxter et al. could identify the mutation in 97 % of PV patients using a more sensitive (2-3%) allele specific PCR method (16). Importantly, the mutation was not detected in secondary erythrocytosis (SE). Following these four initial publications numerous papers have reported varying frequencies of the JAK2 V617F mutation in different PV cohorts (169;170;172;202-204). An increasing amount of work has been done using different real-time quantitative PCR (qPCR) methods increasing the sensitivity to approximately 0.05-0.1 % **(Paper I)** (171;196;205), allowing a detection of very small JAK2 V617F allele burdens and thereby increasing the frequency of occurence in PV patients up to 99% **(Paper III)** (206;207).

Whereas the great majority of patients with PV harbors the JAK2 V617F mutation, it can be detected in approximately half of patients with ET and PMF, although figures varies considerably (23-72% in ET and 39-57% in PMF) (14-17;172;206;207). Apart from the variation in the mutation detection method, another major determinant of the frequency of the JAK2 V617F mutation reported in various cohorts is the use of diagnostic criteria for patient inclusion. Considerable discrepancy in classification of both ET, PV and PMF using either the PVSG or the WHO criteria is evident (24). Mutation analysis is most often performed on peripheral blood, mostly granulocytes, but mononuclear cells, platelets, unfractioned leukocytes or whole blood can be

used. Both qualitative (208;209) and quantitative **(Paper II)** (210;211) detection of the JAK2 V617F mutation can be performed successfully on bone marrow, and equivalent levels of the JAK2 V617F allele burden is present in PB and BM **(Paper II)** (112;211). In large scale studies patients with JAK2 V617F positive ET have been demonstrated to have biochemical and clinical features indicating a PV-like phenotype separating them from JAK2 wildtype ET patients (77). Several studies including the large scale PT1 study cohort have demonstrated that JAK2 V617F positive ET patients have higher hemoglobin concentration **(Paper V)** (23;77;212-215), higher white cell and neutrophil counts (23;77;215), lower plasma ferritin and plasma EPO levels **(Paper V)** (77), as well as lower platelet counts **(Paper V)** (23;77;212;215). While sharing biochemical features of PV, data on thrombosis and JAK2 V617F status in ET patients are to some extend diverging. Some studies report an overall increased prevalence and risk of thrombosis **(Paper V)** (186;214;216;217), and some have reported an increased risk of venous thrombosis only (77;215). These observations are balanced by other studies reporting lack of any association between JAK2 V617F positivity and thrombosis (23;212;213;218). Limited data are available, allowing conclusions on the propensity of ET to transform into PV and mutation status, but suggestions of an association to JAK2 V617F positivity is available (77).

The concept of JAK2 V617F positive ET resembling PV and being a different clonal disorder than the JAK2 V617F negative ET have encouraged studies on bone marrow histomorphology and the association to the JAK2 status. JAK2 V617F positive patients have more pronounced bone marrow erythropoiesis and granulopoiesis (77). However, important bone marrow characteristics such as reticulin fiber content, hyperplasia and especially megakaryocyte morphology and topography, which are essential in the differentiation between "true ET" and prefibrotic PMF or IMF according to the WHO criteria, are not ambigiously correlated to the JAK2 mutational status **Paper IV** (208;219;220).

Regarding PMF, data on biochemical and clinical differences between JAK2 V617F mutated and wildtype patients are limited. One study demonstrated an association of a history of pruritus and thrombosis and JAK2 V617F positivity, but no impact on prognosis (221), contrasting findings of inferior survival, less transfusion requirements during follow up and elevated leukocyte and neutrophil counts in a similar European multicenter study **(suppl. Paper c)** (222). The inferior survival demonstrated in the latter study was partly caused by leukemic transformation, an association which was not confirmed in a later study (223).

The JAK2 V617F mutation *per se* provides excellent diagnostic information, and may as well provide prognostic information in ET and PMF. Since the great majority of PV patients harbor the JAK2 V617F mutation, clinical associations to the presence of the mutation *per se* are irrelevant and only a quantitative assessment of mutant allele burden may provide prognostic information.

The blood cell population in a JAK2 V617F mutated patient is a mixture of non-clonal, heterozygous and homozygous cells. Therefore, only an arbitrary cut-of point of more than 50% mutated alleles allows interpretation of homozygosity in a clinical setting. When quantification is performed on peripheral blood cells, about 30 % of PV patients and only a few percent of ET patients are found to be homozygous **Paper III** (14-17;207;215;217). Clearly, this is an inevitable underestimation, because when the analysis is performed on hematopoietic precursors 90% of PV patients are homozygous, whereas only a minority of ET patients share this feature (198). Whereas an estimation of a homozygous disease state, meaning more than 50 % JAK2 V617F mutated alleles, can be obtained with some uncertainty from DNA sequencing methods, an exact and more reliable quantification of the JAK2 V617F mutant alleles is possible by using real-time qPCR **(Paper III)** (207). The JAK2 V617F allele burden is 16-33% in PV **(Paper III)** (207;210) compared to 5-9% in ET **(Paper III)** (207;215). The JAK2 V617F mutant allele burden may increase with time (210). Only a few studies monitoring the JAK2 V617F allele burden have yet been published and are limited by short follow-up (111;114) Accordingly, a potential suppressive effect of myelosuppressive therapy may have influenced the estimation of the JAK2 V617F allele burden in patients analyzed during ongoing treatment **(Paper III)** (207). In patients with PMF the data on homozygosity and allele burden are limited. The proportion of homozygous PMF patients is in the range of 57-85% **(Paper III)** (171;207) and the median JAK2 V617F allele burden has been reported as high as 67 % (**Paper III)** (207). In PV, regardless of time of mutation analysis (at diagnosis or during follow up), the JAK2 V617F allele burden is very variable, ranging from 1-100% **(Paper III)** (171;207;210;224). An increasing amount of data demonstrates a correlation between the JAK2 V617F allele burden - and in particular the homozygous disease state - and the degree of myeloproliferation, reflected by increasing WBC counts **(Paper III)** (207;210;217;224), LDH levels **(Paper III)** (207;224), CD34+ counts **(Paper III)** (207;225), splenomegaly **(Paper III)** (207;217;224), granulocyte activation (207;224;225) and expression of downstream target genes **(Paper III)** (156;170;171;207;224;226;227). Symptoms relating to the degree of myeloproliferation such as pruritus and constitutional symptoms also correlate with increasing allele burden (210;217;224;227) Data on all these parameters are not ambiguous, but point to a JAK2 V617F allele dosage effect on the degree of myeloproliferation. Regarding Hb levels, a few studies report increasing Hb levels, increasing HCT and decreasing s-epo with increasing JAK2 V617F allele burden (217;224;227), whereas others show no correlation (210), and yet another study points to a possible decrease in HCT and Hb in parallel with increasing spleen size and JAK2 V617F allele burden, suggesting an evolution towards a transitional phenotype **(Paper III)** (207). Data on the influence of the JAK2 V617F

allele burden on thrombotic risk in PV are conflicting and the topic remains unresolved. **(Paper III)** (207;217;224;227). Despite suggestions of increased risk of fibrotic transformation (227) and evolution towards transitional PV, and later progression towards post-polycythemic myelofibrosis **(Paper III)** (207), the existing data do not allow accurate conclusions.

Based upon the existing molecular and clinical data it seems reasonable to suggest a hypothetical model, defining JAK2 V617F positive myeloproliferative disorders as one disease with three different phenotypic presentations - traditionally known as ET, PV and PMF - forming a biological continuum. In this model the key determinant of the phenotypic presentation is the JAK2 V617F allele burden, but yet unknown genetic modifiers, acquisition of other molecular events, physiological modifiers (i.e iron deposits and homeostasis) and probably gender is likely to have considerable influence on the phenotype.

Chapter 4. Objectives of the Study

4.1 Aims of the study

My studies have been focused on the implementation and evaluation of the JAK2 V617F mutation analysis in a cohort of patients diagnosed with or suspected of a CMPD. After implementation of one of the originally published qualitative methods for JAK2 V617F mutation detection and following preliminary investigation of clinical correlations, we decided to design a quantitative real-time Taqman based PCR assay, allowing an assessment of the percentage of JAK2 V617F mutant and JAK2 wildtype alleles. This assay enables a highly specific and sensitive determination of the JAK2 V617F mutation status, and if the mutation is present – an exact and reproducible assessment of the proportion of JAK2 V617F mutated alleles. Accordingly, a determination of the size of the JAK2 V617F mutant clone, and its evolution during ongoing myelosuppressive therapy is possible.

4.2 Hypotheses

Using the qPCR assay our aims were to verify the following key pathogenetic and clinical hypotheses:

1. The JAK2 V617F mutation is an early stem cell event not restricted to myeloid cell lineages
2. The assessment of the JAK2 V617F allele burden in peripheral blood leukocytes is a reliable measurement of true JAK2 V617F clonal size in the bone marrow.
3. The JAK2 V617F mutated chronic myeloproliferative disorders (ET, PV and IMF) are different phenotypic presentations of the same clonal disorder, and reflect a biological continuum.
4. The JAK2 V617F allele burden is associated with the degree of myeloproliferation and myeloid metaplasia.
5. According to the "biological continuum model" JAK2 V617F mutated ET patients may have clinical and biochemical features resembling PV
6. According to the "biological continuum model", JAK2 V617F mutated ET patients may have different bone marrow morphological changes compared to JAK2 wildtype ET patients – does it fit the WHO model of ET versus prefibrotic myelofibrosis ?
7. According to the "biological continuum model" JAK2 V617F mutated PMF patients may have clinical and biochemical features resembling PV.

Chapter 5. The Study Population

5.1 Study population

The total study population comprised a cohort of patients referred to or followed in the outpatient clinic at the Department of Hematology, Odense University Hospital in the period from December 1, 2004 to August 1, 2007. On first visit all referred patients had a blood sample (20 ml of EDTA anti-coagulated blood) collected for molecular analysis together with standard blood tests and a bone marrow biopsy. On patients diagnosed prior to the study, blood sampling was performed during a visit in the outpatient clinic.

The samples were sent to the Department of Clinical Pathology, Laboratory for PCR Analysis at Odense University Hospital for further processing and molecular analysis. In total 349 patients were registered in the study. From the total cohort, 106 patients gave their informed consent to be enrolled in an ongoing prospective study monitoring molecular markers every 4-6 month during ongoing treatment. The study was conducted in accordance with the Helsinki Declaration and after approval by the Regional Danish Ethics Committee (Case no.VF 2004216). An overview of the study population is given in **Figure 1.**

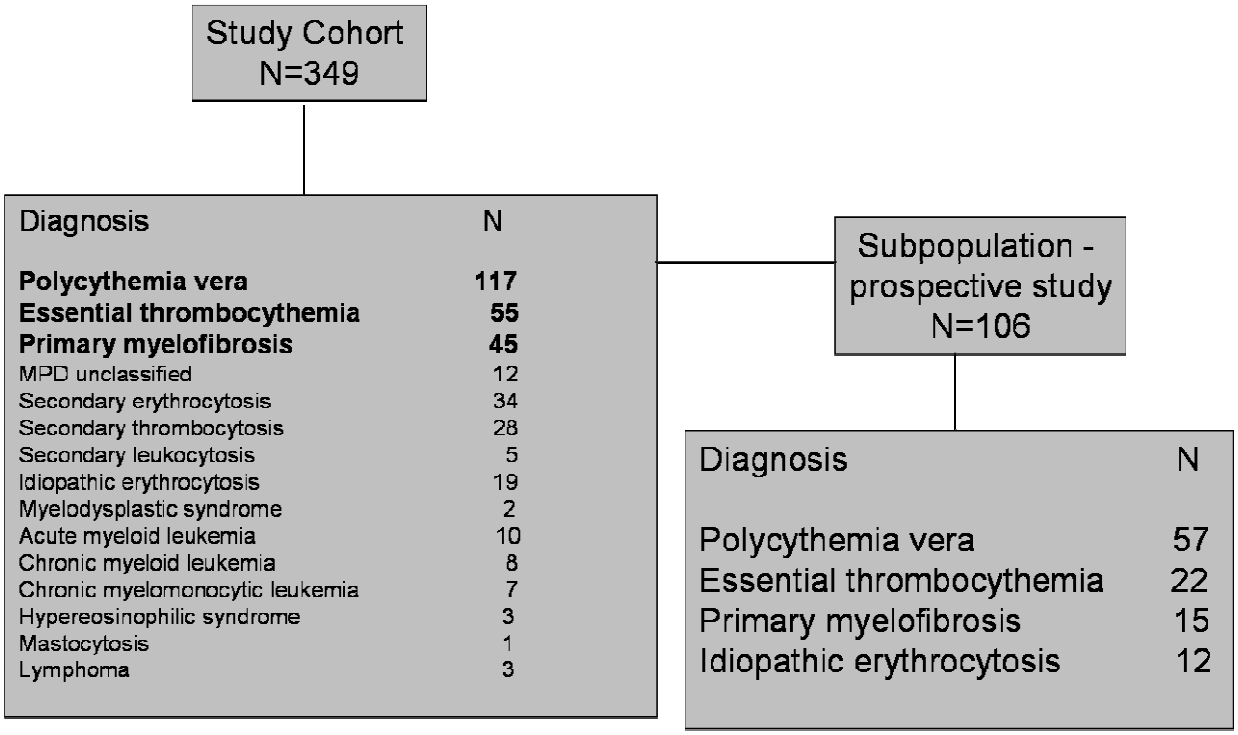


Figure 1. Diagram showing the study population

Chapter 6. Methodological aspects

Multiple different laboratory disciplins (classical biochemistry, immunohistochemical analysis of bone marrow samples, G-band karyotyping, FISH, flowcytometry, FACS, and qPCR) have been applied to describe and analyze this large cohort of patients. In this section a description of the FACS sorting procedure applied in **Paper I** and the allele specific JAK2 mutation analysis assay designed by Baxter J et al. will be given, followed by a general introduction to qPCR and a description of the qPCR assay for *PRV1* expression analysis. However, a special emphasis on the development and improvement of the qPCR assay for JAK2 V617F mutation analysis is the primary scope of this section. Further information regarding different methodological aspects is given in **Paper I-V.**

6.1 Fluorescence activated cell sorting (FACS)

Flowcytometry is a powerful technique for rapid and precise measurements of single cell characteristics in a cell suspension. The basic principle is the application of laser light to a fluid stream containing cells. The light scatter and emission of light from fluorescent dye marked antibodies on the cell surface or cytoplasma is detected by a number of detectors (photomultipliers), which translates the signal for subsequent electronical (software) processing. By passing the fluid stream through a vibrating nozzle, the fluid stream divides into droplets containing one cell each, allowing a sort of droplets, and accordingly cells. Based on the information from the scatter and immunofluorescence emission characteristics a droplet containing a cell with the desired properties can be charged with an electric pulse and directed into the proper tube when passing through an electric field **(Fig 2).**

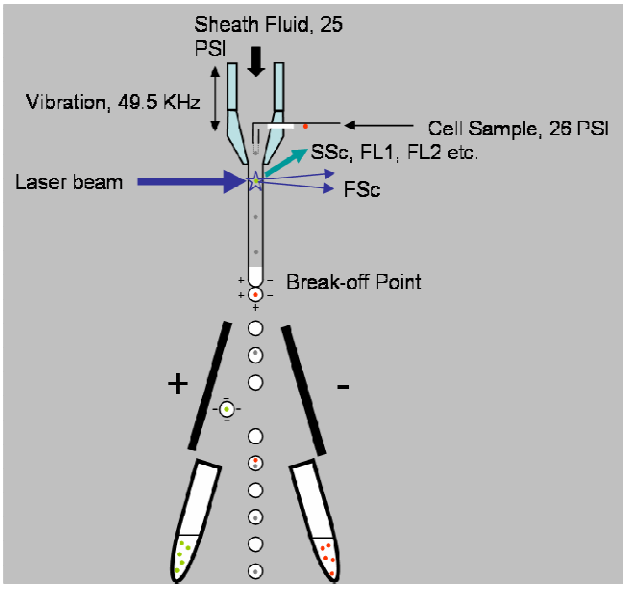


Figure 2: A suspension containing the antibody labeled cells is added to a liquid stream (sheath fluid), which passes through a vibrating nozzle resulting in the fluid stream breaking into droplets. Droplets containing cells of interest, based on the information from forward- and side scatter and the immunofluorescence emission are charged by adding an electric pulse. The charged droplets pass through an electric field, allowing a deflection of the droplets into the relevant collection tubes. It is possible to sort from one to four different cell types by adjustment of the polarity and charge applied. (The illustration was kindly provided from Flow Cytometric Cell Sorting: A Basic Guide. Graham Leslie)

The FACS procedure laying the foundation for **Paper I** was performed on a FACSVantage (BD Biosciences Erembodegem, Belgium) using three lasers (red, green an UV), allowing a four-colour four-way sorting protocol. After red cell lysis with a NH_4Cl lysis buffer and resuspension in New Born Calf Serum (NB) 0.1 µM, the cells were counted on a Sysmex KX-21N (Sysmex GmbH, Norderstedt, Germany) and a volume containing 5 x 10^6 white blood cells (WBC) were collected for antibody labelling. The cells were labelled with CD3-APC, CD19-PE, CD14-PE-Cy7 (BD Biosciences Erembodegem, Belgium) and CD66-FITC (Dako A/S, Glostrup, Denmark) fluorchrome-conjugated monoclonal antibodies according to manufacturers' protocols and incubated at 4℃ for one our.

Before the FACS was performed a compensation for spectral overlapping of the four flourchromes was done using Calibrite (BD Biosciences). Also, an isotype control test using IgG_1 FITC, IgG_1 PE, IgG_1 PE-Cy7 and IgG_1 APC (BD Biosciences) was performed. The cells were sorted in a high purity mode aborting droplets containing doublet cells or more than one cell. The gating strategy allowing a purification of CD3+ T-lymphocytes, CD19+ B-lymphocytes, CD14+ monocytes and CD66+ granulocytes is depicted in **Fig 3**. A post sorting control was performed by running the sorted samples through the flowcytometer once again for confirmation of the purity of the sorted sample **Fig 4.**

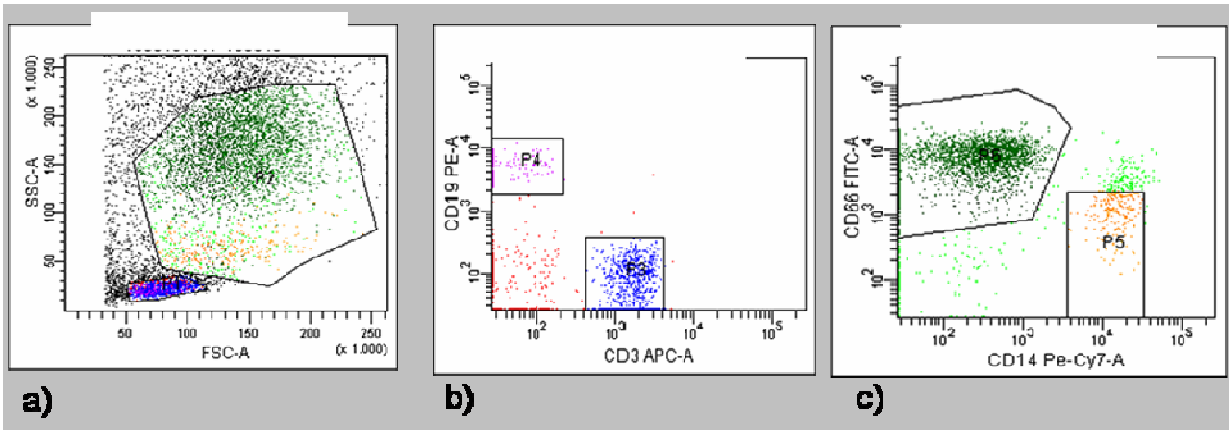


Figure 3. **a)** FSC-SSC scatter plot showing 2 gates P1 (lymphocyte gate) and P2 (monocyt-granulocyte gate), including back-gating with colours indicating the four different populations (CD3+ blue, CD19+ purple, CD14+ orange and CD66+ green).
b): CD3+/CD19+ scatter plot separating the lymphocytic population in CD3+ T-cells (P3) and CD19+ B-cells (P4).
c): CD14+/CD66+ scatter plot separating CD14+ monocytes (P5) from CD66+ granulocytes (P6).

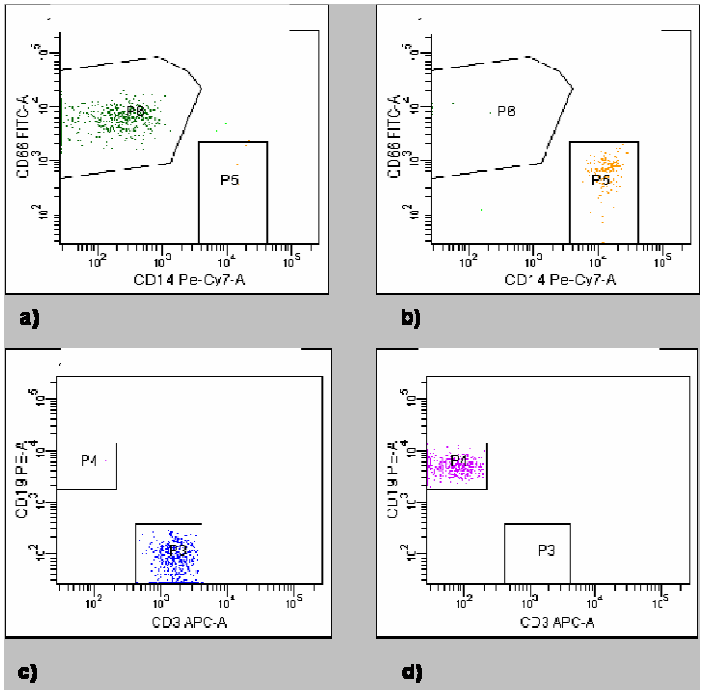


Figure 4: Post-sorting quality control panels showing ~ 99 % purity of: **a)** CD66+ granulocytes, **b)** CD14+ monocytes, **c)** CD3+ T-lymphocytes and **d)** CD19+ B-lymphocytes.

6.2 Cell preparation for DNA and RNA analysis

Twenty milliliters of EDTA anticoagulated blood was collected from the patients. Red cell lysis was performed using an ammoniumchloride (NH_4Cl) lysis buffer 9.8 %. After centrifugation the WBC's were washed and resuspended in Hanks balanced salt solution (Bie & Berntsen, Herlev, Denmark), containing 3 % NB. A cell count was performed on a Sysmex KX-21N (Sysmex GmbH, Norderstedt, Germany). A volume containing 5 x 10^6 cells was collected and 1 ml per 1 x 10^6 cells of MagNa Pure LC mRNA lysis buffer (Roche Diagnostics, Mannheim, Germany) was added. The cell lysates were stored at - 80°C.

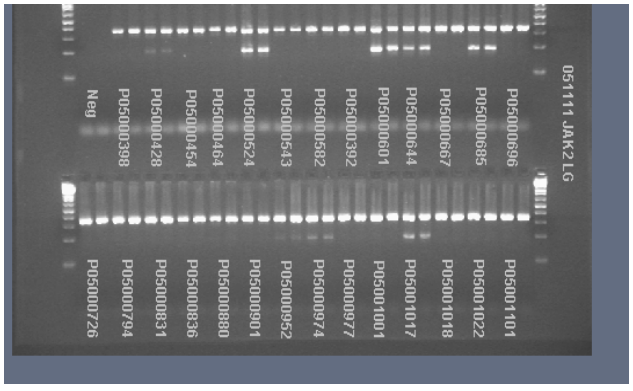
6.3 DNA and RNA extraction

DNA and RNA extraction was performed using a MagnaPure LC© robot (Roche Diagnostics, Mannheim, Germany) according to the manufacturers' protocol. Briefly, the basic principle is the use of silica coated magnetic beads, which bind DNA or total RNA in a solution of different reagents and salts. Intact cells are lysed and accordingly DNA or RNA is released. Proteins are degraded by proteinases. If RNA is extracted, RNases are inhibited and reagents are incubated with DNase to avoid contamination with genomic DNA. The magnetic beads binding the RNA or DNA are washed to remove unbound substances such as PCR inhibitors like Hb. When performed optimally a yield of approximately 1 µg of DNA or RNA eluated in 100 µl is obtained. However, no

quantification of the DNA or RNA was performed routinely, as the standard sample size was held constant at 1 x 10^6 cells and the relative amount of DNA or RNA could be assesed from the cycle threshold values from the PCR reaction (se section 6.5).

