The JAK2 V617F allele burden in essential thrombocytopenia, 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 thrombocytopenia (ET) and primary myelofibrosis (PMF). The three distinct disease entities may be considered as three phenotypic presentations of the same JAK2 V617F positive chronic myeloproliferative 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 assess 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 < 0.0001 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 < 0.0001 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 thrombocythemia; 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 thrombocytopenia (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 thrombembolic 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'-CAG-GTTGCAACCTGCTCAAT-3', a reverse primer: 5'-GCCAAGTTTCCGTGTCAATA-3' and a probe: 5'-Fam-TTGCAGTTCTCGTCAATCCACGGGC- 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 categori- 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
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%) (Table 1). 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 haemoglobin (P = 0.03) 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

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**Figure 1** Box-plots showing the JAK2 V617F allele percentage in essential thrombocytemia (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).
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.

<table>
<thead>
<tr>
<th></th>
<th>WT (total)</th>
<th>V617F (total)</th>
<th>Hetero (qPCR)</th>
<th>Homo (qPCR)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV (total) (n)</td>
<td>1</td>
<td>94</td>
<td>67</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Gender (m/f)</td>
<td>1/0</td>
<td>45/50</td>
<td>29/38</td>
<td>14/9</td>
<td>0.11</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>53</td>
<td>64 (23–89)</td>
<td>63 (23–87)</td>
<td>67 (43–89)</td>
<td>0.38</td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>59</td>
<td>39 (1–379)</td>
<td>37 (1–204)</td>
<td>26 (1–132)</td>
<td>0.56</td>
</tr>
<tr>
<td>Splenomegaly (+/−)</td>
<td>0/1</td>
<td>17/72</td>
<td>7/58</td>
<td>10/11</td>
<td>0.0001</td>
</tr>
<tr>
<td>Haemoglobin (g/dL)</td>
<td>15.5</td>
<td>16.8 (15.8–18.1)</td>
<td>16.8 (15.8–18.1)</td>
<td>16.6 (14.0–20.3)</td>
<td>0.86</td>
</tr>
<tr>
<td>Platelets (x10⁹/L)</td>
<td>575</td>
<td>682 (597–774)</td>
<td>743 (638–840)</td>
<td>492 (347–748)</td>
<td>0.02</td>
</tr>
<tr>
<td>WBC (x10⁹/L)</td>
<td>6.3</td>
<td>11.9 (11.2–13.9)</td>
<td>11.4 (11.0–12.8)</td>
<td>14.6 (11.4–19.8)</td>
<td>0.03</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>405</td>
<td>353 (281–431)</td>
<td>304 (243–400)</td>
<td>480 (342–564)</td>
<td>0.03</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>0.46</td>
<td>53 (50–55)</td>
<td>52 (50–55)</td>
<td>52 (44–63)</td>
<td>0.79</td>
</tr>
<tr>
<td>PRV1 (fold upreg.)</td>
<td>2</td>
<td>34 (20–73)</td>
<td>20 (14–34)</td>
<td>576 (238–1417) &lt;0.00001</td>
<td></td>
</tr>
<tr>
<td>CD34 (x10⁶/L)</td>
<td>2</td>
<td>3 (2–4)</td>
<td>3 (1–3)</td>
<td>6 (2–23)</td>
<td>0.03</td>
</tr>
<tr>
<td>Cytoreductive therapy (+/−)</td>
<td>1/0</td>
<td>47/43</td>
<td>34/33</td>
<td>13/10</td>
<td>0.41</td>
</tr>
<tr>
<td>Thrombosis (+/−)</td>
<td>0/1</td>
<td>40/52</td>
<td>28/39</td>
<td>10/11</td>
<td>0.41</td>
</tr>
</tbody>
</table>

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) | 15.6 (12.6–16.6) | 0.08    |
| Platelets (x10⁹/L)² | (-) | 809 (593–875) | 812 (588–942) | 748 (484–1347) | 0.80    |
| WBC (x10⁹/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 (x10⁶/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.

¹Variable displayed in mean with range in parenthesis.
²Variable 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 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). 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 \textit{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 \textit{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.

References