6.4 Allele specific PCR assessment of JAK2 V617F mutation status (Baxter-method)

Baxter J et al designed a three primer allele specific PCR with a reverse primer, a forward G-T mutation specific primer and a forward internal control primer (16). The mutation specific primer has an intentional mismatch at the third nucleotide from the 3' end, which improves specificity and amplifies a 204-bp product as compared to the internal control primer which generates a 364-bp product. Both forward primers were used in a concentration of 0.5µmol/L, whereas the concentration of the reverse primer was 1.0 µmol/L. A Hotstar Taqman polymerase was used in a 20 µl volume of 15 mM $MgCl_2$ buffer (Qiagen, Hamburg, Germany) and dNTP. The PCR conditions were: 36 cycles of denaturation at 95° C for 15 seconds, annealing at 58° for 30 seconds and extension at 72° for 30 seconds. All reactions were performed in duplicates on a GeneAmp© PCR system 9700 (Applied Biosystems, Foster City, CA, USA). Fifteen µl of the PCR product was loaded on a 3% NuSieve GTG Agarose gel and run at 100 Volts for 45 minutes. An example of PCR products on a gel is given in **Fig 5a**. The sensitivity of this assay is reported to be 2-3 % (172), which was confirmed in our laboratory **Fig. 5b.**



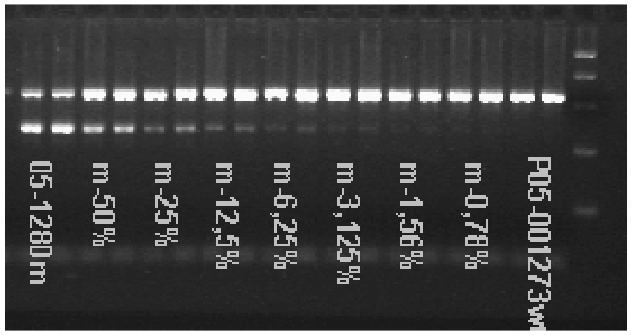


Figure 5a: The allele specific PCR (Baxter et al). Far left a negative control followed by a number of patient samples all in duplicates. All samples (n=27) demonstrate the presence of a 364-bp product (internal control) and some samples have a smaller 204-bp band representing the product of the JAK2 V617F mutation specific primer. Accordingly these patients harbours the JAK2 V617F mutation. Although the density of the mutation specific bands varies to some extend, a semiquantitative assessment is not possible. **5b**: A 2-fold dilution series of JAK2 V617F mutated (~100%) DNA in JAK2 wildtype DNA showing a limit of sensitivity of approximately 3%. Although dependent on the total DNA content accurate interpretations of the JAK2 V617F mutation status in samples containing less than 3% mutated DNA is not possible.

6.5 Real-time quantitative PCR (qPCR) – An introduction

The development of real-time PCR in the mid-1990s allows accurate and reproducible quantification of gene copies without a need for post-PCR handling such as gel-electrophoresis. The method is based on measurement of accumulated PCR product through a Taqman probe. This Taqman probe is a small oligonucleotide labeled with two different fluorescent dyes. In the 5' end a reporter dye (FAM) is attached and in the 3' end a quencher dye (TAMRA) is attached. In the intact form the fluorescent emission from the reporter dye is absorbed by the quencher and no (or low) emission energy is recorded. During the extension phase of the PCR reaction the extension from the 5' end towards the 3' end results in a cleaving of the Taqman probe by the Taq polymerase 5'-3' exonuclease activity. As a result the reporter dye is removed from the quencher and the emission energy is no longer transferred efficiently to the quencher and accordingly an increase in the reporter dye emission is the net result **(Fig 6).** As the PCR reaction proceeds under optimal circumstances an exponentially increase in fluorescent emission will be detected by the system and data processed by computer software, which depicts the data as an amplification plot **(Fig 7)**. A predetermined level in the exponential phase of the fluorescence accumulation is set to be the cycle threshold level (CT), which forms the basis for the quantification (228). All real-time quantitative PCR reactions described in the following were performed on an ABI Prism7900 HT© (Applied Biosystems, Foster City, CA, U.S.A.).

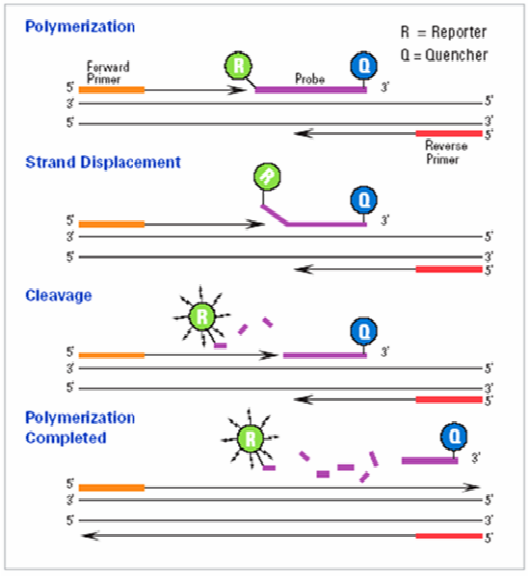


Figure 6: The basic principle of real-time PCR.

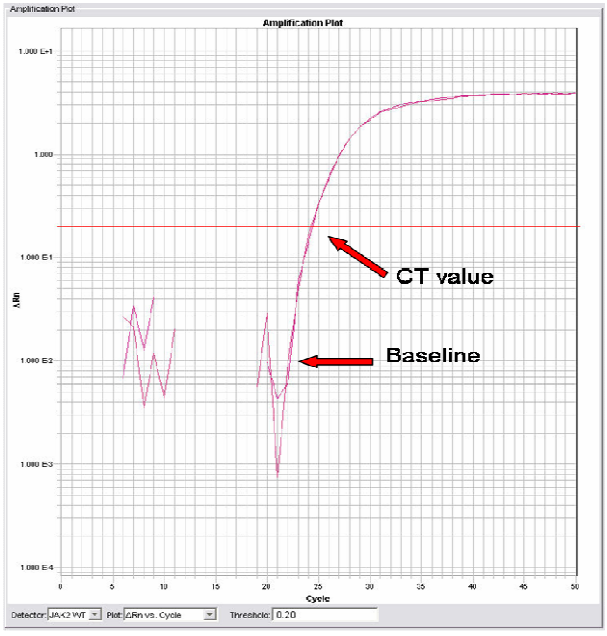


Figure 7: Real-time PCR amplification plot. The baseline fluorescence emission intensity and the cycle threshold (CT) value are marked by red arrows. The red line represents the threshold and should be in the exponential phase of the amplification.

6.6 qPCR analysis of PRV1 gene expression

cDNA synthesis from extracted RNA was performed using random 9 oligonucleotide primers at 25 µM per reaction. RNA and primers were incubated for 5 min at 70 °C. A mixture of 1 mM dNTPs, 1 Unit/µL RNase Inhibitor (Roche), 10 Unit/µL Reverse Transcriptase (Invitrogen Life Technologies, Paisley, UK) and First Strand Buffer x5 (Invitrogen) was added and the material was subsequently incubated for 10 min at 25 °C, followed by 45 min at 37 °C, and finally 5 min at 95 °C.

The *PRV1* qPCR assay comprised a forward primer: 5'-CAGGTTGCAACCTGCTCAAT-3', a reverse primer: 5'-GCCAAGTTTCCGTGTGTC ATAAT-3' and a probe: 5'-Fam-TTGCAGTTC TCAGTCATACCCACGGGC-Tamra-3'. This primer/probe set was cDNA specific since it showed no amplification of 100 ng genomic DNA per well in control experiments. As internal reference the housekeeping genes *beta-glucuronidase (GUS)* and *Abelson* (*ABL*) were used for normalization (229;230). The *PRV1* gene expression was calculated using the ΔΔCycle threshold (CT) method relative to the normalized expression in healthy donors, in whom the level was set to 1 (95% c.i.: 0.3-7, n=38). All qPCR reactions were performed in triplicates.

6.7 qPCR analysis of JAK2 V617F mutation status and assessment of the JAK2 V617F allele burden

We designed the qPCR assay as a 2x2 primer assay. Accordingly, two qPCR reations were performed in parallel with the use of a common Taqman probe and a common forward primer, differing only in the use of a reverse primer specific for either G-T mutated *JAK2* DNA or the wildtype *JAK2* DNA. Both the mutation and wildtype specific primers were designed with an intentional mismatch at the 3' minus 2-position. In order to obtain the best performing assay five generations of primer probe combinations in the aforementioned 2x2 design were tested and optimized. The sequences of this fifth generation of the three primers and the Taqman probe are given in **Table 1**. The primers were designed using the Oligo© Version 6.7 software (Molecular Biology Insights, CO, USA) and the customized primers and probes were manufactured by DNA Technology A/S (Aarhus, Denmark).

Common forward primer: 5'-CCT-TCT-TTG-AAG-CAG-CAA-GTA-TGA-3'
Mutation-specific reverse primer: 5'-GTA-GTT-TTA-CTT-ACT-CTC-GTC-TCC-ACA-tAA-3'
Wildtype-specific reverse primer: 5'-GTA-GTT-TTA-CTT-ACT-CTC-GTC-TCC-ACA-tAC-3'
Taqman probe: 5'-6-FAM-TG-AGC-AAG-CTT-TCT-CAC-AAG-CAT-TTG-GTT-T-TAMRA-3'

Table 1: Primer and probe oligonucleotide sequences of the JAK2 qPCR assay.

The qPCR reaction volume was 25 µl and primer-concentrations were 300 nM, whereas the concentration of the probe was 200 nM. The PCR amplification conditions were: An initial enzyme activation step of 10 minutes at 95° C, followed by 50 cycles of 15 seconds at 95° C and 60 seconds at 60° C. All qPCR reactions were performed in doublicates or triplicates and with a 'no template control', a negative (donor DNA) and a positive control (HEL).

In order to determine the sensitivity of the mutation-specific primer set, a standard curve was created by 5-fold dilution series of homozygous JAK2 V617F mutated DNA initially from a patient with more than 90 % mutated alleles and later confirmed with the homozygous cell line HEL containing 100 % JAK2V617F mutated DNA into donor wildtype DNA. Also from repeated 5-fold dilution series the slopes for both the wildtype-specific and mutation-specific primers were determined to 3.6 and 3.7, respectively **(Fig 8)**. End point limiting dilution series (2-fold) from both the wildtype and mutation specific primer-probe sets were performed using the stochastic multiple tube (8 replicates) approach to determine the Y-intercepts (cycle threshold (Ct) values), which correspond to one copy of the target gene in the sample (231). The assay sensitivity was calculated to 1:10000 **(Fig 9).** However, we defined a ten-fold higher cut-off limit corresponding to 1:1000 to be significant of detecting JAK2 V617F mutated alleles. The copy-number$_{JAK2V617F}$ was calculated as $10^{((Y\text{-interceptJAK2V617F} - \text{mean CtJAK2V617F})/\text{slopeJAK2V617F})}$, and the copy-number$_{JAK2Wildtype}$ as $10^{((Y\text{-interceptJAK2Wildtype} - \text{mean CtJAK2Wildtype})/\text{slopeJAK2Wildtype})}$.

Finally the percentage of JAK2 V617F mutated alleles were calculated as (copy-number$_{JAK2V617F}$/(copy-number$_{JAK2V617F}$ + copy-number$_{Wildtype}$)) x 100. The sensitivity in the individual qPCR reactions were calculated as (1/(copy-number$_{JAK2V617F}$ + copy-number$_{Wildtype}$)) x 100. The qPCR assay performs with a very high reproducibility **(Fig 10).** Direct sequencing was used to confirm the G to T base conversion **(Fig 11).**

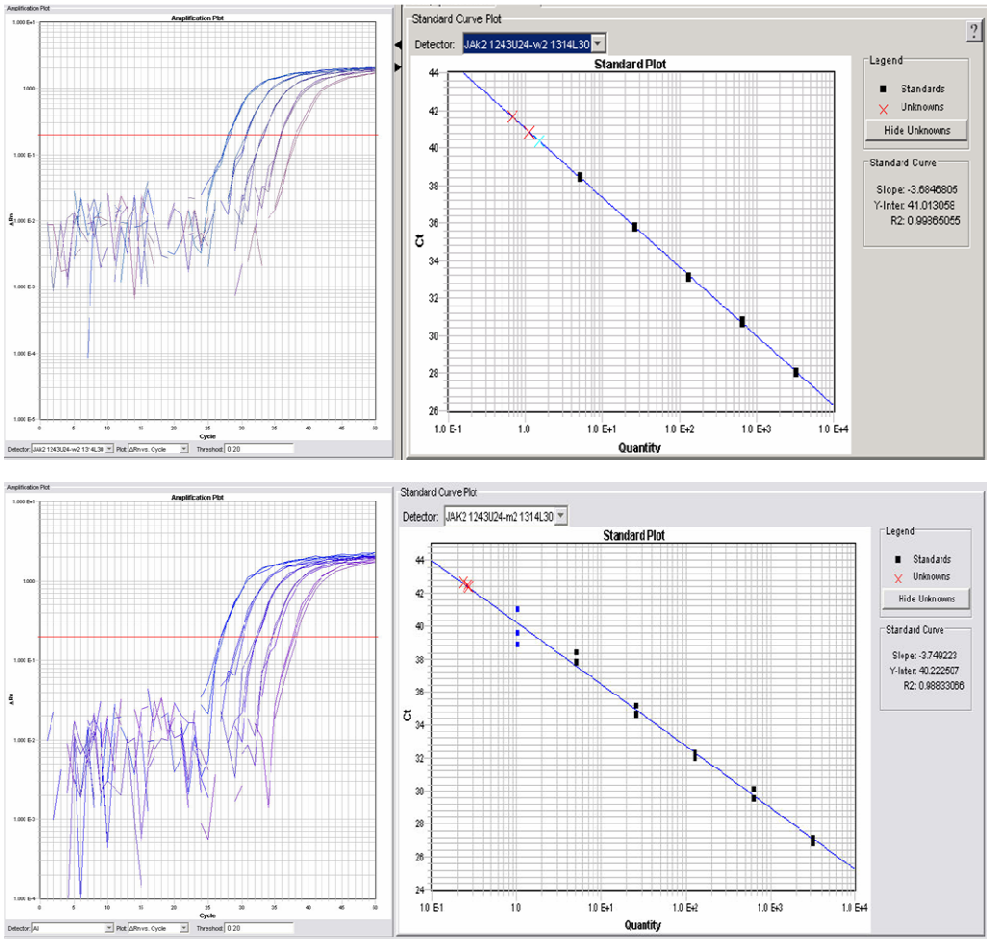


Figure 8: qPCR amplification plots showing a 5-fold dilution series of homozygous JAK2 V617F mutated DNA into donor *wildtype* DNA and corresponding standard curve (upper panel), and a 5-fold dilution series of JAK2 widtype donor DNA in H_2O and the corresponding standard curve (lower panel)

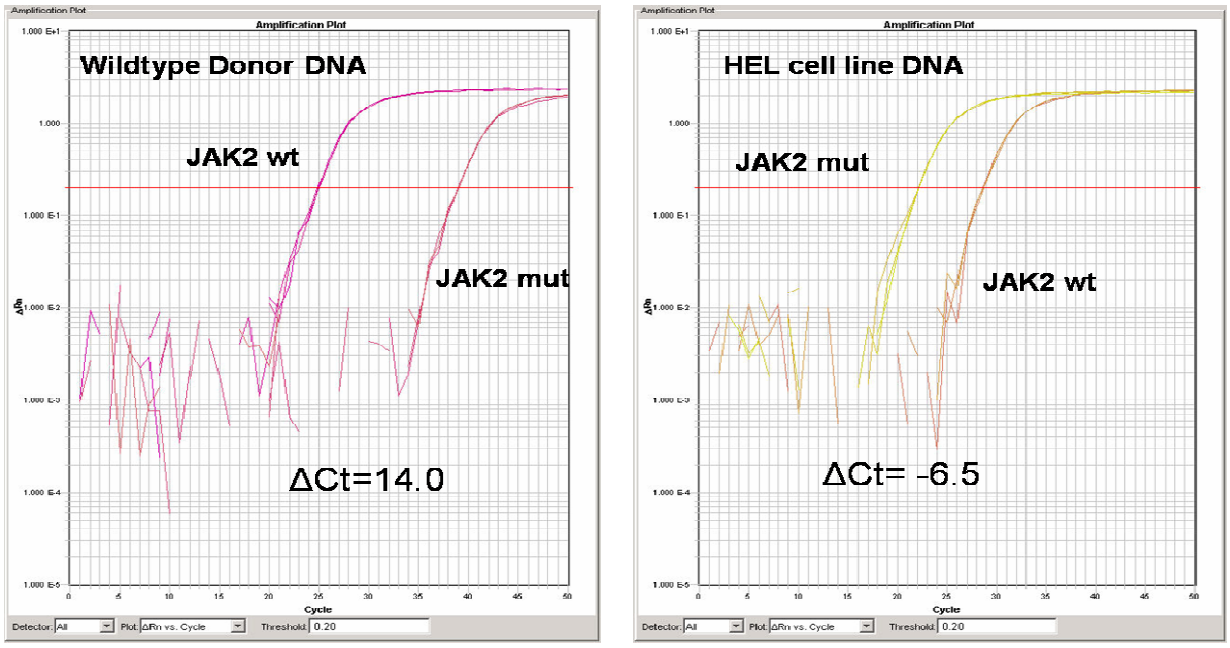


Figure 9: qPCR amplification plots from **left)** a donor wildtype sample and **right)** the HEL cell line showing both the amplification curve of the wildtype primer-set and the amplification curve of the JAK2 V617F mutation specific primer-set. In panel **"Right")** the "JAK2 mut" represents a highly reproducible unspecific amplification of JAK2 wildtype DNA by the JAK2 V617F specific primer. The distance between the two X-intercepts known as the delta cycle threshold value, (ΔCt) was fourteen, which corresponds to a sensitivity of 2^{14}, and accordingly more than 1:10000.

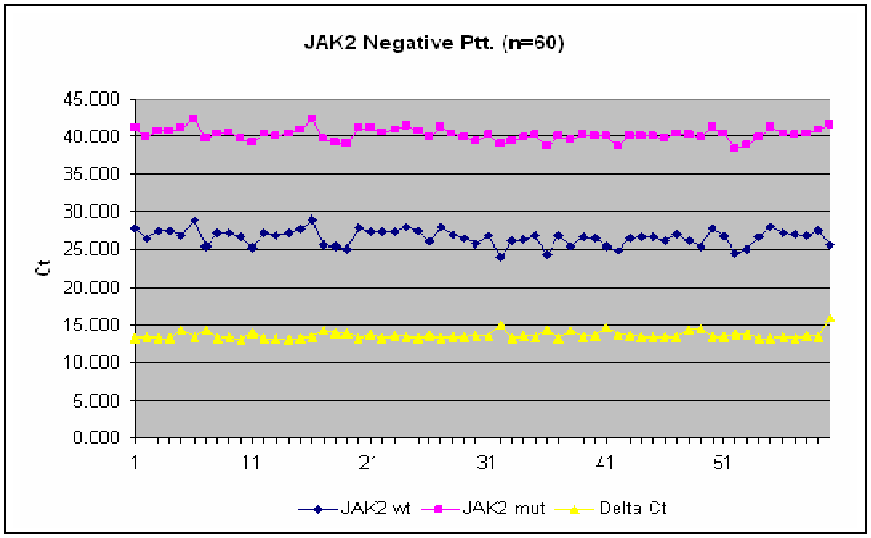


Figure 10: JAK2 qPCR analysis of 60 consecutive JAK2 V617F negative patients demonstrating the very high reproducibility illustrated by a constant ΔCT = 14.

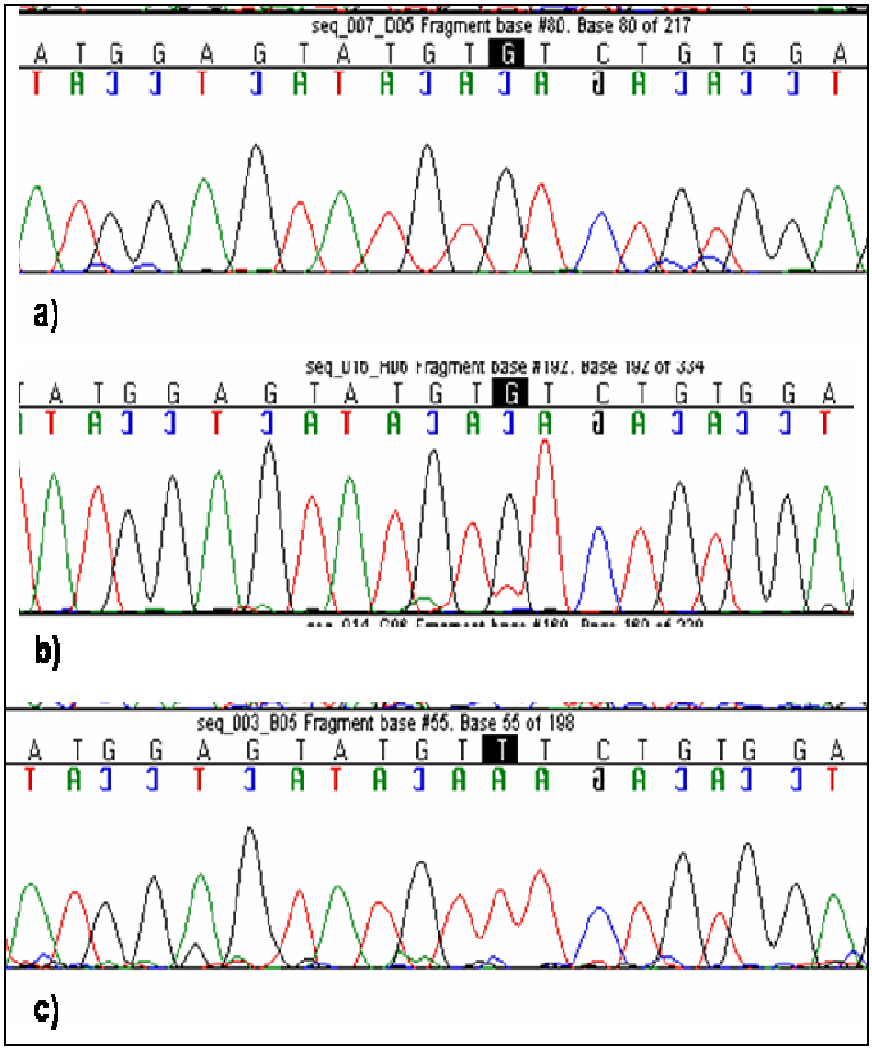


Figure 11: Sequence trace showing **a)** wildtype DNA represented by the "G" peak, **b)** a heterozygous patient represented by a majority of "G" and a small peak of "T" and c) the HEL cell line homozygous for JAK2 V617F mutation, and accordingly only a peak of "T" is present. First the three primer allele specific PCR by Baxter et al was performed for amplification. The upper band from the agarose gel was cut out and DNA was purified by ethanol precipitation. A linear amplification was performed using the forward internal control primer and the reverse primer from the Baxter assay and BigDye Terminator v1.1 Sequencing Standard Kit (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 3100 (Applied Biosystems, Foster City, CA, USA).

Chapter 7. Results and Publications

7.1 A general overview

In total, the JAK2 mutation status was determined in 349 patients. The majority of patients (n=217 (62%)) had a Ph-neg CMPD (ET, PV or PMF). Approximately half of the patients with ET and PMF (53% and 45%, respectively) were positive for the JAK2 V617F mutation. The vast majority of patients with PV harbored the JAK2 V617F mutation (115 out of 117, corresponding to 98.3 %) (**Fig.13)**. Two patients only, with unambiguous features of PV were JAK2 V617F negative on several measurements. A substantial number of patients with reactive/secondary erythro-/thrombo- and leukocytosis (n=34, n=28 and n=5, respectively) were all JAK2 V617F negative, as were 19 patients with idiopathic erythrocytosis, 8 patients with CML, 7 patients with CMML, 2 patients with MDS, 3 patients with HES, 1 patient with mastocytosis and 3 patients with malignant non-Hodgkin lymphoma. Four out of nine patients (44%) with AML and previous CMPD were JAK2 V617F positive at the time of leukemic transformation. Their pre-transforming JAK2 V617F status was only known in two patients (one JAK2 positive PV and one JAK2 negative ET, respectively). Twelve patients were categorized as unclassified CMPD, of whom four patients (33%) harbored the JAK2 V617F mutation.

In the group of JAK2 V617F positive patients with ET, PV and PMF, a total of 29, 112 and 20 patients, respectively had their JAK2 V617F allele burden quantified by qPCR. Because of continuous inclusion of patients after submission of **Paper III,** the figures are slightly different than those reported in **Paper III** (page 55). However, the median allele proportions in the three disease entities obtained from the total study cohort is fully comparable with the results reported in **Paper III** (page 55). The median JAK2 V617F allele burden in ET was 15% (95% c.i.: 6-30 %), (range 0.1-40 %), in PV 29% (95% c.i.: 20-37 %), (range 0.1-92%) and in PMF 63% (95% c.i.: 52-80 %), (range 35-99%) **(Fig 14 left).** If non-newly diagnosed patients are excluded and only patients not treated with myelosuppressive agents are considered, the median JAK2 V617F allele burden in ET was 21% (95% c.i.: 9-33 %), (range 0.1-40%), in PV 34 % (95% c.i.:23-46 %), (range 0.1-92%) and in PMF 59% (95% c.i.:51-75 %), (range 35-97%) **(Fig 14 right).**

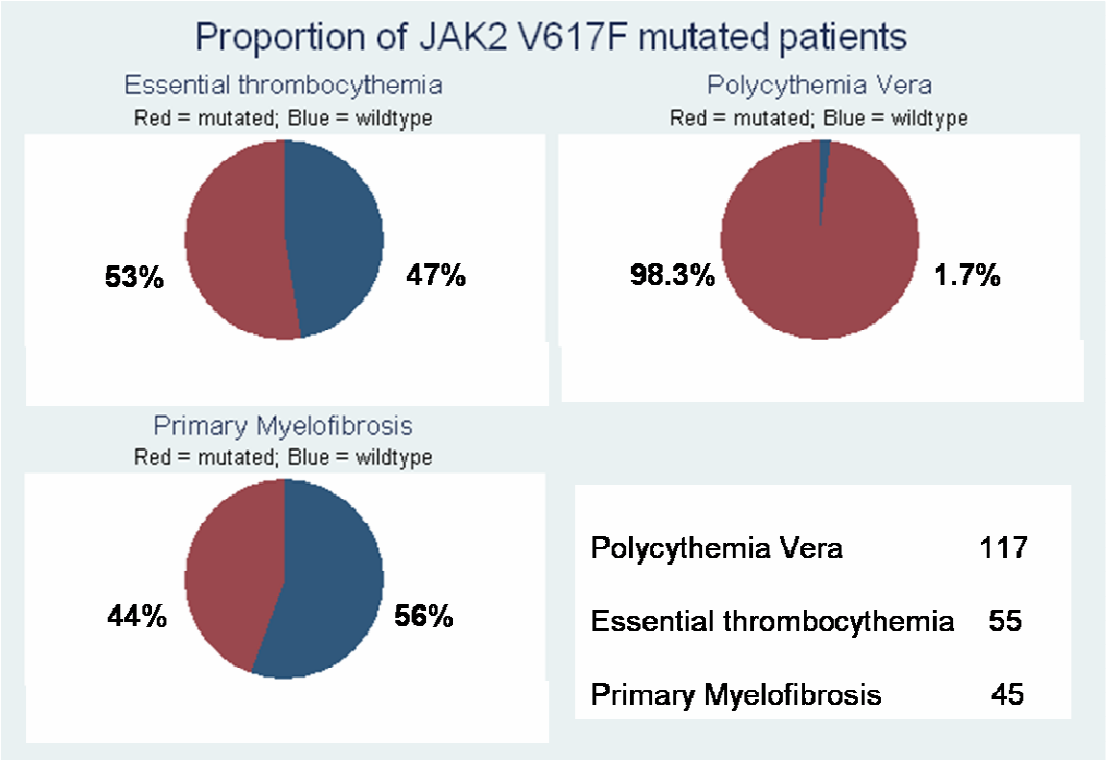


Figure 13: Pie-charts illustrating the percentage of JAK2 V617F mutated patients with ET, PV and PMF.

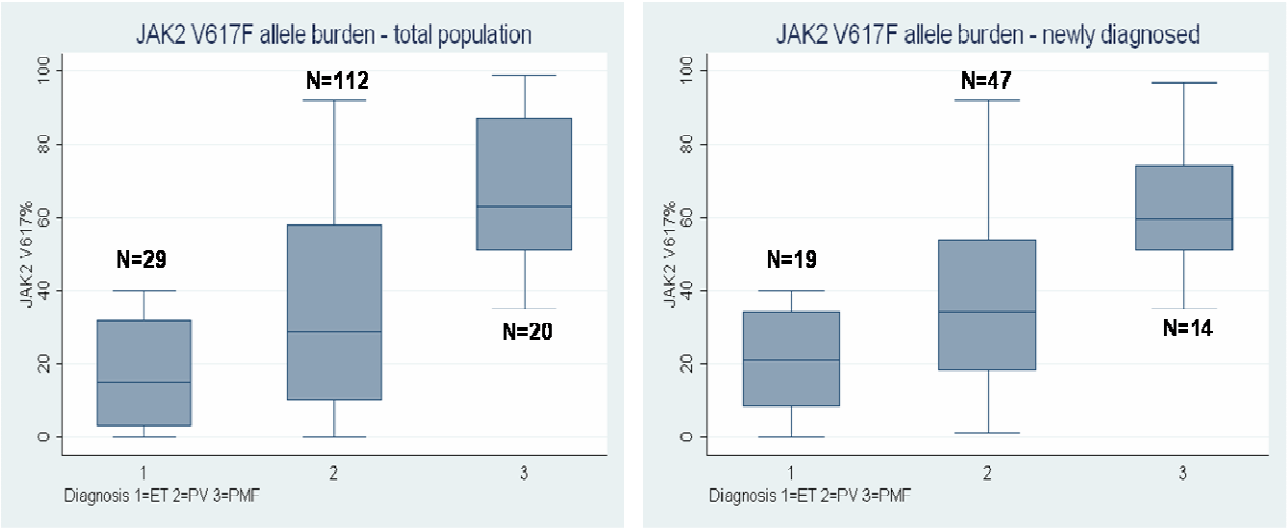


Figure 14: The median JAK2 V617F allele burden in the total population **(left panel)** and newly diagnosed **(right panel)** patients with ET, PV and PMF patients. qPCR assessment of the JAK 2 V617F allele burden could not be performed in 3 patients due to lack of material, and 2 patients were JAK2 V617F negative and therefore excluded. Accordingly, in total 112 of 117 PV patients were examined.

Clinical data on patients with ET, PV and PMF are given in **Table 2.** Cytogenetic data were available in 85 of 117 patients (73%) with PV. Twenty-three (27%) of those had chromomal aberrations. In ET patients, cytogenetic data was available in 44 of 55 patients (80%) and only 2 patients (5%) had an abnormal karyotype. In the PMF group a karyotype was available in 37 out of 45 patients (82%). Nine-teen of 37 (51%) patients had chromosomal aberrations. The karyotypes are given in **Table 3.** A history of thrombosis at time of diagnosis was present in 35 % of ET-patients, in 37 % of PV-patients and 11 % of the PMF-patients. Four patients (7%) with ET of whom 2 had a history of pre-diagnostic thrombosis and 13 patients (11%) with PV of whom 5 had a history of pre-diagnostic thrombosis and one (2%) with PMF experienced thrombotic events during follow-up. An overview of the thrombotic events is given in **Table 4**. It is beyond the scope of this thesis to describe in detail clinical findings in patients with secondary or idiopathic erythrocytosis and reactive thrombocytosis. Briefly, however, some key findings should be given: The median platelet count in patients with reactive thrombocytosis was 545 x 109/l (95% c.i.: 511-649), (range 425-1385). Six out of 28 patients had experienced a previous thrombosis, whereas none had microvascular disturbances. The median Hb concentrations in patients with SE and IE were 10.7 mmol/l (95% c.i.: 10.3-11.0 mmol/l) and 11 mmol/l (95%c.i.:10-12.2 mmol/l), respectively, whereas the Hct was 0.51 (0.49-0.54) and 0.53 (95% c.i.:0.49-0.59), respectively. Five out of 34 patients with SE had a history of previous thrombosis, whereas 6 out of 19 patients with IE had a previous thrombosis. Only 1 patient with IE and none of the patients with SE experienced any microvascular disturbances. Whereas both Hb and HCT were not significantly different in either IE or SE compared to PV, a median s-EPO of 8 (95% c.i.:7-13) in IE and 9 (95% c.i.: 6-12) in SE was significantly higher as compared to PV, median 3 (95 c.i.: 2-5), ($p=0.002$ and $p<0.00005$, respectively). In parallel, higher plasma levels of ferritin (IE: median=74, 95% c.i.: 42-247); (SE: median = 69, 95% c.i.: 33-185) ($p = 0.001$ and $p= 0.006$, respectively) was found in both IE and SE as compared to PV. The exact figures for PV are given in **Table 2.**

Whereas the JAK2 V617F mutation status has a major impact on the phenotype in ET **(Paper V)**, the phenotypic differences between JAK2 V617F mutated and wildtype PMF are modest. JAK2 V617F mutated patients had a higher Hct (median 36%, 95% c.i.:32-39% vs median 30%. 95%c.i.: 29-35%; $p=0.04$), lower s-EPO levels (median 16 IU, 95%c.i.: 5-26 IU vs. median 51 IU, 95% c.i.: 19-179 IU) ($p=0.02$) and lower plasma ferritin levels (median 94, 95% c.i.: 61-261 vs. median 477, 95% c.i.: 145-768) ($p=0.007$). There was no significant difference in Hb, WBC, platelet, and CD34 counts. Neither was there any significant difference in LDH and *PRV1* levels, age, gender, disease duration, thrombotic or microvascular complications, frequency of splenomegaly, occurrence of constitutional symptoms or chromosomal aberrations between JAK2 V617F mutated and wildtype patients.

Diagnosis	Polycythemia vera			Essential thrombocythemia			Primary myelofibrosis		
	Total	JAK2 V617F	JAK2 wt	Total	JAK2 V617F	JAK2 wt	Total	JAK2 V617F	JAK2 wt
Number of patients (%)	117	115 (98.3%)	2 (1.7%)	55	29 (53%)	26 (47%)	45	20 (44%)	25 (56%)
Newly diagnosed y/n (% y)	48/69 (41%)	47/67	1/1	27/28 (49%)	19/10 (66%)	9/17 (35%)	28/17	14/6	14/11 (56%)
Gender m/f (% m)	57/60 (48%)	56/59	1/1	25/30 (45%)	10/19 (34%)	15/11(58%)	25/20	11/9	14/11 (56%)
Age (years)	64 (23-91)	64 (23-91)	44 (34-53)	62 (32-91)	64 43-91)	60 (32-84)	67 (33-89)	65 (33-88)	70 (46-89)
Disease duration (months) *	71 (2-379)	71 (2-379)	59 (59)	64 (2-267)	57 (2-240)	69 (3-267)	68 (4-192)	58 (33-80)	69(50-77)
V617F %	29 (20-37)	29 (20-37)	N.A	N.A	15 (6-30)	N.A	N.A	63 (52-80)	N.A
Homozygous (y/n)	N.A	36/76 (32%)	N.A	N.A	0	N.A	N.A	17/3	N.A
Hemoglobin (mmol/l)	10.3 (9.9-10.8)	10.3 (9.9-10.8)	11 (9.6-12.4)	8.5 (8.1-8.7)	8.7 (8.4-9.2)	8.1 (7.8-8.5)	6.5 (6.1-7.0)	6.9(6.1-7.8)	5.2 (5.9-6.9)
Platelets ($x10^9$/l)	660 (578-755)	673 (591-773)	524 (473-575)	932 (834-1021)	857 (537-1810)	1182 (510-2860)	351 (267-621)	338 (304-539)	388(149-827)
WBC ($x10^9$/l)	12.1 (11.3-13.9)	12.3 (11.3-14.0)	6.8 (6.3-7.4)	9.8 (8.3-10.6)	10.1 (7.9-11.2)	8.9 (7.7-11.0)	10.9 (7.8-14.0)	11.4(8.0-2.5)	10.3(5.5-19.1)
LDH (normal/elevated)	69/35 (66%)	68/34	1/1	26/27 (49%)	19/9 (68%)	8/17 (32%)	37/3	15/2	22/1
HCT (%)	51 (49-54)	51 (49-54)	54 (46-61)	42 (41-43)	42 (41-44)	42 (41-44)	32 (30-37)	36 (32-39)	30 (29-35)
EPO	3 (2-5)	3 (2-5)	1 (1-1)	7 (5-13)	6 (5-8)	13 (6-18)	25 (10-48)	16 (5-26)	51 (19-179)
Ferritin	26 (20-36)	26 (19-35)	67 (67)	53 (40-88)	57 (35-92)	53 (40-113)	183 (91-409)	94 (61-261)	477 (145-768)
PRV1 (fold upreg.)	34 (20-67)	34 (20-80)	23 (2-43)	7 (3-20)	7 (3-32)	9 (2-28)	169 (55-670)	169 (65-3024)	207 (30-635)
CD34 ($x10^6$/l)	3 (2-4)	3 (2-4)	2 (2)	1 (1-4)	1 (0-4)	2 (0-6)	74 (42-254)	83 (49-515)	45 (3-317)
Splenomegaly y/n (% y)	26/86 (23%)	26/84	0/2	5/50 (9%)	1/29 (3%)	4/22 (15%)	22/19 (54%)	11/7 (61%)	11/12 (48%)
History of thrombosis y/n (% y)	43/73 (37%)	43/71	0/2	19/36 (35%)	17/12 (58%)	2/24 (8 %)	5/38 (11%)	3/16 (16%)	2/22 (8%)
Thrombosis follow-up y/n (% y)	13/103 (11%)	13/101	0/2	4/51 (7%)	1/28 (3%)	3/23 (12%)	1/42 (2%)	1/18 (5%)	0/24 (0%)
Microvascular symptoms y/n (% y)	53/60 (47%)	53/58	0/2	20/34 (37%)	9/20 (31%)	11/14 (44%)	5/30 (14%)	4/11 (27%)	1/19 (5%)
Constitutional symptoms y/n (% y)	33/76 (30%)	33/74	0/2	3/49 (6%)	1/23 (4%)	2/24 (8%)	21/12 (64%)	10/5 (67%)	11/7 (61%)
Chromosomal aberrerations y/n/u (% y)	23/63/31 (27%)	22/61/31	0/2/0	2/42/11 (5%)	2/23/4 (8%)	0/19/7 (0%)	19/18/8 (51%)	6/9/5 (40%)	13/9/3 (59%)
Cytoreductive therapy y/n (% y) *	55/10 (85%)	54/10	1/0	21/5 (81%)	9/0 (100%)	12/5 (63%)	7/10 (70%)	4/2 (67%)	3/8 (27%)

Table 2:* Only non-newly diagnosed patients. All parameters are from the time of diagnosis. In non-newly diagnosed patients V617F %, PRV1 expression levels, CD34 counts and cytoreductive therapy information are from the time of the JAK2 V617F analysis. N.A = Not applicable. y=yes, n=no, u=unknown, m=male, f=female. All parameters are given in medians with 95 % confidence intervals in paranthesis except “Age” and “Disease duration”, which are given in means with range in parenthesis.

Polycythemia Vera	JAK2 mut	JAK2 wt
trisomi 8	2	0
trisomi 9	4	0
trisomi 8 + trisomi 9	1	0
der(8;18)(p10;q10)	2	0
der(18)t(9;18)(p13;p11)	1	0
del(13)	2	0
del(7)	2	0
-Y,+14	1	0
add(X)(q26)	1	0
der(22) t(1;22)(q11;p11)	1	0
t(7;16)(q34;p13)	1	0
t(3;9)(p13;p14)	1	0
t(7;13)(p21:q13)	1	0
der(21)(t(1;21)	1	0
del(3)(p13;p21.2)	1	0
inv(11)(p14;q13)	1	0
Total	23	0
Primary Myelofibrosis	**JAK2 mut**	**JAK2 wt**
trisomi 8	0	2
trisomi 9	1	0
der(18)t(9;18)(p13;p11)	1	0
del(13q)	1	3
del(20q)	0	2
del(20q),+19	0	1
dup(1q)	1	1
trisomi 21	0	2
der(1;17)(q10;p10),-7	1	0
del(14)(q21;q32)	1	0
t(12;17)(q24;q22)	0	1
+8,del(6)(q13;q23)	0	1
Total	6	13
Essential Thrombocythemia	**JAK2 mut**	**JAK2 wt**
trisomi 14	1	0
-Y, del(9)(q22;q32)	1	0
Total	2	0

Table 3: Overview of chromosomal aberrations in patients with ET, PV and PMF.

Essential Thrombocythemia	n
Transient ischemic attack	9
Cerebral infarction	8
Acute myocardial infarction	3
Peripheral arterial thromboembolism	3
Total	23
Polycythemia Vera	**n**
Deep venous thrombosis	3
Pulmonary embolism	2
Transient ischemic attack	15
Cerebral infarction	16
Acute myocardial infarction	10
Peripheral arterial thromboembolism	9
Unknown	1
Total	56
Primary Myelofibrosis	**n**
Deep venous thrombosis	1
Cerebral infarction	2
Acute myocardial Infarction	2
Total	5

Table 4: Overview of thrombotic events

7.2 Paper I summary

Reference: ***Larsen TS, Christensen JH, Hasselbalch HC, Pallisgaard N.*** *The JAK2 V617F mutation involves B- and T-lymphocyte lineages in a subgroup of patients with Philadelphia-chromosome negative chronic myeloproliferative disorders.* ***Br J Haematol 2007;136:745-51****.*

Objectives: The primary scope of this paper was to evaluate the hypothesis that the Ph-neg CMPD are clonal stem cell disorders arising at the level of the pluripotent stem cell. The original publications on the JAK2 V617F mutation demonstrated its presence in myeloid cells, but stated that the mutation was absent in lymphocytes. In the 'Pre-JAK2 era' several papers based upon identification of other clonal markers, suggested that both B- and T-cells may be clonal. Using our highly sensitive qPCR assay for identification and quantification of the JAK2 V617F mutation on highly FACS purified granulocytes, monocytes, B-lymphocytes and T-lymphocytes we aimed at demonstrating the presence of the JAK2 V617F mutation in the various cell compartments.

Results: The results clearly demonstrated the presence of the JAK2 V617F mutation in both granulocytes and monocytes as expected, but also in B- and T-lymphocytes in a subgroup of the patients. Accordingly, the JAK2 V617F mutation arises in an early progenitor cell with the capacity of both myeloid and lymphoid proliferation. The JAK2 V617F allele burden was very variable in the different cell compartments. In general, the largest proportion of mutated alleles could be demonstrated in granulocytes, and data suggested a trend towards increasing propensity of lymphoid clonal involvement with increasing myeloid JAK2 V617F allele burden. However, firm conclusions on this aspect were not possible because of the limited patient number.

bjh research paper

The JAK2 V617F mutation involves B- and T-lymphocyte lineages in a subgroup of patients with Philadelphia-chromosome negative chronic myeloproliferative disorders

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Summary

The JAK2 V617F mutation is a frequent genetic event in the three classical Philadelphia-chromosome negative chronic myeloproliferative disorders ($Ph^{neg.}$-CMPD), polycythemia vera (PV), essential thrombocythemia (ET) and idiopathic myelofibrosis (IMF). Its occurrence varies in frequency in regards to phenotype. The mutation is found in the majority of patients with PV and about half of the patients with ET and IMF. These diseases are clonal stem cell disorders arising in an early stem cell progenitor. The level in the stem cell hierarchy on which the initiating genetic events and the JAK2 V617F mutation occurs is not known. The mutation has so far been detected in all cells of the myeloid lineage, whereas the potential clonal involvement of the lymphoid lineage is controversial. In this study, we detected the JAK2 V617F mutation by real-time quantitative PCR (qPCR) in both B-lymphocytes and T-lymphocytes in a subgroup of patients with $Ph^{neg.}$-CMPDs. These results demonstrate the origin of the JAK2 V617F positive disorders in an early stem cell with both lymphoid and myeloid differentiation potential.

Keywords: myeloproliferative, clonality, mutation analysis, real-time quantitative PCR, fluorescence-activated cell sorted.

Most recently a major breakthrough in the understanding of molecular pathogenesis of the Philadelphia chromosome negative chronic myeloproliferative disorders ($Ph^{neg.}$-CMPD) has been achieved with the identification of the V617F mutation in the *Janus Kinase 2* gene *(JAK2)*. The majority of patients with polycythaemia vera (PV) (65–97%) harbor the mutation in their clonal haematopoietic cells, as do approximately half of those with essential thrombocythaemia (ET) and idiopathic myelofibrosis (IMF) (Baxter *et al*, 2005; James *et al*, 2005; Kralovics *et al*, 2005; Levine *et al*, 2005). The V617F mutation occurs in the autoregulatory JH2 domain of the tyrosine kinase, leading to loss of the inhibitory effect of the JH2 domain on the kinase activity, and thereby constitutive activation of JAK2 and downstream signalling molecules, among these STAT5 (signal transducer and activator of transcription 5) and ERK (extracellular signal-regulated kinase) (James *et al*, 2005). *In vitro* studies have provided evidence of the capability of erythroid progenitors habouring the JAK2 V617F to form erythropoietin (EPO)-independent erythroid colonies (EECs), which is a well established hallmark of PV (Casadevall *et al*, 1982; Baxter *et al*, 2005; James *et al*, 2005). Furthermore *in vivo* studies have demonstrated that mice transplanted with murine bone-marrow cells retrovirally transfected with the JAK2 V617F encoding gene develop a PV-like disease that tends to terminate in a myelofibrosis-like phaenotype (James *et al*, 2005; Lacout *et al*, 2006; Wernig *et al*, 2006). In most studies on the JAK2 V617F in $Ph^{neg.}$-CMPD, the mutational analysis was performed on peripheral blood granulocytes and, to some extent, mononuclear cells and unfractionated leucocytes after red cell lysis. The JAK2 V617F mutation is detectable in granulocytes and cells of the myeloid lineage in general and hence originates in a myeloid lineage precursor, which is in line with earlier studies demonstrating the clonality by glucose-6-phosphate dehydrogenase isoenzyme (G6PD) analyses of different myeloid cell types (granulocytes, monocytes, platelets and erythrocytes) (Adamson *et al*, 1976; Jacobson *et al*, 1978; Fialkow *et al*, 1981). However, controversy exists whether the JAK2 V617F clone involves the B- and T-cells of the lymphoid lineage and hence arises in a pluripotent non-commited stem cell. Clonality studies based

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on the same method (G6PD) and later X-linked DNA-analysis have provided evidence of clonal involvement of B-cells in $Ph^{neg.}$-CMPDs, as also shown in chronic myeloid leukaemia (CML) (Fialkow *et al*, 1978; Martin *et al*, 1980; Raskind *et al*, 1985; Anger *et al*, 1990; Gilliland *et al*, 1991; Tsukamoto *et al*, 1994;el-Kassar *et al*, 1997). T-cells have been reported as non-clonal, although a few studies on IMF using RAS mutations and karyotypic abnormalities as clonal markers have found T-cell clonal involvement (Buschle *et al*, 1988; Reeder *et al*, 2003). Likewise, in a subgroup of PV patients evidence of possible clonal T-cells have been demonstrated (Kralovics *et al*, 2002). These studies were all published in the pre-JAK2 era. The identification of the JAK2 V617F mutation has provided a novel tool to be used in studies of clonal involvement in different haematopoietic cell types. The data so far published using the JAK2 V617F mutation as a clonal marker are conflicting in regard to the involvement of lymphoid B- and T-cells in the JAK2 V617F clone (Baxter *et al*, 2005; James *et al*, 2005; Lasho *et al*, 2005; Delhommeau *et al*, 2006; Ishii *et al*, 2006).

In this study, we have performed *JAK2* mutation analysis by a highly sensitive real-time quantitative PCR (qPCR) method on fluorescence-activated cell sorted (FACS) granulocytes, monocytes B-lymphocytes and T-lymphocytes from patients with $Ph^{neg.}$-CMPDs with a known JAK2 V617F mutation to further elucidate this issue.

Materials and methods

Ethylenediaminetetraacetic acid anticoagulated blood (20 ml) was collected from seven healthy controls and 13 patients with a known $Ph^{neg.}$-CMPD (PV = 10, IMF = 2, ET = 1). All patients were known to have a JAK2 V617F mutation identified by the three-primer allele-specific method published by Baxter *et al* (2005). All samples were collected after informed consent according to the Helsinki Declaration and guidelines of the Danish Regional Science Ethics Committee. Patient characteristics are summarised in Table I.

Purification of granulocytes, monocytes, B-lymphocytes and T-lymphocytes

An initial red cell lysis was performed using an NH_4Cl lysis buffer. After centrifugation and resuspension the cells were labelled with CD3-allophycocyanin (APC), CD19-phycoerythrin (PE), CD14-PE-Cy7 (BD Biosciences, Erembodegem, Belgium) and CD66-flurescein isothiocyanate (FITC) (Dako A/S, Glostrup, Denmark) fluorchrome-conjugated monoclonal antibodies according to manufacturers protocols. The cells were sorted using a FACSVantage (BD Biosciences) in a high purity mode, aborting droplets containing doublet cells or more than one cell because of *in-vitro* aggregation, resulting in 99% purity for $CD3^+$ T-cells, $CD19^+$ B-cells, $CD14^+$ monocytes and $CD66^+$ granulocytes. Peripheral blood from one out of the seven healthy controls was sorted as described, while unfractionised leucocytes were used for analysis in the remaining six healthy controls. The FACS gating strategy is depicted in Fig 1.

DNA purification

DNA was extracted from the four different cell isolates using a MagnaPure robot (Roche Diagnostics, Mannheim, Germany) according to the manufacturers protocol.

Real-time quantitative PCR (qPCR)

We designed two qPCR assays with a common forward primer 5′-CTTTCTTTGAAGCAGCAAGTATGA-3′ and a common forward probe 6-FAM-TGAGCAAGCTTTCTCACAAG-CATTTGGTTT-TAMRA.

The reverse primers were designed as a wildtype-specific primer 5′-GTAGTTTTACTTACTCTCGTCTCCACAtAC-3′ or

Table I. Patient characteristics.

Patient	Age (years)	Gender	Disease duration (months)	Cytoreductive treatment at time of present analysis	Previous cytoreductive treatment	Prior thrombosis
PV1	72	Female	17	None	None	No
PV2	66	Female	38	HU and ANA (comb.)	HU, IFN, IMA	No
PV3	53	Male	22	IFN	HU	Yes
PV4	58	Male	17	IFN	HU, ANA	Yes
PV5	58	Female	21	ANA	IFN	Yes
PV6	70	Female	17	HU	None	Yes
PV7	78	Male	14	HU	None	No
PV8	77	Male	75	None	BU	No
PV9	78	Male	157	None	BU	No
PV10	59	Male	118	IFN and HU (comb.)	ANA, HU	Yes
IMF1	67	Male	16	HU	None	Yes
IMF2	74	Female	12	None	None	No
ET	57	Female	10	ANA	None	Yes

HU, hydroxyurea; IFN, interferon-alpha; ANA, anagrelide; BU, busulfan; IMA, imatinib.

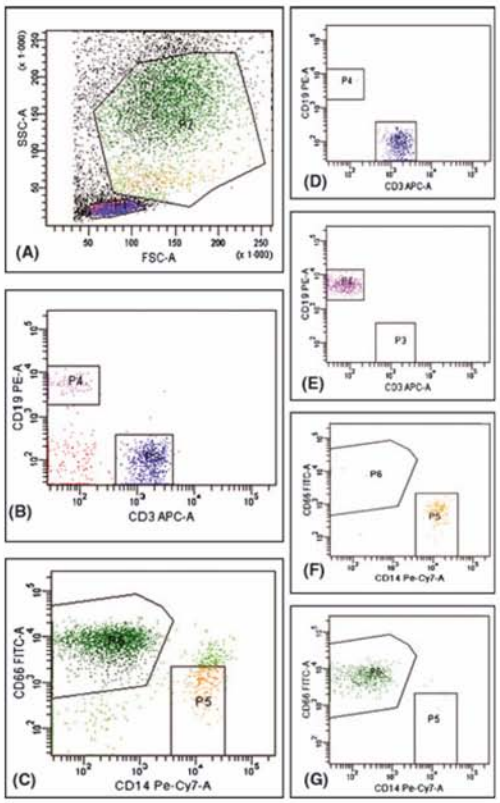

Fig 1. Gating strategy: (A) FSC-SSC scatter plot showing 2 gates P1 (lymphocyte gate) and P2 (monocyte-granulocyte gate), including back-gating with colours indicating the four different populations ($CD3^+$ blue, $CD19^+$ purple, $CD14^+$ orange and $CD66^+$ green). (B) $CD3^+/CD19^+$ scatter plot separating the lymphocytic population in $CD3^+$ T-cells (P3) and $CD19^+$ B-cells (P4). (C) $CD14^+/CD66^+$ scatter plot separating $CD14^+$ monocytes (P5) from $CD66^+$ granulocytes (P6). (D–G) Post-sorting quality control showing 99% purity of the four cell types.

a *JAK2* V617F mutation-specific primer 5′-GTAGTTTT ACTTACTCTCGTCTCCACAtAA-3′, both with an intended mismatch at the 3′-minus 2-position. The qPCR reaction volume was 25 µl and primer-concentrations were 300 nmol/l, whereas the concentration of the probe was 200 nmol/l. The PCR amplification conditions were: An initial enzyme activation step of 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 60 s at 60°C. All qPCR reactions were performed in triplicates on an ABI Prism7900HT (Applied Biosystems, Foster City, CA, USA).

qPCR Data analysis

In order to determine the sensitivity of the mutation-specific primer set, a standard curve was created by fivefold dilution series of homozygous JAK2 V617F mutated DNA into donor wildtype DNA. From standard curves the slope was calculated for both the wildtype-specific (3·6) and mutation-specific (3·7) primer-probe sets. The assay sensitivity was calculated to 1:10 000 (Fig 2). However we defined a 10-fold higher cut-off limit, corresponding to 1:1000, to be significant regarding detection of JAK2 V617F mutated alleles. The Y-intercept [cycle threshold (Ct) value] corresponding to one copy of the target gene in the sample, was calculated from limiting twofold dilution series of both primer sets. The $\text{copy-number}_{\text{JAK2V617F}}$ was calculated as $10^{((\text{Y-intercept}_{\text{JAK2V617F}} - \text{mean Ct}_{\text{JAK2V617F}})/\text{slope}_{\text{JAK2V617F}})}$, and the $\text{copy-number}_{\text{JAK2Wildtype}}$ as $10^{((\text{Y-intercept}_{\text{JAK2Wildtype}} - \text{mean Ct}_{\text{JAK2Wildtype}})/\text{slope}_{\text{JAK2Wildtype}})}$. Finally the percentage of JAK2 V617F mutated alleles were calculated as $[\text{copy-number}_{\text{JAK2V617F}}/(\text{copy-number}_{\text{JAK2V617F}} + \text{copy-number}_{\text{JAK2Wildtype}})] \times 100$.

Results

FACS sorting

Fluorescence-activated cell-sorting was performed in a high purity mode, aborting droplets containing more than one cell leading to a decrease in the sorting efficiency. This was most prominent for the monocytes and granulocytes, median 55% (range 34–78%) and 67% (range 47–88%) respectively, whereas the sorting efficiency was markedly higher in the lymphoid compartments, median 94% (range 90–97%) and 93% (range 89–97%) for the B-cells and T-cells respectively. This decrease in sorting efficiency for the myeloid cells was probably caused by a tendency of *in vivo* and *in vitro* aggregation of monocytes, granulocytes and platelets in the $Ph^{neg.}$-CMPD.

qPCR

As described the assay sensitivity was calculated to 1:10 000, however we defined an assay cut-off limit of 1:1000. The sensitivity of the individual qPCR reactions was solely dependent on the assay sensitivity (1:1000) in reactions with no limitations in the amount of DNA. In qPCR reactions with limited amount of DNA, as it was the case in especially the $CD19^+$ B-lymphocyte and $CD14^+$ monocyte compartment, the sensitivity was calculated as [1/(copy number JAK2 wildtype + copy number JAK2 V617F)] × 100. The individual qPCR reaction sensitivities are given in Fig 3A. As a result of the FACS, efficiency of 99% a lower limit of 1% mutated alleles must be present for a cell compartment to be interpreted as 'JAK2 V617F positive'.

All 13 patients had JAK2 V617F clonal granulocytes and monocytes with a median proportion of mutated alleles of 60%, ranging from 8–96% in the granulocyte compartment, and 35%, range (2–84%) in the monocyte compartment. Eight patients (PV1-PV2, PV6, PV8-PV10 and IMF1-IMF2) had more than 50 % mutated alleles in their granulocytes, indicating

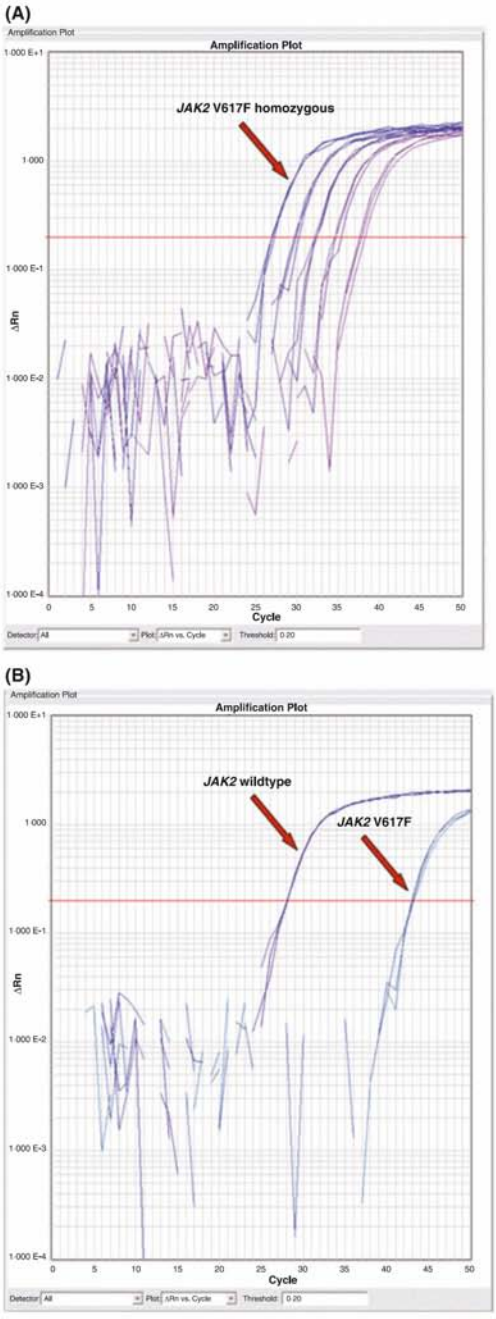

Fig 2. (A) Real-time quantitative PCR (qPCR) amplification plot showing a fivefold dilution series of homozygous JAK2 V617F mutated DNA into donor *wildtype* DNA. (B) qPCR amplification plot from a donor wildtype sample showing both the amplification curve of the wildtype primer-set and the amplification curve of the JAK2 V617F mutation specific primer-set, the latter representing a reproducible unspecific amplification of JAK2 wildtype DNA by the JAK2 V617F specific primer. The distance between the two X-intercepts, known as the delta cycle threshold value, (ΔCt), was 14, which corresponds to a sensitivity of 2^{14}, hence more than 1:10 000.

homozygocity, whereas five patients (PV2, PV8, PV10 and IMF1-IMF2) had JAK2 V617F homozygous monocytes. The median proportion of mutated alleles in T-lymphocytes was 3%, range (0·2–83%). Four patients (PV3, PV4, PV5 and PV7) had 1% or less mutated alleles and were therefore interpreted as 'JAK2-negative' in their T-cell compartment. Nine patients (PV1, PV2, PV6, PV8, PV9, PV10, IMF1, IMF2 and ET) had 3–83% mutated alleles in their T-cells with a reasonably high sensitivity (0·1–0·04). Two of these patients (PV8 and IMF1) were remarkably homozygous with 65% and 83% JAK2 V617F mutated alleles respectively. Six patients (PV2, PV3, PV7, PV8, PV10 and IMF1) had JAK2 V617F mutated alleles in their B-cells, but in all but one patient (PV2 with 46% mutated alleles) at relatively low levels (2–6%). None had JAK2 V617F homozygous B-lymphocytes. The median proportion of JAK2 V617F mutated alleles in B-cells was 3·5 %, range (1–46%). The qPCR data are summarised in Fig 3.

Discussion

Two of the first published papers on the JAK2 V617F mutation described the absence of the mutation in T-cells within all three $Ph^{neg.}$-CMPDs (Baxter *et al*, 2005; James *et al*, 2005), which has later been confirmed. In addition, the absence of JAK2 V617F mutation in the B-cell compartment in one PV patient and two myelofibrosis patients with previous ET and PV respectively, was demonstrated (Lasho *et al*, 2005). Accordingly, it was concluded, that the JAK2 V617F mutation is restricted to a myeloid precursor cell. The same group has previously shown that both B- and T-cells may be of clonal origin in IMF (Reeder *et al*, 2003). This observation does not exclude the possibility that the JAK2 V617F mutation is a secondary event in an already existing clone with a possible other genetic marker e.g. the del 20q, as it has been proposed by others (Kralovics *et al*, 2006). All these data were based on sequencing methods, which are known to have a limited sensitivity (Campbell *et al*, 2005). Small B- and T-cell JAK2 V617F clones could have been undetectable because of the limited sensitivity. Most recently, strict myeloid lineage involvement of the JAK2 V617F mutation has been challenged by the findings of the JAK2 V617F mutation in both $CD19^+$ B- and $CD3^+$ T cells in one patient and JAK2 V617F clonal B-cells but not T-cells in another patient by qPCR on FACS sorted cells in a group of ten patients with PV (Ishii *et al*, 2006). A similar pattern of heterogeneous lympho-myeloid JAK2 V617F clonal involvement in addition to involvement of NK-cells in both PV and the majority of IMF patients has been confirmed in a most recent study (Delhommeau *et al*, 2006).

(A)

Patient No.	CD66+		CD14+		CD3+		CD19+	
	V617F %	Sensitivity %	V617F %	Sensitivity %	V617F %	Sensitivity %	V617F %	Sensitivity %
PV1	73	0·1	31	4	3	0·1	0	5
PV2	96	0·03	84	0·3	3	0·06	46	1
PV3	28	0·2	17	1	0·2	0·08	6	0·8
PV4	38	0·02	23	2	1	0·04	0	2
PV5	23	0·09	20	1	1	0·1	0	5
PV6	63	0·05	35	0·4	4	0·06	0·5	0·3
PV7	8	0·03	6	0·3	1	0·04	2	0·4
PV8	93	0·03	78	1·1	65	0·8	4	0·2
PV9	52	0·04	13	0·04	3	0·05	1	0·7
PV10	94	0·03	65	0·7	11	0·05	3	0·4
IMF1	82	0·03	74	0·7	83	0·7	3	0·06
IMF2	60	0·2	82	5	7	0·05	0	4
ET	13	0·07	2	0·7	12	0·6	0·3	0·05

(B)

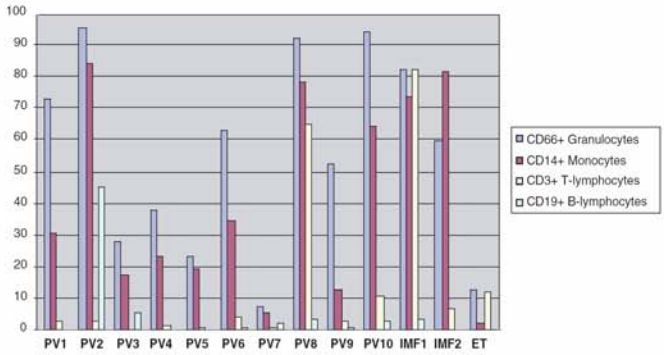

Fig 3. (A) Real-time quantitative PCR (qPCR) data: the percentage of JAK2 V617F alleles and the corresponding specific sensitivity in each qPCR reaction calculated as: $[1/(\text{copy-number}_{JAK2V617F} + \text{copy-number}_{Wildtype})] \times 100$. (B) qPCR data: Histogram plot showing the percentages of JAK2 V617F alleles in CD66$^+$ granulocytes, CD14$^+$ monocytes, CD3$^+$ T-cells and CD19$^+$ B-cells, respectively, in patients PV1-PV10, IMF1-IMF2 and ET.

In the present study we designed a qPCR assay with a very high sensitivity of at least 1:1000. Because of limitations in cell numbers, and hence DNA amount in the CD19$^+$ B-cell and CD14$^+$ monocyte compartments, this high sensitivity could not be reached in all qPCR reactions, and thus was calculated for each qPCR reaction. In addition to the expected findings of the JAK2 V617F mutation in granulocytes and monocytes, the results clearly demonstrated detectable levels of JAK2 V617F alleles in both CD19$^+$ B-lymphocytes and CD3$^+$ T-lymphocytes in a subgroup of patients, in total six (PV2, PV3, PV7, PV8, PV10, IMF1 and IMF2) and nine (PV1, PV2, PV6, PV8, PV9, PV10, IMF1, IMF2 and ET) respectively. Both IMF patients had JAK2 V617F clonal T-cells, one of them (IMF1) had a majority (83%) of mutated alleles, which definitely should be detectable with sequencing techniques, and this is also likely to be the case for patient PV8. In the other patients (PV1, PV2, PV6, PV9, PV10, IMF2 and ET) the T-cell clone would probably have been missed by sequencing because less than 13% mutated alleles were detected. One of the patients (PV2) with B-lymphocyte clonal involvement had 46% JAK2 V617F mutated alleles and should therefore be detectable by sequencing. In the present study, four (PV2, PV8, PV10 and IMF1) out of the 13 patients had JAK2 V617F clonal involvement of all four cell compartments analysed (granulocytes, monocytes, B-lymphocytes and T-lymphocytes). JAK2 V617F clonal involvement of all four cell compartments has previously been demonstrated in IMF patients (Delhommeau *et al*, 2006). Interestingly the three PV patients (PV2, PV8 and PV10) with detectable JAK2 V617F levels in all four cell compartments had very high proportions of mutated alleles in their monocytes and granulocytes as well as a significantly longer disease duration than the other PV patients in this study. The results of the present study are comparable with previously published data (Delhommeau *et al*, 2006; Ishii *et al*, 2006), and provide further evidence that the JAK2 V617F mutation occurs in a lympho-myeloid progenitor within the Ph^{neg}-CMPDs. In the present study though, JAK2 V617F clonal involvement of T-cells were detected in nine out of 13 patients, which is a considerably higher frequency than reported by Delhommeau *et al* (2006), whereas the proportion of patients with JAK2 V617F clonal B-cells were comparable. The reason for this finding is not clear, but in the present study the detection of potential JAK2 V617F 'positive' B-cells could have been missed regardless of the use of a highly sensitive

qPCR assay (>0·1%), because of limitations in DNA amount. On the other hand, both Delhommeau *et al* (2006) and Ishii *et al* (2006) used another qPCR method with competitive mutation-specific probes and reported a sensitivity of 2% (Delhommeau *et al*, 2006). Some of the discrepancies between the studies could therefore be related to technical differences. Nevertheless, the proportion of JAK2 V617F alleles seems to be at much lower levels in lymphoid cells than in myeloid cells in the majority of patients. T-cells are long-living cells, but they are produced throughout adult life by periodically importation of haematopoietic stem cells from the bone marrow to the thymus (Schwarz & Bhandoola, 2006). There is some evidence that the haematopoietic stem cells trafficking from the bone marrow to the thymus are progenitor cells with both myeloid and lymphoid differentiation potential (Katsura, 2002), which could explain the more scarce occurrence of T-cell JAK2 V617F clonal involvement as recorded in the present and previous studies (Delhommeau *et al*, 2006; Ishii *et al*, 2006). The identification of progenitors capable of producing B-cells and differentiating into cells of the myeloid lineage has further challenged our classical understanding of lineage-specific progenitors (Hou *et al*, 2005). It is intriguing to consider the possibility that the JAK2 V617F clone has a proliferative advantage in the myeloid lineage. During progression of the disease the percentage of JAK2 V617F cells increases, myeloid cells become homozygous for the JAK2 V617F mutation and in parallel, the possibility of the JAK2 V617F clone to proliferate in lymphoid lineage may increase. The percentage of JAK2 V617F alleles in progenitors seems to increase from ET to PV (Scott *et al*, 2006), and it is likely that JAK2 V617F positive CMPDs could be considered as a biological continuum from ET over PV to myelofibrosis (Campbell *et al*, 2005; Wolanskyj *et al*, 2005). Although the numbers are too small to draw firm conclusions, a trend towards increasing lymphoid involvement in late PV and IMF was recorded in the present study. A similar association has been described most recently (Delhommeau *et al*, 2006). It is important to notice that the JAK2 V617F clonal involvement is very heterogenous, probably partly reflecting biological variation among patients, although previous and current cytoreductive therapy might contribute. Moreover, it is of interest to note that T-cell involvement was actually observed in all disease entities (ET, PV and IMF), supporting the model of a biological continuum within the JAK2 V617F-CMPDs. In conclusion, this study provides further evidence that the JAK2 V617F mutation in the Ph^{neg}-CMPDs occurs in a lympho-myeloid progenitor cell. Future studies in a large series of untreated patients are needed to further delineate precisely the level in the stem cell hierarchy at which the JAK2 V617F mutation occurs.

Authors' contributions

Thomas Stauffer Larsen organised the studies, performed the FACS sort and *JAK2* analysis and wrote the manuscript. Niels Pallisgaard designed the qPCR assay and contributed to the study design and the final manuscript. Jacob Haaber Christensen contributed with his expertise on FACS sorting and in reviewing the manuscript. Hans Carl Hasselbalch followed the patients, contributed to the study design, organised the sample collection and reviewed the manuscript.

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7.3 Paper II summary

Reference: ***Larsen TS, Pallisgaard N, Møller MB, Hasselbalch HC.*** *Quantitative assessment of the JAK2 V617F allele burden: equivalent levels in peripheral blood and bone marrow.* ***Leukemia 2008;22:194-195.***

Objectives: In order to evaluate the value of the JAK2 qPCR assay in assessment of the bone marrow JAK2 V617F allele burden and to investigate the correlation between the JAK2 V617F allele burden in bone marrow and peripheral blood leukocytes, we conducted a study on 11 consecutive patients, who were referred for a bone marrow biopsy on suspicion of an Ph-neg CMPD. Accordingly, analogically with the monitoring of the BCR-ABL fusion gene in CML we wanted to investigate whether the quantification of the JAK2 V617F allele burden in peripheral blood leukocytes is a reliable measurement of the true "tumor burden" in the bone marrow.

Results: The qPCR assay performed well on bone marrow material. There was a highly significant correlation between the JAK2 V617F allele burden in bone marrow (biopsy and aspirate) and peripheral blood leucocytes. Because none of the 11 patients had a JAK2 V617F allele burden below 21 %, a firm conclusion on the correlation at low allele burdens was not possible. However, we concluded that qPCR assessment of the JAK2 V617F mutational load is a reliable measurement of the true JAK2 V617F "tumor burden".

Quantitative assessment of the JAK2 V617F allele burden: equivalent levels in peripheral blood and bone marrow

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The detection of the JAK2 V617F mutation has become a key component in the diagnostic procedure in patients suspected of a chronic myeloproliferative disorder. The proportion of patients with polycythemia vera (PV) harboring the mutation is at least 97% with sensitive PCR-based methods, whereas approximately half of the patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF) are JAK2 V617F-positive.[1,2] The source used for DNA or RNA mutation analysis is peripheral blood (PB), mostly granulocytes. It is evident that the JAK2 V617F mutation is detectable also in bone marrow (BM) specimens.[3,4] A growing body of evidence suggests that the JAK2 V617F allele burden and in particular the state of homozygosity in PV has an impact on disease phenotype as regards the degree of elevated hemoglobin levels, pruritus[4–6] leukocytosis,[4,5] microvascular symptoms,[4] thrombocytosis, thrombosis, splenomegaly and propensity to develop secondary myelofibrosis.[5] Furthermore, the JAK2 V617F allele burden in PB increases from ET over PV to primary myelofibrosis (PMF), which might reflect a biological continuum, the phenotypic presentation in part influenced by the mutant allele burden. However, the size of the JAK2 V617F allele burden varies considerably within a given phenotype, that is in PV ranging from a few percent to almost 100%.[4,7] No clear correlation between disease duration or the need for cytoreductive treatment and JAK2 V617F allele burden has been reported.[4,5] Despite a relatively low allele burden, patients may have a proliferative disease with a need for cytoreductive treatment as well as frequent phlebotomies in patients with PV. Thus, one could speculate that the JAK2 V617F allele burden measured in PB does not reflect the true size of the JAK2 V617F mutant clone, which may account for a larger proportion in the BM.

To date, a correlation between the JAK2 V617F allele burden in PB and BM has not been reported. In the present study, we provide evidence of a highly significant correlation between the JAK2 V617F allele burden as assessed by quantitative real-time PCR (qPCR), measured in unfractionized white blood cells (WBCs) from PB and BM biopsies.

In the period from January to March 2007, 11 consecutive patients (ET = 2, PV = 8, PMF = 1) (Table 1) were enrolled in the study, which was conducted according to the Helsinki Declaration and the guidelines of the Danish Regional Science Ethics Committee. Twenty milliliters of ethylenediaminetetraacetic acid-anticoagulated blood was collected together with a BM biopsy and aspirate. Nine patients (patient nos. 1–8 and 11) were newly diagnosed, whereas two patients (patient nos. 10–11) were followed for a period of 12 and 60 months respectively before collection of the BM and PB used in this study. Patient no. 9 had a 60 months history of discrete bilinear cytopenia (thrombocytopenia and leucopenia) and slight myelodysplastic BM changes. Both the cytopenia and dysplastic changes resolved and a more proliferative phenotype evolved and accordingly a new BM biopsy was performed. Patient nos. 10 was diagnosed with slight anemia and BM features suggesting PMF 12 months before the sample collection for the present study. None of these two patients received cytoreductive therapy before this study. Unfractionized WBCs from PB were used as the DNA source. Paraffin-embedded BM trephines were fixated in formaldehyde for 24 h, followed by decalcification with formic acid exposition for 6 h. DNA extraction was performed using a MagnaPure robot (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. qPCR was performed as described previously.[8] Briefly, two real-time qPCRs were performed in parallel with a common forward primer and a Taqman probe and only differed in the use of a reverse primer specific for the JAK2 wild type and the V617F mutated DNA respectively. The JAK2 V617F proportion was calculated from standard curves and end point determination from limiting dilution series of JAK2 wild-type donor DNA and the homozygous JAK2 cell line HEL. All qPCRs were performed in triplicates.

The 11 patients had a median JAK2 V617F% of 54 and 53 in PB and BM, respectively (range: PB, 21–92%; BM, 30–85%).

Table 1 Patient number, diagnosis and the JAK2 V617F% in PB, BM biopsy and aspirate

Patient. no.	*JAK2 V617F%*			
	Diagnosis	*PB*	*BM biopsy*	*BM aspirate*
1	PV	91	82	89
2	ET	21	42	(–)
3	PV	32	30	(–)
4	PV	54	56	58
5	PV	49	52	50
6	ET	41	36	(–)
7	PV	76	77	79
8	PV	32	39	38
9	PV	57	38	40
10	PMF	92	85	(–)
11	PV	59	50	48

Abbreviations: BM, bone marrow; ET, essential thrombocythemia; PB, peripheral blood; PMF, primary myelofibrosis; PV, polycythemia vera.

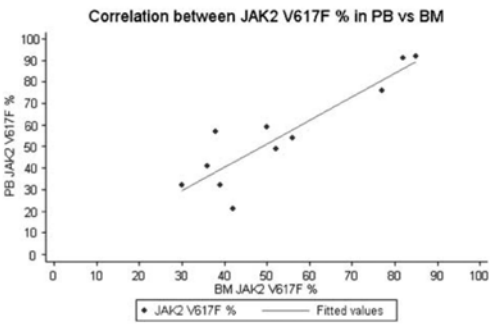

Figure 1 Correlation between JAK2 V617F% in PB unfractionized WBC and BM. Spearman's $r = 0.76$ ($P = 0.007$). The line represents the fitted regression line with a regression coefficient of 1.09 (95% confidence interval: 0.6932764–1.483659, $P < 0.0001$). BM, bone marrow; PB, peripheral blood.

calculated from standard curves and end point determination from limiting dilution series of JAK2 wild-type donor DNA and the homozygous JAK2 cell line HEL. All qPCRs were performed in triplicates.

The 11 patients had a median JAK2 V617F% of 54 and 53 in PB and BM, respectively (range: PB, 21–92%; BM, 30–85%). The highest JAK2 V617F percentage was recorded in the patient with PMF, which is in line with previous data (unpublished). A highly significant correlation was found between levels of JAK2 V617F percentages in PB and BM, (Spearman's $r=0.76$; $P=0.007$) (Figure 1). Furthermore, in the seven patients from whom both a BM biopsy and a representative BM aspirate were available, the JAK2 V617F proportion determined in the two different BM samples was equivalent (Spearman's $r=0.96$; $P=0.0005$). The amount of extracted DNA, reflected by the cycle threshold (C_t) value, was higher in the BM aspirates compared to the BM biopsies (data not shown). This finding might be explained by degradation of DNA caused by the formic acid decalcification procedure. It is evident that some patients, in particular with ET, have rather small JAK2 V617F allele burdens below 10%.[4,7] None of the patients in the present study had such low levels of JAK2 V617F alleles. However, it is interesting that patient no. 2 with 21% JAK2 V617F alleles in PB is the only patient in this series with a significantly larger JAK2 mutant burden in BM. This may be an indication that patients with low levels of detectable JAK2 V617F allele burden in PB may have harbored a significantly larger mutant burden in BM. Future studies of 'low-burden' JAK2 V617F-positive patients could elucidate a theoretical model of increasing propensity of the mutant cells to egress from the BM as the mutant burden in BM increases, as it is observed with CD34-positive cells in IMF. In this study, we have demonstrated that the levels of the JAK2 V617F alleles in PB and BM is equivalent, at least in patients with a mutant clone in PB accounting for more than 30%. The quantification of the JAK2 V617F mutant clone in PB unfractionized WBCs is an exact and reliable determinant of disease burden at diagnosis. As the clonal involvement of various mature hematopoietic cells is very heterogenous and varies between patients, and a separation of, for example, granulocytes is sometimes difficult because of *in vivo* and *in vitro* aggregation of monocytes, granulocytes and platelets,[8] the use of unfractionized WBC as DNA source seems rational. Hereby the time-to-time uncertainty and potential bias from an unsuccessful separation procedure are eliminated. A prospective trial elucidating the dynamics of the JAK2 V617F clone with serial measurements of the JAK2 V617F allele burden in both PB and BM during ongoing cytoreductive therapy with, for example, hydroxyurea, interferon-α or a future JAK2 inhibitor is warranted.

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7.4 Paper III summary

Reference: ***Larsen TS, Pallisgaard N, Møller MB, Hasselbalch HC.*** *The JAK2 V617F allele burden in essential thrombocythemia, polycythemia vera and primary myelofibrosis – Impact on disease phenotype.* ***Eur J Haematol 2007;79:508-515***

Objectives: The JAK2 V617F mutation is present in virtually all patients with PV, but also in approximately half of the patients with ET and PMF. Accordingly, one mutation, sufficient to cause a PV-like disease in animal models, is associated with three different phenotypes in humans. A gene dosage model has been proposed and speculations on whether the three distinct phenotypes represent different stages of the same JAK2 V617F mutated disease are intriguing. This model implies that the JAK2 V617F mutant allele burden increases from ET over PV to PMF. In line with this hypothesis, we wanted to do a large scale assessment of the JAK2 V617F allele burden by qPCR in our Ph-neg CMPD cohort and to evaluate correlations with a variety of clinical parameters.

Results: This study provided solid evidence of an increasing JAK2 V617F allele burden from ET over PV to PMF, supporting the hypothesis that the three JAK2 V617F positive disorders (ET, PV and PMF) represent three different phenotypic presentations of a JAK2 V617F positive chronic myeloproliferative disorder. Moreover, the JAK2 V617F allele burden is a key determinant of the degree of myeloproliferation and myeloid metaplasia. However, there is a large variation in the JAK2 V617F allele burden in patients with PV ranging from one to nearly 100 %. In general, independent of diagnosis, males seem to have larger JAK2 V617F allele burdens compared to females. No correlation was found between previous thrombosis and JAK2 V617F allele burden (homozygosity) in PV.

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ORIGINAL ARTICLE

The JAK2 V617F allele burden in essential thrombocythemia, polycythemia vera and primary myelofibrosis – impact on disease phenotype

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Abstract

Background and objectives: The JAK2 V617F tyrosine kinase mutation is present in the great majority of patients with polycythemia vera (PV), and approximately half of the patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF). The three distinct disease entities may be considered as three phenotypic presentations of the same JAK2 V617F positive chronic myleoproliferative disorder. Together with physiological and genetic modifiers the phenotype may be determined by the JAK2 V617F allele burden. In the present study, we aimed to asses the JAK2 mutational load and its impact on phenotype. *Methods:* A highly sensitive real-time quantitative PCR (qPCR) assay was used for quantification of the JAK2 V617F mutational load in 165 patients with Philadelphia chromosome negative chronic myeloproliferative disorders (ET = 40, PV = 95, PMF = 30). *Results:* We provide evidence of increasing JAK2 V617F allele burden from ET, over PV to PMF ($P = 0.001$ and $P < 0.00001$ respectively). The present data suggests the JAK2 V617F allele burden as a key determinant of the degree of myeloproliferation and myeloid metaplasia reflected by significantly higher levels of white blood cell counts (WBC) ($P = 0.03$), CD34 counts ($P = 0.03$), lactate dehydrogenase and Polycythemia Rubra Vera gene 1 levels ($P = 0.03$ and $P < 0.00001$ respectively), as well as lower platelet counts ($P = 0.02$) and more cases of splenomegaly ($P = 0.001$) in homozygous PV patients compared to their heterozygous counterparts. *Conclusion:* The present study support the concept of the JAK2 V617F positive chronic myeloproliferative disorders as a biological continuum with phenotypic presentation in part influenced by JAK2 V617F mutational load.

Key words essential trombocythemia; polycythemia vera; primary myelofibrosis; JAK2; quantitative PCR

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The V617F mutation of the *Janus Kinase 2* gene (*JAK2*) is present in haematopoietic cells in the vast majority of patients with polycythemia vera (PV). With the most sensitive methods for mutation detection the JAK2 V617F mutation is detected in more than 97% of patients with PV (1). The mutant JAK2 tyrosine kinase is constitutively active and confers tyrosine kinase growth factor independent proliferation of *in vitro* cultured cells (2) – a phenomenon recognised decades ago and known as erythropoietin independent colony (EEC) growth (3). *In vivo* studies have demonstrated that mutant *JAK2* transfected into animal models is sufficient to develop a chronic myeloproliferative disorder mimicking PV which tends to terminate in myelofibrosis (2, 4, 5). Other mutations upstream in exon 12 of the *JAK2* gene have recently been identified, probably accounting for a significant proportion of the JAK2 V617F negative patients with a PV phenotype (6).

In essential thrombocythemia (ET) and primary myelofibrosis (PMF) the JAK2 V617F mutation can be identified in approximately 50% of the patients (1, 2, 7, 8). A growing body of evidence suggests a phenotypic relationship of JAK2 V617F positive ET and PV. Thus, ET patients harbouring the JAK2 V617F mutation have higher levels of haemoglobin (9–12), higher white cell and neutrophil counts, lower platelet counts, lower mean

red cell volume and lower plasma ferritin and plasma erythropoietin levels (9). Although data on thromboembolic risk to some extent are diverging, most studies have shown an increased risk of thrombosis in JAK2 V617F positive ET patients (9, 12–15). Information on the impact of the JAK2 V617F mutation on clinical phenotype in PMF is limited. Although data on prognosis and clinical parameters such as transfusion requirements are conflicting there are indications of phenotypic differences of JAK2 mutated vs. wildtype PMF patient (16, 17).

Homozygosity for the JAK2 V617F is a result of mitotic recombination (7) and it is rarely observed in peripheral blood cells from patients with ET, whereas about one-third of patients with PV and PMF are reported to be homozygous (1, 2, 7, 8). Increasing JAK2 V617F allele burden is associated with increased expression of downstream target genes (18–20), and granulocyte activation (21). An impact on several clinical parameters such as haemoglobin concentration, WBC counts, platelet counts, spleen size, pruritus and thrombosis have been demonstrated in patients with ET and PV (15, 19, 22). Accordingly the JAK2 V617F allele burden and the decrease in the number of wildtype alleles are likely to have a major impact on disease phenotype. Whereas evidence of higher JAK2 V617F allele burden in PV than ET have been reported in a few studies (15, 21–23), data on allelic burden in PMF remains to be validated.

In this study, we have performed an exact quantitative real time PCR (qPCR) determination of the JAK2 V617F allele burden in a cohort of patients with ET, PV and PMF diagnosed according to the WHO criteria (24) and followed in a single institution to investigate clinical correlates and clarify the impact of the JAK2 V617F allele burden on disease phenotype.

Patients and methods

In the period from March 2004 to November 2006, 20 mL of ethylenediaminetetraacetic acid anti-coagulated blood was collected from 165 patients fulfilling the WHO criteria of a Philadelphia chromosome negative chronic myeloproliferative disorder (CMPD) (ET = 40, PV = 95, PMF = 30). The patients were diagnosed and followed in a single institution. Seventy-eight of the patients had their JAK2 V617F allele proportion determined at diagnosis and before administration of any cytoreductive therapy (ET = 18, PV = 42, PMF = 18), whereas 87 patients (ET = 22, PV = 53, PMF = 12) with a mean disease duration of 64 months (ET = 59, PV = 70, PMF = 48) range (ET: 2–240; PV: 2–379; PMF: 4–132) were diagnosed and treated prior to the sampling for mutation analysis. All samples were collected after informed consent according to the Helsinki Declaration and the guidelines of the Danish Regional Science Ethics Committee. After red cell lysis with ammonium chloride lysis buffer, DNA was extracted from un-fractionized leucocytes using a MagnaPure Robot (Roche Diagnostics, Mannheim, Germany) according to the manufacturers' protocol. The JAK2 V617F mutation status was determined by the three-primer allele specific PCR assay described by Baxter and colleagues (1), and the proportion of JAK2 V617F mutated alleles was determined by a highly sensitive (0.1%) (qPCR) assay developed by our own group and described in detail elsewhere (25). Briefly, two real-time qPCR reactions were performed in parallel with a common forward primer and Taqman probe and only differing in the use of a reverse primer specific for the JAK2 wildtype and the V617F mutated DNA respectively. The JAK2 V617F proportion was calculated from standard curves and end point determination from limiting dilution series of JAK2 wildtype donor DNA and the homozygous JAK2 cell line HEL. Patients were interpreted as homozygous for the JAK2 V617F mutation if their mutational load exceeded 50%. All qPCR reactions were performed in triplicates. *Polycythemia Vera Rubra gene 1* (*PRV1*) qRT-PCR was performed using standard conditions on a ABI 7900HT system (Applied Biosystems, Foster City, CA, USA) and a forward primer: 5′-CAGGTTGCAACCTGCTCAAT-3′, a reverse primer: 5′-GCCAAGTTTCCGTGTGTCATAAT-3′ and a probe: 5′-Fam-TTGCAGTTCTCAGTCATACCCACGGGC-Tamra-3′. This primer/probe set was cDNA specific as it showed no amplification of 100 ng genomic DNA per well in control experiments. As internal reference the housekeeping genes *beta-glucuronidase (GUS)* and *Abelson* (*ABL*) were used for normalization (26, 27). The normalized *PRV1* expression was calculated relative to the normalized expression in healthy donors, in whom the level was set to 1 (95% CI: 0.3–7, $n = 38$). All qPCR reactions were performed in triplicates. The chi-square and Fisher's exact test was used for comparing categorical variables, whereas the Wilcoxon rank sum test was used for continuous variables. Correlation coefficients were calculated using Spearman rank correlation. All statistical calculations were performed using the STATA© Statistics/Data analysis 9.0 (College Station, TX, USA) software.

Results

Polycythemia vera

The vast majority (94/95) corresponding to 99% of patients with PV were JAK2 V617F positive. Of the 95 patients with PV 90 were examined with qPCR. The median JAK2 V617F proportion was 23% (95% CI: 17–34%), range (1–92%), (Fig. 1 upper left). Twelve patients

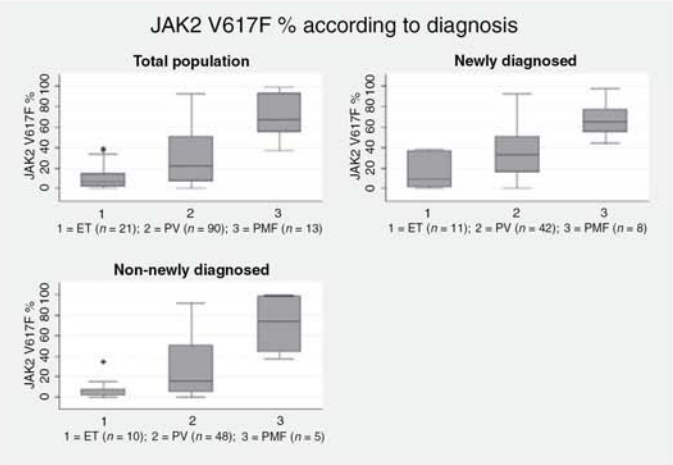

Figure 1 Box-plots showing the JAK2 V617F allele percentage in essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF) for (upper left) the total JAK2 V617F positive population analysed with qPCR (n = 124). The difference in JAK2 V617F allele burden was highly significant between the disease entities (ET vs. PV P = 0.001 and PV vs. PMF P < 0.00001); Upper right: newly diagnosed patients (n = 61) (ET vs. PV P = 0.02 and PV vs. PMF P = 0.0008). Lower left: non-newly diagnosed patients (n = 63).

with JAK2 V617F positive PV had a mutated allele burden of less than 3%, median 1%, range (0.1–2.0%). Eight of these 12 patients were previously examined with the Baxter assay (1) and interpreted JAK2 V617F negative, whereas three patients were positive by both assays (all had 2% mutated alleles), and one patient was only examined by qPCR (1% mutated alleles). By using the highly sensitive qPCR method, the proportion of JAK2 V617F mutated PV patients increased from 91.5% to 99%. When the analysis was restricted to newly diagnosed patients, not treated with cytoreductive drugs (n = 42), the median proportion of mutated alleles was higher: 33% (95% CI: 20–40%), range (1–92%) (Fig. 1 upper right) compared with 17% (95% CI: 8–29), range (1–92) (Fig. 1 lower left), although not reaching significance (P = 0.09). In total, 23 of 90 (26%) patients had 50% or more JAK2 V617F mutated alleles and thus categorised as homozygous. In the group of newly diagnosed patients 11 of 42 (26%) were categorised as homozygous, and likewise in the group of patients with longer disease duration 12 of 48 patients (25%) were homozygous. Spleen size was recorded in 86 of the 90 patients with a quantified JAK2 V617F allele proportion. Seventeen patients (20%) had splenomegaly at the time of diagnosis. These 17 patients had significantly higher median JAK2 V617F allele proportion of 65% (95% CI: 14–84%), range (1–90%) when compared with a median of 20% (95% CI: 12–30%), range (1–92%) in patients without splenomegaly (P = 0.006). This finding was sustained when the analysis was restricted to newly diagnosed patients in whom nine cases of splenomegaly were recorded in a total of 41 [median JAK2 V617F %: 60, (95% CI: 8–87%), range (1–90%), vs. 30% (95% CI: 20–34%), range (0.1–54%), (P = 0.05)]. There was a significant correlation between JAK2 V617F % and the expression of the *PRV1* (Spearman's correlation coefficient = 0.57 and 0.53 in newly diagnosed patients and the total PV population, respectively (P = 0.0001) (Fig. 2). When data were analysed in regard to whether the patients were heterozygous or homozygous for the JAK2 V617F mutation significant more cases of splenomegaly (P = 0.001), lower platelet (P = 0.02), higher leucocyte (P = 0.03), and CD34 (P = 0.03) counts were recorded in the homozygous patients. The LDH and *PRV1* levels were also significantly higher in the homozygous patients (P = 0.03 and P < 0.00001 respectively). No significant differences were observed in regard to age, gender, disease duration, cytoreductive therapy or thrombosis between the hetero- and homozygous PV patients (Table 1). When restricted to newly diagnosed patients a similar pattern was recorded, although the lower platelet count in the homozygous patients did not reach statistical significance. Interestingly, lower levels of the haematocrit (P = 0.05) and a trend towards lower haemoglobin was recorded in the homozygous patients (P = 0.08) (Table 1).

Essential thrombocythemia

Twenty-one of 40 patients (53%) were JAK2 V617F positive by qPCR, and a female preponderance (male/females: 6/15) was recorded. All the patients with JAK2 V617F positive ET had their JAK2 V617F allele burden quantified. The median proportion of JAK2 V617F mutated alleles was 7% (95% CI: 2–15%), range (1–39%), (Fig. 1 upper left). Of the 21 patients, nine had a low median JAK2 V617F allele proportion of 0.7%, range (0.1–3.0) and were initially considered JAK2

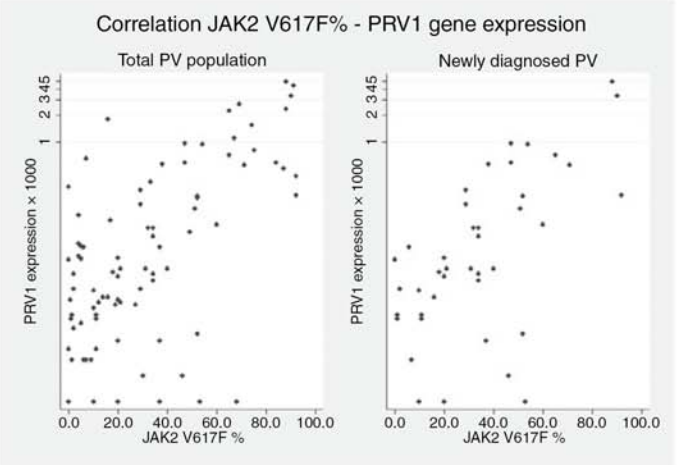

Figure 2 Scatter plots showing the correlation between the JAK2 V617F allele percentages and the over-expression of *PRV1* for the total polycythemia vera (PV) population (n = 90) analysed with qPCR (left panel), Spearman correlation coefficient r = 0.53 (P = 0.0001). Right panel: newly diagnosed PV patients (n = 42). Spearman correlation coefficient = 0.57 (P = 0.0001).

Table 1 Clinical and biochemical parameters from PV patients and their association to the JAK2 V617F hetero- vs. homozygous disease state. Univariate *P*-values are displayed. Significant *P*-values are shown in bold.

JAK2	WT (total)	V617F (total)	Hetero (qPCR)	Homo (qPCR)	*P*-value
PV (total) (*n*)	1	94	67	23	
Gender (m/f)	1/0	45/50	29/38	14/9	0.11
Age (yr)	53	64 (23–89)	63 (23–87)	67 (43–89)	0.38
Disease duration (months)	59	39 (1–379)	37 (1–204)	26 (1–132)	0.56
Splenomegaly (+/–)	0/1	17/72	7/58	10/11	**0.001**
Haemoglobin (g/dL)	15.5	16.8 (15.8–18.1)	16.8 (15.8–18.1)	16.6 (14.0–20.3)	0.86
Platelets ($\times10^9$/L)	575	682 (597–774)	743 (638–840)	492 (347–748)	**0.02**
WBC ($\times10^9$/L)	6.3	11.9 (11.2–13.9)	11.4 (11.0–12.8)	14.6 (11.4–19.8)	**0.03**
LDH (U/L)	405	353 (281–431)	304 (243–400)	480 (342–564)	**0.03**
HCT (%)	0.46	53 (50–55)	52 (50–55)	52 (44–63)	0.79
PRV1 (fold upreg.)	2	34 (20–73)	20 (14–34)	576 (238–1417)	**<0.00001**
CD34 ($\times10^6$/L)	2	3 (2–4)	3(1–3)	6 (2–23)	**0.03**
Cytoreductive therapy (+/–)	1/0	47/43	34/33	13/10	0.41
Thrombosis (+/–)	0/1	40/52	28/39	10/11	0.41
PV (newly diagnosed) (*n*)	0	42	21	11	
Gender (m/f)	(–)	18/24	12/9	6/5	0.36
Age (yr)[1]	(–)	70 (62–74)	64 (28–87)	72 (56–89)	0.13
Splenomegaly (+/–)	(–)	9/32	4/27	5/5	**0.03**
Haemoglobin (g/dL)[2]	(–)	16.0 (15.0–16.8)	16.0 (15.2–18.2)	15.0 (12.6–16.6)	0.08
Platelets ($\times10^9$/L)[2]	(–)	809 (593–875)	812 (588–942)	748 (464–1347)	0.80
WBC ($\times10^9$/L)[2]	(–)	11.8 (11.2–14.0)	11.3 (10.1–12.1)	18.4 (12.8–22.4)	**0.007**
LDH (U/L)[2]	(–)	226 (192–262)	197 (186–240)	345 (237–537)	**0.001**
HCT (%)[2]	(–)	50 (47–54)	51 (47–57)	47 (40–53)	**0.05**
PRV1 (fold upreg.)[2]	(–)	34 (24–103)	31 (18–44)	240 (80–1632)	**0.01**
CD34 ($\times10^6$/L)[2]	(–)	3 (0–7)	3 (0–7)	6 (0–28)	0.59
Thrombosis (+/–)	(–)	15/26	11/20	4/6	0.54

PV, polycythemia vera; WBC, white blood cell counts; LDH, lactate dehydrogenase; HCT, haematocrit; PRV1, Polycythemia Rubra Vera gene 1.
[1]Variable displayed in mean with range in parenthesis.
[2]Variable displayed in median with 95% confidence intervals in parenthesis.

negative when using the conventional three-primer allele-specific non-quantitative PCR with a sensitivity of approximately 2–3% (1). Accordingly the proportion of JAK2 V617F positive patients as assessed by qPCR increased from 32.5% to 52.5%. In newly diagnosed JAK2 positive ET patients (n = 11) the median proportion of mutated alleles was not significantly higher [9%, (95% CI: 1–37), range (1–39) (Fig. 1 upper right) vs. 2.5%, (95% CI: 0.9–37), range (0.1–37), (P = 0.20)] (Fig. 1 lower left). None of the 21 patients were homozygous.

Primary myelofibrosis

Thirteen of 30 (43%) patients with PMF were JAK2 V617F positive by qPCR. Eight of 13 patients were males. The median proportion of JAK2 V617F mutated alleles was 67%, (95% CI: 52–95%, range 37–99%), (Fig. 1 upper left). Of the 13 patients, eight were newly diagnosed. In this group, the median proportion of JAK2 V617F mutated alleles was 66% (95% CI: 51–86%), range (44–97) (Fig. 1 upper right). The vast majority were homozygous for the JAK2 V617F mutation (11/13 = 85%). No significant differences in any of the clinical parameters was recorded between JAK2 V617F positive and negative patients, although there was a trend towards higher WBC counts (P = 0.12) in the JAK2 positive patients.

Total cohort

The difference in JAK2 V617F allele burden was highly significant between the disease entities (ET vs. PV P = 0.001 and PV vs. PMF P < 0.00001). When the analysis was restricted to newly diagnosed patients only, this significant difference was still apparent (ET vs. PV P = 0.02 and likewise PV vs. PMF P = 0.0008). Box-plots of JAK2 V617F allele burden in the total cohort of patients and in the subgroup of newly and previously diagnosed patients are shown in Fig. 1. In the total cohort of JAK2 V617F positive patients, regardless of diagnosis, males had a significantly higher proportion of mutated alleles. The 57 males had a median JAK2 V617F % of 31, (95% CI: 20–49%), range (1–99), whereas the corresponding figures in females were median 17% (95% CI: 10–33%), range (1–97%) (P = 0.04). In the newly diagnosed JAK2 V617F positive patients, regardless of diagnosis, a highly significant correlation was recorded between the JAK2 V617F % and LDH levels r = 0.74 (P < 0.00001) (Fig. 3).

Discussion

The identification of the JAK2 V617F mutation has unravelled several important clinical and biochemical

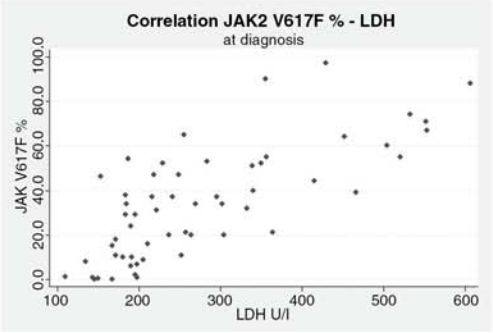

Figure 3 Scatter plot showing the correlation between the JAK2 V617F allele percentages and lactate dehydrogenase (LDH) in newly diagnosed patients (n = 61). Spearman Correlation coefficient r = 0.74 (P < 0.00001).

correlates which have great impact upon the future classification of the Philadelphia negative chronic myeloproliferative disorders. In regard to ET, a series of studies have demonstrated differences between JAK2 V617F mutated and wild type ET indicating that the two entities most likely are distinct disorders (9). The same may be true for PMF, although concise data is lacking. Considering JAK2 V617F positive disease, a gene dosage model, in which the JAK2 V617F mutation burden is a key determinant of phenotypic presentation, has been proposed (28).

Most previous studies have dealt with the presence or absence of the JAK2 V617F mutation and clinical correlates, whereas a few studies have addressed phenotypic characteristics related to the JAK2 V617F mutational load, in particular the homozygous disease state (15, 29). The results are ambiguous. Homozygosity seems to be related to an increased risk of transformation into myelofibrosis, elevated haemoglobin levels (15, 29) and systemic symptoms whereas the data on WBC counts, splenomegaly as well as thrombotic risk are conflicting.

Being homozygous for the JAK2 V617F mutation, it is important to underscore that some patients may harbour wildtype, heterozygous as well as homozygous cells in a mixture with a total of JAK2 V617F mutated alleles below 50% (30). In the present single institution study, we have done an exact quantification of the percentage of JAK2 V617F mutated alleles vs. wildtype alleles, which allows a precise interpretation of the impact on the JAK2 V617F allele burden on clinical phenotype. A highly significant difference in the JAK2 V617F allele burden between patients with ET, PV and PMF was found, the highest levels being recorded in PMF. The increase in JAK2 V617F allele burden was reflected – not only in the proliferation marker LDH and the WBC

as well as $CD34^+$ count – but also in the increasingly expressed downstream target gene *PRV1*. Accordingly these data add further evidence to the model of a biological continuum of the JAK2 V617F mutated disorders.

It is quite intriguing that males seem to have larger JAK2 V617F allele burdens than females. It has previously been suggested that sex might influence the phenotypic presentation of JAK2 V617F positive disease as PV is more frequent in men (31) and JAK2 V617F positive ET is more common in women (9). In this series, we also recorded more JAK2 V617F positive women with ET, whereas the homozygous disease state in PV and PMF were more common in males, although not reaching statistical significance. Moreover, a trend towards higher JAK2 V617F allele burden was found at diagnosis in ET and PV compared to patients with the same diagnosis but longer disease duration. This is in contrast to previously reported larger mutational load in PV patients with a disease duration of more than 5 yr (22). It may reflect the suppressive effect of cytoreductive therapy on the JAK2 V617F clone. Although numbers are too small to draw firm conclusions, it is interesting that the reverse phenomenon of lower mutational load at diagnosis was recorded in the PMF patients.

The large majority of patients with JAK2 V617F positive ET are heterozygous, which was confirmed in the present study. Some of the few homozygous ET patients previously reported (1, 2, 7, 8) may have been misclassified PV patients, at least in one study, in which some of these 'ET' patients needed phlebotomy (15). The present data demonstrated a significant increase in the proportion of JAK2 V617F positive ET patients from 32.5% to 52.5% when using the qPCR assay, underlining a potential risk of bias in data based on less sensitive mutation detection methods. Clinical data (unpublished) on the JAK2 V617F positive ET patients indicate in line with previously published data (9, 12–15) a phenotype resembling PV. In contrast, the vast majority (85%) of patients with JAK2 V617F positive PMF were homozygous, which is a considerably larger proportion than previously published (1, 2, 7, 8). In the PV population, regardless of disease duration, the proportion of JAK2 V617F mutated alleles varied from almost undetectable levels to more than 90%, equal to those recorded in PMF. The very sensitive qPCR assay allowed us to determine down to 0.1% mutated alleles, which resulted in the highest percentage (99%) of JAK2 V617F mutated PV patients ever recorded. The reason for the very pronounced variation in JAK2 V617F allele burden remains to be elucidated. Although the categorisation of patients as homozygous for the JAK2 V617F mutation is based on a, at least partly, arbitrary limit of 50% mutated alleles, this cut off value seems to separate the patients in two clinically relevant subgroups. The homozygous PV patients which accounted for 26% of the total – a similar proportion as published by others (1, 2, 7, 8, 15), displayed overall higher WBC counts, LDH and *PRV1* levels as well as higher CD34 counts and more patients had enlargement of the spleen, whereas the platelet counts as previously reported were significantly lower (15, 19, 32). However, the haemoglobin concentration and the haematocrit were actually lower in the newly diagnosed homozygous PV patients. This observation has not been reported previously, but indeed supports the model of a biological continuum. As the JAK2 V617F allele burden increases the phenotype becomes more proliferative with increasing WBC counts, LDH levels, splenomegaly and consequently a decrease in haemoglobin levels – a phenotype resembling a transitional myeloproliferative disorder between PV and postpolycythemic myelofibrosis. This model implies that JAK2 V617F positive PMF patients with very high JAK2 V617F allele burden (median 67%) might have lived several years with a PV-like subclinical, and accordingly undiagnosed phase without overt symptoms before entering the advanced 'burn out' phase of the disease with myelofibrosis and myeloid metaplasia. This stage of JAK2 positive PMF in which a large proportion of patients are diagnosed resembles JAK2 negative PMF, thus explaining the lack of consistent data on phenotypic differences between JAK2 positive PMF patients and their JAK2 negative counterparts in this and previously published larger series (16, 17).

In conclusion, the present single institution study on a well-defined cohort adds further solid support to the concept that the JAK2 V617F positive myeloproliferative disorders – ET, PV and PMF – may reflect different phenotypes of the same disorder. In addition to yet unknown genetic and physiologic modifiers such as gender, this phenotype is determined by the JAK2 V617F allelic burden. The homozygous disease state reflect a more proliferative phenotype of PV with increasing proliferation and myeloid metaplasia, which in some cases may resemble transitional PV developing into manifest postpolycythemic myelofibrosis.

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7.5 Paper IV summary

Reference: ***Larsen TS, Hasselbalch HC, Pallisgaard N, Møller MB****. Bone marrow histomorphology and JAK2 mutation status in essential thrombocythemia.* ***Acta Pathologica, Microbiologica et Immunologica Scandinavica (APMIS) 2007;115:1267-1273.***

Objectives: An increasing amount of data suggests that JAK2 V617F negative and positive ET are two different clonal myeloproliferative disorders. The JAK2 V617F positive ET patients have several features resembling PV, supporting the model of a biological continuum of the JAK2 V617F positive CMPD. Previous data have demonstrated the presence of more pronounced bone marrow erythroid and granulocyte hyperplasia in JAK2 V617F positive ET. Data from the pre JAK2 era have shown that patients appearing to have ET can be subdivided into "true ET" and early prefibrotic myelofibrosis. This concept entered the WHO criteria from 2001. The basis for identifying prefibrotic myelofibrosis is a careful evaluation of several distinct bone marrow parameters with special emphasis on megakaryocyte morphology. To evaluate how the new molecular classification of ET fits the morphological WHO criteria, we did a re-evaluation of bone marrow morphology on 38 bone marrow biopsies, using a semi-quantitative scoring system, and looked for any correlations between revised diagnosis, individual bone marrow parameters and the JAK2 V617F mutation status.

Results: Based on bone marrow cellularity and megakaryocyte morphology a clear distinction between ET and prefibrotic myelofibrosis was possible. However, no correlation between revised diagnosis, or the individual bone marrow parameters and the JAK2 V617F status could be identified. Regardless of diagnosis ("true"ET or prefibrotic myelofibrosis) the JAK2 V617F mutation was associated with higher hemoglobin and lower platelet levels, which are features resembling PV. However, the molecular classification does not seem to fit into the concept of ET and prefibrotic myelofibrosis as two distinct disorders.

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Bone marrow histomorphology and JAK2 mutation status in essential thrombocythemia

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Larsen TS, Hasselbalch HC, Pallisgaard N, Møller MB. Bone marrow histomorphology and JAK2 mutation status in essential thrombocythemia. APMIS 2007;115:1267–1273.

A retrospective study of 38 essential thrombocythemia (ET) patients was conducted, reviewing bone marrow biopsies according to WHO criteria using a semiquantitative scoring system. Four patients did not fulfil the WHO criteria for a myeloproliferative disorder and one biopsy was insufficient for evaluation. 14 patients were reclassified as having prefibrotic idiopathic myelofibrosis (IMF), whilst the ET diagnosis was sustained in 19 patients. The individual bone marrow parameters of the reviewed diagnosis showed no correlation with JAK2 V617F mutation status, which was determined by a highly sensitive quantitative real-time PCR (qPCR) method. However, we could confirm previous findings of higher haemoglobin and lower platelet levels in the JAK2 V617F positive patients. Thus, the well-established phenotypic relationship of JAK2-positive ET and PV at the biochemical and molecular level was not recorded as regards bone marrow morphology according to the WHO criteria. Accordingly, the WHO concept of two distinct entities, ET and prefibrotic IMF, does not seem to fit the model of JAK2-positive ET as part of a biological continuum of JAK2 V617F-positive chronic myeloproliferative disorders.

Key words: Essential thrombocythemia; idiopathic myelofibrosis; prefibrotic; JAK2; qPCR.

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The classic Philadelphia chromosome-negative chronic myeloproliferative disorders (Ph⁻ CMPDs) comprise essential thrombocythemia (ET), polycythemia vera (PV) and idiopathic myelofibrosis (IMF). Our knowledge of a phenotypic relationship regarding many clinical, morphological and biological features within this group of disorders has been well recognized for decades. With the recent identification of the V617F mutation in the tyrosine kinase *Janus Kinase 2* (JAK2), important new insights into the pathogenesis at the molecular level have been achieved. The JAK2 V617F mutation results in a constitutively active tyrosine kinase, which confers growth factor-independent cell proliferation (1–4). Mutant *JAK2* transfected into animal models is sufficient to develop a chronic myeloproliferative disorder which mimicks PV and may terminate in myelofibrosis (1, 5, 6). The JAK2 V617F mutation can be detected in almost all patients with PV and approximately half the patients with ET and IMF (7). Accordingly, a large group of patients with ET and IMF have obvious biochemical and bone marrow changes, compatible with CMPD, but lack the JAK2 V617F mutation.

The introduction of the JAK2 V617F mutation allows an identification of molecularly defined subgroups of ET that might represent early or atypical phases of PV (8). Before 2001, the diagnosis of ET was based on the PVSG

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criteria, which do not allow a distinction between possible PV without yet significant erythrocytosis, early chronic idiopathic myelofibrosis (CIMF) without a leukoerythroblastic peripheral blood smear and what is often referred to as true ET (9). With the introduction of the WHO criteria the concept of prefibrotic IMF was introduced (10). This new concept describes a condition with bone marrow hypercellularity, neutrophilic proliferation, megakaryocytic proliferation and atypia, and minimal or absent reticulin fibrosis. Together with these bone marrow characteristics, IMF often but not always presents with a peripheral blood picture with slight anaemia, mild-to-moderate leukocytosis and moderate-to-marked thrombocytosis as the most prominent feature. Prefibrotic IMF should be interpreted as a prephase (cellular phase) of the classic IMF with marked fibrosis ranging from pronounced reticulin fibrosis to extensive collagen fibrosis with or without osteosclerosis, together with prominent megakaryocytic proliferation, leukoerythroblastic blood smear, and splenomegaly. The concept of prefibrotic IMF and fibrotic IMF as two stages of a biological continuum seems reasonable in a biological perspective (11). The key to identification of the prefibrotic IMF is the abnormal, atypical megakaryocytic morphology and topography typically in a hypercellular bone marrow, compared to a more restricted proliferation of mature enlarged megakaryocytes in ET (12). A number of retrospective studies have reviewed bone marrow histomorphological characteristics and clinical parameters of ET patients (13–15). These studies evaluated the WHO criteria in cohorts of ET patients and concluded that a significant proportion of ET patients fulfilled the revised PVSG criteria for prefibrotic IMF. Patients with prefibrotic IMF have been reported to have a significantly inferior prognosis compared with ET (13). Recently the incorporation of a semiquantitative scoring system of myelofibrosis (MF grade 1–3) has been proposed in order to evaluate the dynamics of the fibrotic process (16, 17). However, controversy continues to persist concerning the validity of the WHO histomorphological criteria. So far only a few studies of ET patients have dealt with the influence of the JAK2 V617F mutation on bone marrow morphology. The megakaryocyte morphology does not allow a distinction between JAK2 V617F-mutated and wild-type ET patients (18, 19), whereas a significantly more pronounced bone marrow erythro- and granulopoiesis in JAK2 V617F-mutated patients was reported from the MRC PT1 trial data, based on patients enrolled according to the PVSG criteria (19).

In order to analyse the relationship between the JAK2 V617F mutation and bone marrow morphology in patients with ET and prefibrotic IMF according to the WHO classification we conducted a retrospective study on bone marrow biopsies collected at diagnosis from 38 patients and correlated the findings with the JAK2 V617F status as assessed by a highly sensitive real-time quantitative PCR assay (qPCR) (20).

MATERIALS AND METHODS

Bone marrow biopsies from 38 patients diagnosed with ET in the period 1984–2006 were reviewed by an expert haematopathologist, who was blinded to the JAK2 mutation status and peripheral blood biochemical data relating to the bone marrow trephines. All bone marrow biopsies were collected on admission preceding any cytoreductive therapy. The ET diagnosis was based on the PVSG criteria for patients diagnosed before 2001 (n=11) and on the WHO criteria (n=27) for patients diagnosed from 2001 and onwards. Trephine biopsy materials and bone marrow aspirates were fixed in 4% neutral-buffered formaldehyde, decalcified in formic acid, and embedded in paraffin. Paraffin sections of 2 μm thickness were stained using H&E, Giemsa, silver stain for reticulin fibres and Perl's stain for haemosiderin. For immunohistochemical studies trephine biopsy sections were stained for CD34 (clone QBEND/10, Novocastra Laboratories, Newcastle Upon Tyne, UK), CD117 (polyclonal rabbit anti-human antibody, Dako, Glostrup, Denmark) and CD61 (clone 2f2, Novocastra). The EnVision™+ System (Dako) was used for visualisation. Appropriate control slides were stained in parallel with test slides. For quantification of blasts in trephine sections, a semiquantitative approach based on number of CD34 or CD117 expressing non-endothelial cells per 40× high-power field (hpf) was used: 0–5 positive cells/hpf was normal; 6–10 positive cells/hpf was a minimal increase; 11–20 positive cells/hpf was a moderate increase; >20 positive cells/hpf was an overt increase.

The bone marrow biopsies were reviewed on the basis of the semiquantitative scoring system developed by the Cologne group (21). This semiquantitative scoring system evaluates several parameters with regards to cellularity, megakaryopoiesis, granulopoiesis, erythropoiesis, fibres, macrophages, stroma and sinus-

oids. The different parameters were graded as minimal increase=(+1), moderate increase=(+2) and overt increase=(+3), and likewise minimal reduction=(−1), moderate reduction=(−2) and overt reduction=(−3). If the parameter is considered normal (or not present) it is scored=0. For statistical comparison the results were converted to a binary outcome as follows: +/−1 and 0 corresponding to minimal or absent increase/decrease versus +/−2 to 3, corresponding to moderate or overt increase/decrease. The clinical characteristics of the patients are depicted in Table 1. A two-sided Fisher's exact test was used for comparing variables with small expected frequencies.

The JAK2 V617F mutation analysis was performed on DNA extracted from peripheral blood unfractionated leukocytes by the allele-specific PCR method described by Baxter and co-workers (2) and verified with a more sensitive real-time quantitative PCR (qPCR) developed by our own group as described elsewhere (20).

RESULTS

Of the 38 bone marrow biopsies, 37 were sufficient for an evaluation. By reviewing the diagnosis according to the WHO criteria using the semiquantitative scoring system developed by the Cologne group, 4 of the 37 patients did not fulfil the bone marrow pathological criteria of an MPD. 14 patients (14/33=42%) were reclassified as having prefibrotic IMF, with fibrosis grade 0: n=4 and grade 1: n=10. In 19 (58%) cases the ET diagnosis was sustained (Table 1). The parameters that were significantly different between the ET and IMF group were bone marrow cellularity (p=0.001), dense clustering of megakaryocytes (p=0.002), megakaryocyte maturation defects (p=0.001), megakaryocyte bulky nuclei (p<0.0005), granulocyte quantity (p=0.001), sinusoidal proliferation (p=0.009) and intrasinusoidal haematopoiesis (p=0.02). Examples of bone marrow morphology findings are given in Fig. 1. All four patients not fulfilling the diagnosis of a MPD were JAK2 V617F negative. In the IMF group, eight patients were JAK2 positive (57%). In the ET group, 9 patients were JAK2 V617F positive (47%), whereas 10 patients were JAK2 V617F negative. Thus there was no difference in JAK2 V617F mutation frequency between ET and IMF patients. Interestingly, in the IMF patients the presence of the JAK2 mutation was correlated with higher haemoglobin concentration at diagnosis: median 14.7 g/dl (95% c.i: 13.4–15.8) versus 12.3 g/dl (11.3–15.0) (p=0.02). In the ET group, the same trend toward higher levels of haemoglobin was observed: JAK2 V617F positive: median 14.0 g/dl (95% c.i: 12.3–15.0) versus 12.6 (10.5–13.7) in the JAK2 V617F-negative group (p=0.08). Regardless of the revised WHO diagnosis the median haemoglobin concentration was significantly higher 14.7 g/dl (13.7–15.2) versus 12.6 g/dl (11.6–13.2) (p=0.002), and the platelet was significantly lower in the JAK2 V617F-positive patients 806 $\times 10^9$/l (646–961)) versus 1027$\times 10^9$/l (838–1363) (p=0.03)). This difference was confirmed in the ET group (p=0.04), but not present in the IMF group. The proportion of patients presenting with elevated levels of LDH was significantly higher among patients with JAK2 V617F-negative versus -positive ET (p=0.005). There was a trend towards a higher leukocyte count at diagnosis in the IMF group (10.6$\times 10^9$/l (9.2–12.5) versus

TABLE 1.
Clinical characteristics of patients

WHO diagnosis	ET			IMF			Total		
JAK2	V617F	wt	p-value	V617F	wt	p-value	V617F	wt	p-value
Number of patients	9	10		8	6		17	16	
Age (years)*	55 (44–83)	67 (32–84)	NS	67 (49–91)	58 (47–84)	NS	57 (44–91)	64 (32–84)	N.S
Gender (m/f)	0/9	4/6	0.05	4/4	2/4	NS	4/13	6/10	N.S
Disease duration (months)**	32 (1–240)	45 (1–206)	NS	15 (1–88)	34 (1–82)	NS	34 (1–240)	25 (1–206)	N.S
Hb (g/dl)***	14.0 (11.8–15.5)	12.6 (10.5–13.7)	0.08	14.7 (13.4–15.8)	12.3 (11.3–15.0)	0.02	14.7 (13.7–15.2)	12.6 (11.6–13.2)	0.002
Platelets (10E9/l)***	682 (629–954)	978 (749–1629)	0.04	945 (567–1254)	1170 (540–2009)	NS	806 (646–961)	1027 (838–1363)	0.03
Leuk (10E9/l)***	7.9 (6.3–12)	8.6 (7.3–10.0)	NS	10.7 (8.8–15.2)	10.4 (6.4–13.3)	NS	10.4 (7.1–11.3)	8.7 (7.4–10.6)	N.S
LDH (normal/elevated)	8/1	2/8	0.005	2/6	3/3	NS	10/7	5/11	N.S

* Variables displayed as median with range in parentheses; ** variables displayed as mean with range in parentheses; *** variables displayed in median with 95% confidence intervals in parentheses. Abbreviations: NS=Nonsignificant.

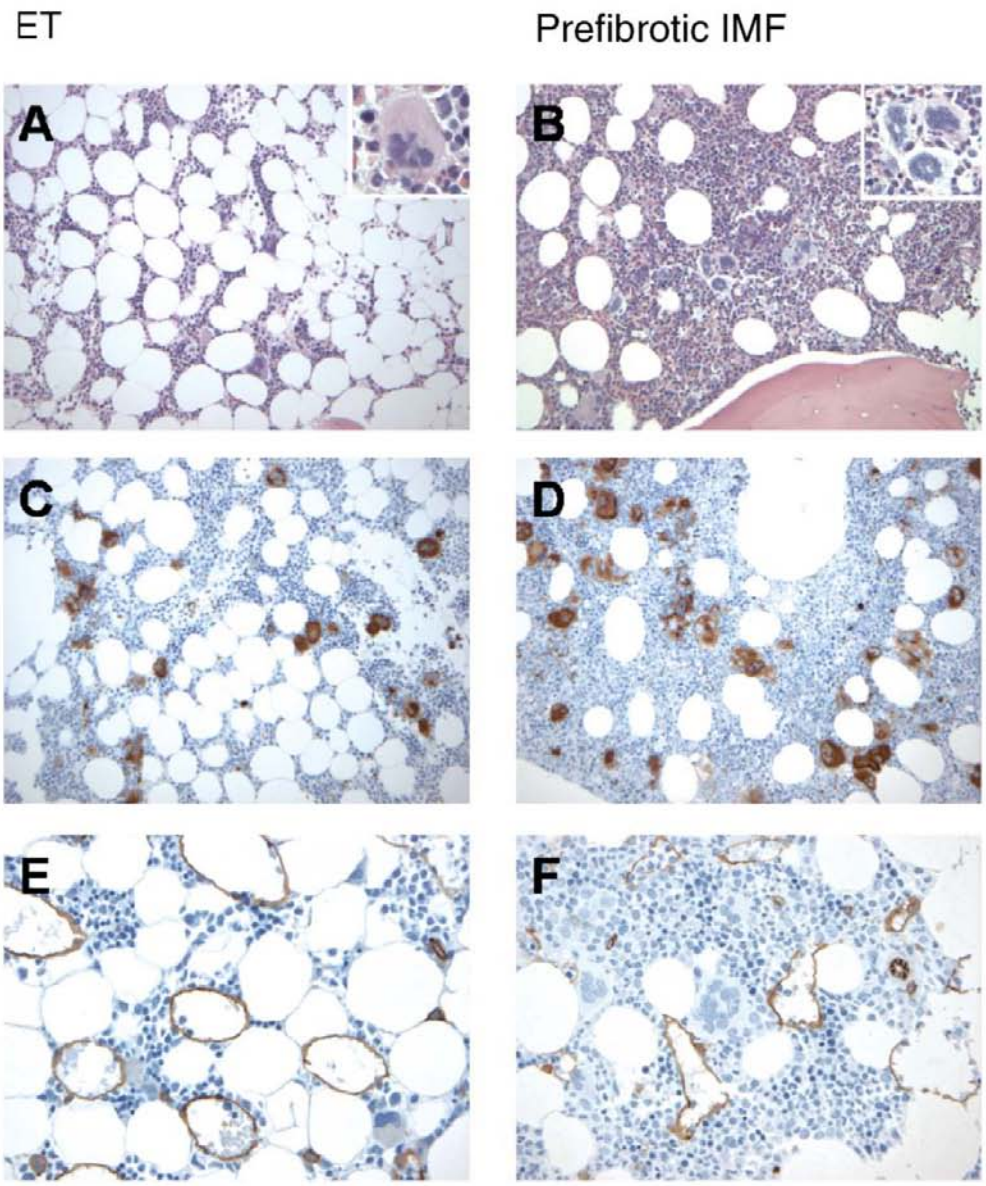

Fig. 1. Bone marrow biopsy sections from a case of essential thrombocythaemia (ET; A, C, E) and a case of prefibrotic chronic idiopathic myelofibrosis (IMF; B, D, F). H&E staining shows a normocellular bone marrow (A) with scattered enlarged megakaryocytes (insert) in ET, and in IMF the marrow is hypercellular (B) with clusters of abnormal, bulky megakaryocytes (insert). Staining for CD61 highlights the megakaryocytes (C, D). The endothelial lining of the sinusoids expresses CD34, but there is no increase in CD34-positive blasts (E, F).

8.6×10^9/l (7.2–10.5) (p=0.07)). No associations between leukocyte count at diagnosis and disease duration and JAK2 V617F mutation status within the two groups were observed. Interestingly, all the JAK2 V617F-positive patients with ET were women. The individual bone marrow parameters and their correlation with JAK2 V617F mutation status are listed in Table 2. No significant differences in the distribution of the semiquantitative gradings with regard to JAK2 V617F mutation status were observed.

DISCUSSION

Since the publication of the WHO classification of the Ph$^-$ CMPDs in 2001 the concept of prefibrotic IMF has challenged both haematologists and haematopathologists, and its existence has been questioned. Reviewing cohorts of ET patients originally diagnosed according to the PVSG criteria, several studies have shown that a significant proportion of ET patients are indeed reclassified as early IMF, ranging from

TABLE 2. *Semiquantitative evaluation of bone marrow histomorphological features*

Morphological parameters JAK2 wildtype/V617F	Decrease	Normal or slight increase +1	Moderate or marked increase +2–3	p-value
Cellularity	0/0	13/13	3/4	0.54
Megakaryopoiesis				
Quantity	0/0	0/2	16/15	0.26
Loose clusters	0/0	4/3	12/14	0.46
Dense clusters	0/0	11/13	5/4	0.48
Size (small)	0/0	13/14	3/3	0.64
Size (large to giant)	0/0	1/0	15/17	0.49
Maturation defects	0/0	8/13	8/4	0.11
Nuclear lobulation	0/0	7/8	9/9	0.56
Bulky nuclei (quantity)	0/0	9/14	7/3	0.11
Naked nuclei	0/0	10/14	6/3	0.19
Granulopoiesis				
Quantity	0/0	12/13	3/4	0.58
Left shift	0/0	14/17	1/0	0.47
Maturation defects	0/0	15/17	0/0	
Blasts CD34	0/0	15/16	0/1	0.53
CD117	0/0	15/17	0/0	
Eosinophils	0/0	15/17	0/0	
Erythropoiesis				
Quantity	0/0	14/15	1/2	0.55
Left shift	0/0	14/17	1/0	0.47
Maturation defects	0/0	15/16	0/1	0.53
Fibers				
Score	0/0	15/17	0/0	
Osteosclerosis	0/0	0/0	0/0	
Macrophages				
Iron deposits	5/10	5/5	0/0	0.34
Pseudo-Gaucher cells	0/0	14/17	1/0	0.47
Stroma				
Perivascular plasma cells	0/0	15/16	0/1	0.53
Cell debris	NE	NE	NE	NE
Lymphoid nodules	0/0	14/16	2/1	0.48
Sinusoids				
Proliferation	0/0	5/8	10/9	0.36
Dilatation	0/0	14/16	2/1	0.48
Intrasinusoidal hematopoiesis	0/0	13/16	3/1	0.28

It was not possible to evaluate some of the parameters in all the biopsies. Abbreviations: NE=Not evaluated.

prefibrotic IMF with grade 0 reticulin fibres to grade 2 with reticulin and collagen fibrosis according to the WHO criteria combined with the European consensus criteria for bone marrow fibrosis (13–15). The concept of prefibrotic IMF was introduced in the pre-JAK2 era and suggests that probably more than 50% of formerly classified ET should be classified as prefibrotic IMF with varying degrees (mostly grade 0–1) of fibrosis. How does this concept fit with the fact that approximately 50% of ET patients harbour the JAK2 V617F mutation and the indications of these patients as a forme fruste of PV (19)? These two new concepts are to some extent conflicting. Both statements could be biased by questionable validity of the initial diagnosis with a tendency to misclassification due to the insufficiency of the PVSG criteria with regard to the identification of early PV and early IMF.

In the present study we systematically reviewed 37 bone marrow biopsies from patients initially diagnosed as having ET. When using the semiquantitative scoring system developed by the Cologne group, about half of the cases were reclassified as early IMF with cellularity and abnormal megakaryocyte morphology and topography being the significant parameters of discrimination. In parallel, and with the haematopathologist blinded, we did the JAK2 V617F mutation analysis using a conventional qualitative assay and a highly sensitive qPCR assay. 17 out of 37 patients were JAK2 V617F positive. In all but one of the 37 patients, the JAK2 V617F allelic burden was determined by a highly sensitive qPCR method. In 9 out of the 17 patients, the JAK2 V617F allele burden was $\leq$=5%. Five of the patients had $\leq$=1 % mutated alleles. Most likely these patients would have been regarded as JAK2 negative if the mutation analysis had been performed by sequencing or allele-specific PCR, which are the methods that are used in most laboratories. However, the JAK2 V617F-positive patients were evenly distributed in the ET and IMF group: 8/14 and 9/19, respectively. When evaluating the individual bone marrow parameters, no correlation with the JAK2 V617F status could be identified. Taking into account that JAK2-positive ET patients show several distinct laboratory features resembling PV one could expect PV-like bone marrow changes as well. Thus, the finding of no significant correlation between important parameters of bone marrow histomorphology and the JAK2 V617F mutation was somewhat surprising, although in line with previously published data (18). Despite the limited number of patients we could nevertheless confirm a correlation between higher haemoglobin and lower platelet levels in the JAK2-positive patients in the total cohort of 37 patients. This significant difference was also seen in the prefibrotic IMF patients and there was borderline significance in the ET group, whereas the finding of lower platelet counts in the JAK2 V617F-positive patients was eliminated when the patients were divided into ET and prefibrotic IMF. In this context it is noteworthy that none of the 37 patients were reclassified as PV according to the WHO criteria. When only histomorphology is taken into account, our study supports the concept of an early stage of IMF encompassing about half those patients initially categorized as having ET according to the PVSG criteria. It is important to recognize this group of patients in the early stage of the disease when cytoreductive therapy should be considered to postpone or inhibit the development of progressive myelofibrosis, debilitating bone marrow failure, and myeloid metaplasia. However, the total lack of correlation between what appears to be two morphologically distinct entities and JAK2 V617F mutation status is somewhat troublesome. One could speculate that different mutations in proteins involved in receptor-JAK/STAT signalling, such as the W515K/L in the thrombopoietin receptor MPL (22, 23) and other as yet unknown mutations, could lead to the same bone marrow histomorphological changes. A large proportion of the patients are still molecularly undefined. In this context, bone marrow histology evaluated according to reproducible semiquantitative criteria is of the utmost importance and a prerequisite for a modern classification of these disorders based upon a combination of histomorphology and molecular analyses.

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7.6 Paper V summary

Reference: ***Larsen TS, Pallisgaard N, Møller MB, Hasselbalch HC.*** *High prevalence of arterial thrombosis in JAK2 mutated essential thrombocythemia:Independence of the V617F allele burden..* ***Hematology 2007 (In press).***

Objectives: Approximately half of the patients with essential thrombocythemia (ET) harbor the JAK2 V617F mutation. Despite evidence of a phenotypic mimicry of JAK2 V617F positive ET and PV, data on thromboembolic risk and correlation to JAK2 mutation status are ambiguous. On a strictly WHO defined ET cohort comprising 55 patients we evaluated possible clinical correlations including a history of previous thrombosis to the JAK2 V617F mutation status and allele burden

Results: In our cohort the JAK2 V617F positive patients account for approximately 50 % when assessed by qPCR. A subgroup of patients has low levels of JAK2 V617F alleles below the detection limit of some of the most used methods. The JAK2 V617F mutated patients have biochemical features resembling PV such as higher levels of Hb and lower levels of platelets and s-EPO. We demonstrated a significantly higher prevalence of arterial thrombosis in the JAK2 V617F mutated patients compared to their JAK2 wildtype counterparts. However, as there is a strong association between the JAK2 V617F mutation *per se* and arterial thrombosis, no association between the exact allele burden and thrombosis was recorded. Accordingly, we have added further support to the suggestion of the JAK2 V617F mutation *per se* as a marker of an increased risk of thrombosis in patients with essential thrombocythemia

High prevalence of arterial thrombosis in JAK2 mutated essential thrombocythaemia: independence of the V617F allele burden

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Approximately half of the patients with essential thrombocythaemia (ET) harbor the JAK2 V617F mutation. Despite a phenotypic mimicry of JAK2 V617F positive ET and polycythaemia vera (PV), the data on thromboembolic risk and correlation to JAK2 mutation status are ambiguous. On a strictly WHO defined ET cohort we evaluated possible clinical correlations to the JAK2 mutation status including a history of previous thrombosis. We used a highly sensitive quantitative real-time PCR (qPCR) assay for JAK2 V617F detection and allele burden quantification in a single institution study of 55 patients. A significantly increased prevalence of arterial thrombosis was recorded in JAK2 positive ET (p=0·001). There was no association between the mutational load and thrombosis. As compared to their JAK2 V617F negative counterparts, the JAK2 V617F positive patients had PV-like biochemical characteristics such as higher haemoglobin levels (p=0·02), lower platelet counts (p=0·002) and lower plasma EPO levels (p=0·04). The JAK2 V617F mutation *per se* but not the mutational load in patients with ET is associated with a PV-like phenotype and a higher prevalence of previous arterial thrombosis. This study adds further support to the contention of the JAK2 V617F mutation as a marker of increased risk of thrombosis.

Keywords: Essential thrombocythaemia, thrombosis, JAK2, qPCR

Introduction

Within the last two years important new insight into the molecular pathogenesis of the Philadelphia chromosome negative chronic myeloproliferative disorders (Ph^- CMPD) have been achieved with the identification of the JAK2 V617F mutation. The mutation is sufficient to cause cytokine independent cell growth of cultured bone marrow cells (1) and mutant JAK2 transfected into animal models is sufficient to develop a chronic myeloproliferative disorder mimicking polycythaemia vera (PV) which tends to terminate in myelofibrosis.[1–3] The JAK2 V617F mutation can be detected in virtually all patients with PV and approximately half of the patients with essential thrombocythaemia (ET) and primary myelofibrosis (PMF).[1,4–6] A substantial amount of data indicates a phenotypic mimicry of JAK2 V617F positive ET and PV. JAK2 positive ET has been referred to as a *forme fruste* of PV and the concept of a biological continuum of JAK2 positive myeloproliferative disorders from ET over PV to myelofibrosis has been introduced.[7] This model is supported by the fact that a large proportion of *in vitro* haematopoietic colonies from patients with PV are homozygous for the JAK2 V617F mutation, whereas the homozygous state, caused by mitotic recombination[5] is exceedingly rare in patients with ET.[8] Moreover, it has been demonstrated that the three phenotypes ET, PV and PMF have significantly

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different levels of mutated JAK2 with the highest levels being recorded in patients with PMF.[9,10] Recent data seem to indicate that not only the mutation status – positive or negative – but also the JAK2 V617F allele burden has an impact upon disease phenotype.[10–12] Following the hypothesis of a biological continuum of JAK2 V617F positive disorders, one might expect JAK2 V617F positive ET patients to have a higher risk of thrombosis, which may increase with increasing JAK2 V617F allele burden. However, the data on thrombosis risk and JAK2 V617F mutation status are conflicting, despite parallel observations of biochemical characteristics suggesting a PV-like phenotype in the same studies. ET patients harbouring the JAK2 V617F mutation have a PV-like phenotype with higher levels of haemoglobin,[13–16] higher white cell and neutrophil counts, lower mean red cell volume, lower plasma ferritin and plasma erythropoietin levels[13] and interestingly lower platelet counts.[13,14] Patients with JAK2 V617F negative ET do not seem to develop the JAK2 mutation during disease progression.[17] Regarding thrombotic risk, data are diverging, as some studies have reported an overall increased risk of thrombosis,[11,13,16,18,19] and some have reported increased risk of venous thrombosis only[13,20] whereas others did not record any difference in thromboembolic risk.[14,15,21,22] The results so far reported may be biased by the use of PVSG criteria, which are insufficient in discrimination between ET and early or latent PV with not yet significant erythrocytosis.[23]

Moreover ET patients may carry only a very discrete JAK2 V617F allele burden,[10] and accordingly, interpreted as JAK2 V617F negative if analyzed with sequencing, qualitative or semi-quantitative PCR assays with limited sensitivity. Using a highly sensitive real-time quantitative PCR assay (qPCR),[24] allowing detection of very small (down to 0·1%) JAK2 V617F allele burdens, we provide further data that support the notion of increased risk of arterial thrombosis in JAK2 V617F positive ET. This risk seems to be associated to the presence of the JAK2 V617F *per se* and not the JAK2 V617F allele burden.

Materials and methods

We conducted a prevalence study on patients registered in our out-patient clinic with the diagnosis ET. A total of 59 patients were identified but only 55 fulfilled the WHO criteria of ET and were included in the study after careful evaluation including revision of bone marrow morphology. This single-institution study was conducted in accordance with the Helsinki Declaration and approved by the Regional Danish Ethics Committee. Twenty-seven patients were newly diagnosed, whereas 28 patients had a longer disease duration, median 64 months, range (2–267). Peripheral blood leukocytes collected in the period March 2004 to June 2007 were used as the DNA source for detection of the JAK2 V617F mutation. All samples were analyzed by highly sensitive (0·1%) qPCR to determine the proportion of JAK2 V617F

Table 1 Patient characteristics

	All	JAK2 positive	JAK2 negative	*p*-value
Number	55	29 (53%)	26 (47%)	ND
Newly diagnosed (y/n)	27/28	19/10	9/17	ND
Gender male/female	25/30	10/19	15/11	0·08
Age at diagnosis (years)*	62 (32–91)	64 (43–91)	60 (32–84)	0·36
Disease duration (months)*	64 (2–267)	57 (2–240)	69 (3–267)	0·32
Haemoglobin (g/dl)**	13·7 (13·1–14·0)	14·0 (13·5–14·8)	13·1 (12·6–13·7)	**0·02**
WBC ($\times 10^9$/l)**	9·8 (8·3–10·6)	10·1 (7·9–11·2)	8·9 (7·7–11·0)	0·91
Platelets ($\times 10^9$/l)**	932 (834–1021)	857 (537–1810)	1182 (510–2860)	**0·002**
LDH (normal/elevated)	26/27	19/9	8/17	**0·009**
HCT**	0·42 (0·41–0·43)	0·42 (0·41–0·44)	0·42 (0·41–0·44)	0·71
MCV**	90 (89–93)	90 (88–94)	91 (89–94)	0·73
Ferritin**	53 (40–88)	57 (35–92)	53 (40–113)	0·63
EPO**	7 (5–13)	6 (5–8)	13 (6–18)	**0·04**
PRV–1 (fold upreg.)**	7 (3–20)	7 (3–32)	9 (2–28)	0·22
CD34+ ($\times 10^6$/l)	1 (1–4)	1 (0–4)	2 (0–6)	0·53
Microvasc. symptoms (y/n)	20/34	9/20	11/14	0·33
Const. symptoms (y/n)	3/49	1/23	2/26	0·65
Cytoreductive therapy (±)	21/5	9/0	12/5	0·07

WBC, white blood cell count; LDH, lactate dehydrogenase; HCT, Haematocrit; MCV, mean corpuscular volume. PRV–1=polycythaemia rubra vera gene 1.
*Variable displayed in mean with range given in parentheses.
**Variable displayed in median with 95% confidence intervals given in parentheses.

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mutated alleles. The method is previously described by our own group.[24] Briefly, two real-time qPCR reactions were performed in parallel with a common forward primer and Taqman probe and only differing in the use of a reverse primer specific for the JAK2 wildtype and the V617F mutated DNA, respectively. The JAK2 V617F proportion was calculated from standard curves and end point determination from limiting dilution series of JAK2 wildtype donor DNA and the homozygous JAK2 cell line HEL. All qPCR reactions were performed in duplicates. Forty patients were initially screened for the mutation by the allele-specific three-primer PCR method described by Baxter and co-workers[4] before we were able to quantify the amount of JAK2 V617F mutated alleles. The Chi-squared and Fisher's exact test were used for comparing categorical variables, whereas the Wilcoxon ranksum test was used for continuous variables. Multivariate analysis was performed using logistic regression. All statistical calculations were performed using the STATA Statistics/Data analysis 9·0 (Texas, US) software.

Results

Twenty-nine out of 55 patients (53%) harboured the JAK2 V617F mutation. It was interesting however, that among the 40 patients originally examined with the allele specific assay by Baxter *et al.*[4] nine patients primarily interpreted JAK2 V617F negative turned out to have a small JAK2 V617F allele burden of 0·7%, range (0·1– 3·0), which is below, or at the border of the detection limit of the allele specific method described by Baxter *et al.*[4]

In the total group of JAK2 V617F positive patients the mean JAK2 V617F allele proportion was 15 %, (95 c.i.: 6·4 –29·9). The highest observed JAK2 V617F mutational load was 40% and accordingly none could be categorized as homozygous. A number of different clinical parameters and their correlation to the JAK2 status were investigated (Table 1). The median haemoglobin (Hb) concentration in the JAK2 V617F positive patients was 14·0 g/dl (95% c.i.: 13·5–14·8), whereas it was 13·1 g/dl (95% c.i.: 12·6–13·7) in the JAK2 V617F negative patients (p=0·02). This correlation remained significant when adjusted for gender (p=0·01). The platelet count was significantly lower in the JAK2 V617F positive patients: median 857×10^9/l (95% c.i.: 537–1810) versus 1182×10^9/l (95% c.i.: 510–2860) in the JAK2 V617F negative patients (p=0·002). The plasma-EPO levels were significantly lower in the JAK2 V617F positive patients: median 6 (95% c.i.: 5–8) versus 13 (95% c.i.: 6–18) (p=0·04), as were the proportion of patients with elevated LDH levels at diagnosis (p=0·009). A trend towards more female JAK2 V617F positive patients were recorded (p=0·08) and interestingly, among the JAK2 V617F positive patients the JAK2 V617F allele burden was significantly higher in men [32% (95% c.i.:12–38%)] than in women [8% (95% c.i.:2–22%)] (p=0·02). No significant differences were recorded between mutated and wildtype patients in regard to leucocyte count, hematocrit (HCT), plasma-ferritin, mean corpuscular volume (MCV), peripheral blood CD34 counts, PRV–1 gene expression or the occurrence of microvascular or constitutional symptoms. In this cohort of 55 patients with WHO defined ET, 23 cases of thrombosis were recorded. Nineteen patients (35%) had a history of arterial thrombosis. Eighteen of these thromboses occurred just prior to diagnosis and together with the finding of thrombocytosis and subsequent referral to our haematological department. One case, a female patient, had an acute myocardial infarction (AMI) 2 years prior to diagnosis. From her medical record obtained at referral for her AMI it was verified that she had an elevated platelet count of 575×10^9/l. However, no follow up was initiated. Accordingly, all thrombotic events prior to diagnosis could be linked to thrombosis and a diagnosis of ET. There were four thrombotic events on follow up (7%). Eighteen cases of arterial thrombosis was recorded in 17 of the 29 patients with JAK2 V617F positive ET, compared to 5 cases in 4 out of 26 patients with JAK2 negative ET (p=0·001) (Table 2). No cases of venous thrombosis were recorded. The relative risk of thrombosis in the JAK2 V617F positive ET patients was 2·3 (95 c.i.: 1·4–3·8) compared to JAK2 V617F negative ET.

Table 2 Characteristics of thromboembolic events

Thromboembolic event	JAK2 V617F positive	JAK2 V617F negative	All
Acute myocardial infarction	2	1	3
Cerebral infarction	8	1	9
Transient ischaemic attack	5	3	8
Peripheral arterial embolism	3	0	3
Total	18	5	23

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There was no difference in the JAK2 V617F allele burden in JAK2 V617F positive patients with or without thrombosis (JAK2 V617F: 15·0% (95%c.i.:3·5–31%) versus JAK2 wildtype 15·5% (95% c.i.: 2–32%) (p=0·98). When the overall prevalence of arterial thrombosis was adjusted for age, gender, haemoglobin concentration, platelet count and leukocyte count the JAK2 V617F mutation *per se* remained an individual significant risk factor in multivariate analysis (p=0·004, 95% c.i.: 0·8–4·4) (Table 3).

Discussion

In this cohort study of patients with ET we have confirmed previous findings of biochemical features in the 29 (53%) JAK2 V617F mutated ET patients resembling a PV phenotype. Also in line with previous studies we found that the JAK2 V617F allele burden is relatively low in ET patients and it seems to be lower in women compared to men.[21] No patients in this cohort were homozygous for the JAK2 V617F mutation. Following the hypothesis of the biological continuum of JAK2 V617F positive disorders, one might expect JAK2 V617F positive ET patients to have a higher risk of thrombosis and that this risk might increase with increasing JAK2 V617F allele burden.[11] Despite the limited patient number a highly significant association between JAK2 V617F positivity and a history of arterial thrombosis was demonstrated. This finding is in line with most data,[11,13,16,18,19] although questioned by others.[14,15,21,22] Some of these discrepancies may be explained by inconsistency in diagnosis due to the use of different or insufficient diagnostic criteria. Furthermore, the use of different methods for detection of the JAK2 V617F mutation may have a considerable impact on the results, since the JAK2 V617F mutation may escape detection when less sensitive methods are used like direct sequencing (sensitivity ~10–25%)[1,5,6] or allele specific (ASO) or amplification-refractory mutation sequencing (ARMS) PCR assays which have a sensitivity limit of about 3%.[25] This issue is underlined by the results of the present study showing that nine patients initially interpreted JAK2 V617F negative by the ASO assay, turned out to be JAK2 positive using the qPCR assay. Thus, nine out of 29 JAK2 V617F mutated patients in our mixed cohort of both newly diagnosed patients and patients with a longer disease duration and possible influence of previous and ongoing cytoreductive treatment, had a low allele burden (median 0·7%), undetectable in most conventional assays. Of note, however, out of these nine patients, four had a history of thrombosis. The prevalence of previous arterial thrombosis (35%) is among the highest previously reported.[26] A substantial proportion of these events (eight out of 23 cases) were transitory ischaemic attacks (TIAs). The definition of TIAs in some previous publications has been vague and sometimes lacking. We used the definition of TIAs as a sudden occurrence of neurological symptoms resembling a stroke, lasting from a few minutes up to 24 hours and with complete disappearance and no radiological signs of stroke on CT or MR scans. Six of these eight patients were diagnosed as having TIA on admittance to a neurological department, whereas two cases were based upon information from the patient record only. It is obvious that unclear definitions and or exclusion of TIAs in some previous publications may explain the broad variation in the reported frequencies of thrombotic events.[26] The fact that no venous events were recorded may be explained by the fact that venous thromboembolism is considerably more infrequent in ET than arterial events.[26] In the largest study so far by Campbell *et al.*, thirteen cases of venous thrombotic events were recorded at diagnosis in an ET cohort comprising 776 patients, correspond-

Table 3 Clinical parameters and relation to thrombosis

			p-value	
	Thrombosis +	Thrombosis −	Univariate	Multivariate
Number	21	34	ND	ND
JAK2 (wildtype/V617F)	4/17	22/12	**0·001**	**0·004**
Gender (m/f)	8/13	17/17	0·39	0·88
Age at diagnosis (years)*	60 (44–91)	64 (32–84)	0·29	0·18
Platelet count ($\times 10^9$/l)**	752 (639–1148)	961(835–1104)	0·11	0·97
WBC ($\times 10^9$/l)**	8·6 (7·9–9·6)	8·3 (7·8–8·8)	0·83	0·80
Haemoglobin (g/dl)**	13·9 (12·7–15·5)	13·4(12·6–14·2)	0·48	0·44

WBC, white blood cell count.
*Variable displayed in mean with range given in parentheses.
**Variable displayed in median with 95% confidence intervals given in parentheses.

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ing to approximately 1%.[13] Accordingly, one would expect less than one case of venous thrombotic event at diagnosis in this series. Although not having major impact on the outcome of thromboembolic risk in our series of patients, it is obvious that a lack of discrimination at low JAK2 allelic burden may explain some of the diversities of previously published results in regard to the power of the JAK2 V617F mutation as a predictor of an increased thrombotic risk. The data supporting the leukocyte count as an independent thrombotic risk factor,[22,27] and the suggestion that the proportion of mutated alleles in addition to the mutation status itself (negative or positive) may influence the thrombotic risk in ET,[11] was interestingly not confirmed in the present study. This finding is in agreement with the results published by our own group, which failed to demonstrate an association between thrombosis and the mutational load in PV.[10] Accordingly, even in patients with a small JAK2 V617F clone accounting for less than 3%, a high prevalence of previous thrombosis was recorded, which is in line with recent data demonstrating the presence of a low JAK2 V617F burden in patients with relapsing thrombotic events without clinical or laboratory features of a underlying myeloproliferative disorder.[28] Altogether, these observations suggest a major contributing role of the JAK2 V617F mutation – even at low levels – in eliciting the thrombogenic state in these patients.[28] The very low JAK2 V617F allele burden may also reflect a possible suppressive effect on mutational load by current cytoreductive therapy in the non-newly diagnosed patients. Moreover one must keep in mind that the proportion of JAK2 V617F mutated alleles may be different in the various cell compartments, a higher mutational load being present in platelets compared to granulocytes.[21,29,30]

Conclusion

In conclusion, using a highly sensitive qPCR assay for determination of JAK2 status in this strictly WHO defined cohort of ET patients, we have added further evidence to support the value of a molecular marker – the JAK2 V617F mutation – as a potential thrombogenic factor in patients with ET. However, despite a highly significant association with the JAK2 V&17F mutation *per se*, the mutational load does not seem to have impact on thrombotic risk. Moreover, we confirmed that JAK2 V617F mutated ET patients display biochemical features resembling PV. A large scale prospective study of the JAK2 V617F allele burden in high risk ET patients on cytoreductive therapy is warranted in order to evaluate the impact of therapy on the JAK2 V617F allele burden and the risk of thrombosis.

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Chapter 8. Discussion

The emergence of the chronic myeloproliferative disorders is often associated with deregulated activity of various tyrosine kinases. Until the discovery of the JAK2 V617F mutation in 2005 the molecular pathogenesis behind the largest group of these disorders, namely PV, ET and PMF was largely unknown. Besides proving the existence of clonal myeloproliferation, the identification of this mutation has provided crucial insights into molecular pathogenesis of these disorders. Although several questions remain unanswered, an important new tool in the diagnostic procedure is now available. In several series of patients including our own, it has been demonstrated that the JAK2 V617F mutation does not occur in secondary or spurious polycythemia or in conditions with leuko- or thrombocytosis on a reactive basis. Accordingly, the JAK2 V617F mutation analysis fulfills the important task of demonstrating clonality, but it is not at present diagnostic of a specific CMPD. Historically, the classification of CMPD is based on consensus criteria. These criteria are changing within these years in parallel with our increasing insights into pathogenesis at the molecular level. However, an attempt to evaluate important new molecularly based knowledge of the CMPD in the context of the consensus based criteria (PVSG or WHO) has an obvious inherent weakness. Our interpretation of ET, PV and PMF as three distinct disease entities is historically based, and an attempt to try and fit the JAK2 V617F mutation into a classification system based on the existing criteria may prohibit a sound reclassification based on molecular pathogenesis. On the other hand it is important not to underestimate the validity of clinical and biochemical findings as well as bone marrow morphology. However, bone marrow histomorphological changes are not always ambiguous and interpretations are subjective. With the introduction of the WHO defined concept of prefibrotic myelofibrosis, hematopathologists working in the field of myeloproliferative disorders have been challenged even further. Although biologically meaningful, this new concept has not been widely accepted. In our hands no significant associations between individual bone marrow parameters and the JAK2 V617F mutation status could be identified in a cohort of WHO defined ET patients. However, a substantial proportion of these patients fulfilled the WHO criteria of prefibrotic myelofibrosis and the bone marrow morphology in regard to cellularity and megakaryocyte clustering and maturation was significantly different from what appeared to be “true ET” **(Paper IV)**. Accordingly, a molecularly based classification of ET and the observation that JAK2 positive ET in many aspects resembles PV, does not ambiguously seem to suit the concept of prefibrotic myelofibrosis **(Paper IV).**

A correct classification of CMPDs using the

JAK2 V617F mutation as a major criterion would necessitate an accurate and sensitive method for detection of the mutation. Our development of a qPCR based assay not only allows a detection of mutant clones as small as 0.1%, but also an exact quantification of the JAK2 V617F allele burden **(Paper I).** Because the size of the JAK2 V617F clone is very variable in individual patients and some patients only habor only a very small JAK2 V617F clone that may remain undetectable for non-quantitative PCR and sequencing methods, a reliable assay with a high sensitivity is of importance. Besides being a reliable marker of clonality the JAK2 mutation status *per se* provides important prognostic information in regards to thrombotic risk in ET **(Paper V).** This important aspect should be considered in future risk stratification models, as should other new important findings such as the presence or absence of leukocytosis, which might prove to be associated to the JAK2 V617F mutation.

The JAK2 V617F allele burden seems to be a key determinant of the phenotypic presentation of these disorders. The JAK2 V617F allele burden increases from ET over PV to PMF and high JAK2 V617F allele burden and in particular homozygosity for the JAK2 V617F allele is associated with increasing myeloproliferation and myeloid metaplasia. These observations fit the 'biological continuum model' in which the three JAK2 V617F positive myeloproliferative disorders (ET, PV and PMF) represents different phenotypic presentations of the same JAK2 V617F mutated CMPD **(Paper III).** Patients may present at different time points along this axis when diagnosed. One must expect that the nature of these diseases involves an evolution towards the myelofibrotic stage with high JAK2 V617F allele burden. However, this natural evolution proceeds over several years, in some cases even decades, and as patients are often diagnosed at a relatively high age, this full span of the nature of the disease is often not evolving. Moreover, it is likely that physiological (e.g iron metabolism and gender) and genetic (concomitant mutations and other genetic or epigenetic alterations) may modify the disease presentation and evolution. The model implies that JAK2 V617F negative ET and PMF are caused by different genetic alterations, which most likely may be localized in other components of the JAK-STAT signaling pathway like e.g the MPLW515K/L mutations. Following the "biological continuum" theorem, a rational treatment approach might be to aim at reducing the JAK2 V617F allele burden and hopefully thereby reducing the primary morbidity of thrombohemorrhagic complications and the natural evolution towards myelofibrosis and ultimately transformation to AML. In this context a qPCR assay for assessment of JAK2 V617F clonal size at diagnosis and during ongoing treatment is warranted. Several groups including our own have published different, mainly qPCR based assays allowing an assessment of the JAK2 allele burden. The JAK V617F clone arises in a stem cell with both myeloid and lymphoid differentiation potential and the clonal involvement of various cell lineages seems to be rather heterogeneous in individual patients **(Paper I)**. We have demonstrated a highly significant

correlation between the JAK2 V617F allele burdens in peripheral blood unfractionated leukocytes and bone marrow **(Paper II).** Accordingly, we believe that the total white blood cell population is the most correct and moreover the most easily accessible compartment for the assessment of the JAK2 V617F allele burden. However, an international standardization in line with what has been achieved with the BCR-ABL monitoring in CML is needed.

Chapter 9. Concluding remarks – perspectives and future research

Translational medical research is the phrase used for the disciplin of building bridges between basic laboratory research and daily clinical practice. In that context this Ph.D project serves as a good example. Within the last 2-3 years exciting revolutionary accomplishments have provided insights into the molecular etiology of the CMPDs. This research field has been, and is to date, extremely competitive. Nevertheless, we feel confident that we have succeded playing a role in the field with relevant scientific contributions, which are a result of a most fruitful cooperation in our own institution at Odense University Hospital and University of Southern Denmark, but also through collaborative studies with the Department of Haematology, Cambridge Institute for Medical Research, University of Cambridge, United Kingdom and other European collaborators.

We will continue our research in this exciting field and hopefully be able to provide further contributions to the accumulating insights into these enigmatic disorders. We and others postulate that the "biological continuum model" will lay the foundation for a future classification of the chronic myeloproliferative disorders. Accordingly, the classical stringent diversion into distinct although related disease entities based on the Dameshek concept is likely to change as data accumulates. Moreover, continuous monitoring of the JAK2 V617F allele burden will become of increasing importance because of the ongoing development of tyrosine kinase inhibitors targeting the JAK2 tyrosine kinase. This may very well lead to a change of dogma in our therapeutic approach to these disorders from the "first do no harm" principle towards a more active strategy with early initiation of therapy in order to minimize the malignant clone and thereby hopefully also reducing thrombohemorrhagic complications and evolution towards the myelofibrotic stage and ultimately the transformation to AML. In this context it is highly interesting that treatment with interferon-alpha seems to reduce the JAK2 V617F allele burden, in some patients even to very low levels. Initiatives have been taken to collect peripheral blood and bone marrow samples from patients in Denmark who have been on long-term treatment with interferon-alpha to further characterize those patients who really benefit from this treatment as defined by normalization of peripheral blood values and bone marrow morphology in concert with obtaining major molecular responses like in CML. In addition, it would be of outstanding value if one could predict patients at high risk of developing the myelofibrosis state in order to direct more targeted and undoubtly expensive future therapies to this high-risk group. To do this, a search for relevant target genes or epigenetic alterations driving the fibrotic process is

nessessary. Important information regarding the fibrotic process in the context of our new molecular knowledge may also be obtained from studying collagen and extracellular matrix metabolism. Looking back at the progress made in the field of Philadelphia chromosome negative chronic myeloproliferative disorders within the last 2-3 years, it is intriguing to raise the prospect that the exciting history of CML will repeat itself in these related disorders, leaving the patients with a good opportunity of living a virtually normal life with minimal medical intervention and a prognosis approaching the population in general.

Chapter 10. English Summary

The identification of an acquired somatic point mutation in the *Janus Kinase 2 (JAK2)* gene in the majority of patients with polycythemia vera (PV) and approximately half of patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF) has lead to a crucial breakthrough in our understanding of the molecular pathogenesis of these disorders. The introduction of genotyping in patients suspected of a chronic myeloproliferative disorder (CMPD) has opened up new opportunities in regards to achieving a correct diagnosis, improving classification and perhaps also in regards to predicting prognosis. The *JAK2* gene translates into a receptor-associated cytoplasmatic tyrosine kinase, which plays a key role in regulating normal hematopoiesis. A change in the nucleic acid sequence at position 1849 in exon 14 (guanine to thymine) results in an amino acid substitution of valine for phenylalanine in codon 617 (V617F).

This Ph.D study is a translational study, which seeks to implement and validate new molecular markers within the CMPDs. In a cohort of patients with CMPDs and allied conditions (n=349) we have elucidated different clinical aspects of the JAK2 V617F mutation with the emphasis on the impact of the mutation on the clinical phenotype. We have developed a highly sensitive quantitative real-time PCR (qPCR) assay, which allows us to determine the proportion of JAK2 V617F mutated and wildtype alleles.

Despite the general opinion that these disorders originate at the pluripotent stem cell level, the question of whether lymphoid cells are involved in the clonal expansion have remained controversial. The high sensitivity of our qPCR assay (0.1%) allowed us to demonstrate the presence of the JAK2 V617F mutation in lymphoid cells in a subgroup of patients and in some cases in a substantial proportion especially in T-cells. The clonal involvement of different hematopoietic cells varies between individual patients **(Paper I)**. We have provided evidence that a quantitative assessment of the JAK2 V617F allele burden in unfractionated peripheral blood leucocytes is a reliable and precise measurement of the size of the JAK2 V617F clone. The use of this compartment for quantification seems rational both biologically and technically **(Paper II)**. Numerous studies have demonstrated that approximately half of patients with ET and PMF habours the JAK2 V617F mutation. These figures have been confirmed in the present study. Furthermore, evidence is accumulating that clear phenotypic differences exist between JAK2 V617F mutated and wildtype ET patients. JAK2 V617F mutated ET patients have distinct biochemical features and a thrombotic risk profile resembling PV **(Paper V)**. In contrast, solid associations between specific bone marrow morphological features in ET and the JAK2 V617F mutational status seem to be lacking. In addition, no correlation between the WHO defined conditions ET and prefibrotic

myelofibrosis and the presence or absence of the JAK2 V617F mutation seem to exist (**Paper IV**). Only a few studies have tried to elucidate phenotypic differences between JAK2 mutated and wildtype PMF patients. In this cohort the number of PMF patients is limited and only vague indications of PV-like features are provided. A multicenter study has demonstrated increased leukocytosis, lower transfusion needs and poorer survival in JAK2 V617F positive PMF patients (**Suppl. Paper C**). When, in addition to the evaluation of the JAK2 mutation status *per se,* a quantification of the JAK2 V617F allele burden is performed, clear indications of a close biological relationship between JAK2 V617F mutated PMF and PV emerge. PMF patients have the highest JAK2 V617F allele burden as compared to both ET and PV. This observation supports the concept that the JAK2 V617F mutated chronic myeloproliferative disorders represent different phenotypic presentations of the same JAK2 V617F mutated disorder. This biological continuum is to some extent determined by the JAK2 V617F allele burden, which increases from ET over PV to PMF and seem to be directly associated with the degree of myeloproliferation (**Paper III**). An increasing amount of evidence supports this "biological continuum model" and as a consequence an implementation of a molecularly based classification system without ignoring morphological and biochemical parameters is likely to be introduced. However, a prerequisite for a successfull outcome is the willingness to settle with the traditional interpretation of ET, PV and PMF as individual entities. This would enable Willian Dameshek's concept of a close relationship between the different chronic myeloproliferative disorders introduced in 1951 to come of age in a classification system based on molecular knowledge.

Chapter 11. Dansk Resumé

Identifikationen af en erhvervet somatisk punktmutation i *Janus Kinase 2* (*JAK2*) genet hos størstedelen af patienter med polycythemia vera (PV) og ca. halvdelen af patienter med essentiel thrombocytose (ET) og primær myelofibrose (PMF) har medført betydelige fremskridt i vores forståelse af patogenesen ved disse sygdomme. Endvidere er der med anvendelsen af genotypning af patienter mistænkt for kroniske myeloproliferative sygdomme åbnet nye muligheder for en mere sikker diagnostik, klassifikation og måske prognosevurdering. *JAK2* genet koder for en receptor-associeret cytoplasmatisk tyrosinkinase, som spiller en afgørende rolle i reguleringen af den normale hæmatopoiese. Ændringen i nukleinsyresekvensen i exon 14 af *JAK2* genet fra guanin til thymin i position 1849 medfører en aminosyresubstitution af valin til phenylalanin i codon 617 (V617F). Herved tabes den interne autoregulatoriske funktion af JH2 domænet på det katalytiske JH1 domæne i tyrosin kinasen, som bliver konstitutivt aktivt og dermed afgørende for den øgede sensitivitet og autonomitet overfor forskellige hæmatopoietiske vækstfaktorer, som betinger dysreguleret autonom proliferation af myeloide celler.

Ph.d afhandlingen er et translationelt studie, som tager sit udgangspunkt i implementering og validering af nye molekylære markører ved de kroniske myeloproliferative sygdomme. I en kohorte af patienter med kroniske myeloproliferative sygdomme og beslægtede tilstande (n = 349) har vi belyst kliniske aspekter af JAK2 V617F mutationen, idet hovedvægten er lagt på mutationens betydning for den kliniske fænotype. Vi har i denne proces udviklet et kvantitativt real-time PCR (qPCR) assay, som med høj sensitivitet og reproducerbarhed kan bestemme det procentuelle forhold mellem JAK2 wildtype og V617F muterede alleler.

På trods af opfattelsen af, at disse sygdomme har deres oprindelse i den multipotente stamcelle har spørgsmålet om lymfoide cellers involvering i den klonale ekspansion til stadighed været kontroversielt. Den meget høje sensitivitet (0.1 %) i vores qPCR assay har gjort det muligt at påvise JAK2 V617F mutationen i lymfoide celler i en subgruppe af patienter, hvoraf enkelte havde endog ganske stor JAK2 V617F mutationsbyrde i specielt T-lymfoide celler. Fraktionen af JAK2 V617F muterede alleler i de enkelte cellekompartments synes at variere betydeligt fra patient til patient **(Publikation I)**. Vi har dokumenteret, at en kvantitering af JAK2 V617F allelbyrden i ufraktionerede leukocytter fra perifert blod er et godt og reproducerbart mål for JAK2 V617F klonens størrelse, og anvendelsen af dette kompartment synes at være såvel biologisk som teknisk rationelt **(Publikation II)**. Talrige studier har vist, at ca. halvdelen af patienter med ET og PMF har JAK2 V617F mutationen. Dette bekræftes i denne studiekohorte. Ydermere er der en tiltagende mængde data, som dokumenterer

klare fænotypiske forskelle mellem JAK2 V617F muterede og wildtype ET patienter, idet de muterede patienter har biokemiske træk og en tromboserisikoprofil, som imiterer PV **(Publikation V)**. I modsætning hertil synes der at mangle tydelige sammenhænge mellem specifikke knoglemarvsmorfologiske forandringer ved ET og forekomsten af JAK2 V617F mutationen. Det har ydermere vist sig vanskeligt at påvise korrelationer mellem de morfologisk forskellige WHO definerede tilstande ET og præfibrotisk myelofibrose og tilstedeværelsen af JAK2 V617F mutaionen **(Publikation IV)**. Kun ganske få studier har belyst fænotypiske forskelle mellem JAK2 wildtype og muterede PMF patienter. I vores kohorte er antallet af PMF patienter begrænset og der er kun sparsomme indikationer i retning af at de JAK2 V617F muterede PMF patienter har PV lignende træk. I et multicenterstudie er der fundet øget forekomst af leukocytose, nedsat transfusionsbehov og dårligere overlevelse hos de JAK2 muterede PMF patienter **(Suppl. Publikation C)**. Hvis man udover at betragte mutationstatus *per se* kvantiterer JAK2 V617F klonens størrelse, synes der at være en klar indikation af en tæt biologisk kobling mellem JAK2 V617F muteret PMF og PV, idet de muterede PMF patienter har den højeste JAK2 V617F allelbyrde sammenlignet med ET og PV. Derigennem understøttes hypotesen om, at de JAK2 V617F muterede kroniske myeloproliferative patienter repræsenterer forskellige fænotypiske præsentationer af den samme JAK2 V617F muterede kroniske myeloproliferative sygdom. Dette biologiske kontinuum er til en vis grad bestemt af JAK2 V617F allelbyrden, som tiltager fra ET over PV til PMF og synes direkte associeret til graden af myeloproliferation **(Publikation III)**. Mængden af data, som understøtter denne "biologiske kontinuum model" er tiltagende. Som en logisk konsekvens heraf kunne en implementering af et klassifikationssystem med et molekylærbiologisk udgangspunkt uden en tilsidesættelse af morfologiske eller biokemiske parametre måske umiddelbart synes nært forestående. Hvis denne udvikling ikke skal kompromitteres, forudsættes en vilje til et brud med den traditionelle opdeling af disse sygdomme i ET, PV og PMF. Således vil William Dameshek's epokegørende koncept introduceret i 1951 om disse sygdommes unikke slægtskab vise sig at blive central i vores klassifikation af disse sygdomme på et velfunderet molekylærpatogenetisk grundlag.

Chapter 12. References

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Chapter 13. Appendix

13.1 Supplementary paper A

Thomas Stauffer Larsen, Hans Carl Hasselbalch, Gitte Birk Kerndrup.
A der(18)t(9;18)(p13;p11) and a der(9;18)(p10;q10) in polycythemia vera associated with a hyperproliferative phenotype in transformation to postpolycythemic myelofibrosis. ***Cancer Genet Cytogenet. 2007;172:107-12.***

13.2 Supplementary paper B

Peter J Campbell, E Joanna Baxter, Philip Beer; Linda Scott, Anthony J Bench, Brian JP Huntly, Wendy N Erber, Rajko Kusec, **Thomas Stauffer Larsen**; Stéphane Giraudier, Marie-Caroline Le Bousse-Kerdilés, Martin Griesshammer, John T Reilly, Claire N Harrison, Anthony R Green.
Mutation of JAK2 in the myeloproliferative disorders: timing, clonality studies, cytogenetic associations, and role in leukemic transformation.***Blood 2006;108:3548-55***.

13.3 Supplementary paper C

Peter J Campbell, Martin Griesshammer, Konstanze Döhner, Harmut Döhner, Rajko Kusec , Hans C Hasselbalch, **Thomas Stauffer Larsen** , Niels Pallisgaard, Stéphane Giraudier, Marie-Caroline Le Bousse-Kerdilès, Christophe Desterke, Bernadette Guerton, Brigitte Dupriez , Dominique Bordessoule, Pierre Fenaux , Jean-Jacques Kiladjian , Jean-François Viallard, Jean Brière, Claire N Harrison. Anthony R Green, John T Reilly. **V617F mutation in JAK2 is associated with poorer survival in idiopathic myelofibrosis.** ***Blood 2006;107:2098-100.***