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ORIGINAL ARTICLE

High Risk Janus Kinase 2 V617F Allele Burden in a Seven-Year Cohort of Patients with Myeloproliferative Neoplasms

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SUMMARY

Background: **JAK2 V617F is the most common somatic mutation associated with the classical Philadelphia (Ph) chromosome negative myeloproliferative neoplasms (MPNs), which include essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF). JAK2 V617F allele burden may be used for establishing the diagnosis, determining prognosis, and monitoring progression in these diseases. Limited data is available regarding the epidemiology of MPNs in Africa, and there is paucity of data on demographic, laboratory, and clinical features of MPNs in South Africa. This study determined the JAK2 V617F allele burden in a seven-year retrospective cohort of patients diagnosed with MPNs and described the characteristics of these diseases in a South African setting.**

Methods: **A laboratory database search was performed to identify patients diagnosed with ET, PV or PMF and a positive JAK2 V617F mutation, diagnosed qualitatively on Fluorescence Resonance Energy Transfer (FRET) real-time PCR and melting curve analysis. The allele burden for these patients was measured on archived residual DNA samples using a quantitative allele specific amplification (QUASA) assay. Demographic data and relevant laboratory results at presentation were analyzed.**

Results: **The search identified 87 patients who tested positive for JAK2 V617F mutation and fulfilled the diagnostic criteria for ET, PV or PMF from 2012 to 2018. Median age at diagnosis was 64 years with a male: female ratio of 1.2:1. ET, PV and PMF accounted for 11.5%, 44.8%, and 43.7% of the MPNs, respectively. Median allele burden for ET, PV, and PMF was 24.9%, 71.1%, and 55.8%, respectively. Allele burden was significantly lower in ET compared to PV ($p = 0.0003$) and PMF ($p = 0.0023$) and correlated with leukocytosis, neutrophilia, eosinophilia, and low erythrocyte mean cell volume ($p < 0.05$).**

Conclusions: **JAK2 V617F-positive MPNs occurred predominantly in older patients with approximately equal gender ratio. ET was the least common MPN and there was a higher proportion of PMF cases than described in studies in Europe and America. Allele burden was also relatively high for all three subtypes of MPNs when compared to other published data, which may predispose to poorer prognosis.**

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KEY WORDS

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INTRODUCTION

The World Health Organization (WHO) identifies polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) as the classical Phila-

delphia (Ph) chromosome negative myeloproliferative neoplasms (MPNs) [1]. These are clonal myeloid disorders characterized by autonomous proliferation of hematopoietic precursor cells leading to increased mature blood cells [2]. As a result, patients have significant disease burden including splenomegaly, hepatomegaly, increased thrombotic and bleeding complications, and risk of progression to myelofibrosis and acute leukemia [1]. These diseases appear to form a continuum with ET being the more benign and overt PMF the more aggressive subtype, the latter of which may be preceded by a prefibrotic/early stage (pre-PMF) [1,3].

The Janus kinase 2 V617F mutation (hereafter referred to as V617F) is the most common driver mutation implicated in Ph chromosome negative MPNs, reportedly present in over 95% of PV and 50 - 60% of ET and PMF patients [4]. The mutation causes a nucleotide change from guanine to thymine at base 1,849 on exon 14 of the JAK2 gene, resulting in substitution of valine for phenylalanine at codon 617. This activates the JAK2 kinase and leads to enhanced and dysregulated cell signaling via the JAK/STAT (signal transducers and activators of transcription) pathway [5].

V617F-associated MPNs are biologically related [6,7] with mutant allele burden playing an important role in determination of diagnosis, clinical phenotype, prognosis, and monitoring of therapy [8-10]. To diagnose an MPN, the suggested limit is an allele burden of 1 - 3%, with allele burden less than 1% constituting clonal hematopoiesis of indeterminate potential (CHIP) [11]. V617F allele burden is reportedly low in ET patients at diagnosis and does not frequently exceed 20% [12], while PV and PMF are associated with higher baseline allele burden than ET. The higher the V617F allele burden, the worse the clinical phenotype and prognosis [13, 14]. ET patients with an allele burden above 25% have a higher risk of arterial thrombosis and splenomegaly [15,16] and the allele burden typically increases with transformation to myelofibrosis or acute leukemia. Mutant allele burdens greater than 75% in PV patients are associated with pruritus, three-fold higher risk of thrombosis, and higher frequency of clinically detectable and massive splenomegaly [17,18]. PMF patients with allele burden above 50% frequently present with leukocytosis, massive splenomegaly, higher requirements for splenectomy and chemotherapy, and higher risk of acute leukemic transformation [14,19]. V617F allele burden may also be utilized to monitor therapy such as targeted JAK1/JAK2 inhibitors in MPNs, as well as to determine the adequacy of pre-transplant conditioning and monitor for disease relapse in transplanted V617F positive myelofibrosis patients [20].

The incidence and type of MPNs is known to vary with age, gender, and geographic location [1,21,22]. Notably, limited data is available regarding the epidemiology of MPNs in Africa and there is a paucity of data on demographic, laboratory, and clinical features of MPNs in South Africa [23,24]. To the best of our knowledge, there are no published data on V617F allele burden in South African cohorts. We aimed to determine demographic and laboratory characteristics of a cohort of patients diagnosed with ET, PV and PMF, as well as to measure V617F allele burden and assess the concomitant patient and disease features in this cohort.

MATERIALS AND METHODS

Ethics approval

Ethics approval was granted by University of the Witwatersrand Human Research Ethics Committee (Ethics clearance certificate number: M170589).

Study setting

The study was performed in the Molecular Haematology and Virology laboratory (MHVL), Department of Molecular Medicine and Haematology, National Health Laboratory Service (NHLS) at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) and University of the Witwatersrand.

Study population and data collection

All JAK2 V617F positive patients above 18 years of age with a confirmed diagnosis of ET, PV or PMF (based on World Health Organization (WHO) guidelines) [1,25] during the study period (January 2012 to December 2018) who had complete demographic and laboratory results were included in the data analysis. Demographic data and relevant laboratory results at presentation were obtained from a database search of the NHLS laboratory information system (TrakCare, InterSystems Corporation, Cambridge) and the NHLS Central Data Warehouse. The allele burden for ET, PV, and PMF patients was measured on archived residual DNA samples that had been submitted at presentation for JAK2 V617F Fluorescence Resonance Energy Transfer (FRET) real-time PCR and melting curve analysis. Quality and quantity of DNA samples was assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific®, Wilmington, DE, USA).

Quantitative PCR using a Quantitative Allele Specific Amplification (QUASA) assay

A JAK2 V617F Quantitative Allele Specific Amplification (QUASA) assay kit (Primerdesign® Ltd., Chandler's Ford, United Kingdom) was used to measure the allele burden. This technique detects and amplifies both the wild type (WT) JAK 2 and mutant JAK 2 V617F by quantitative polymerase chain reaction (PCR) and quantifies the proportion of the mutant to the wild type JAK2 gene with a detection limit of 0.1%. This kit was successfully verified prior to use in this study utilizing WHO standards with known allele burdens of 0%, 0.1%, 1%, 10%, 90%, and 100%. The allele burdens of the WHO standards had been pre-determined using a droplet digital PCR technique [26].

In brief, the PCR was set-up in a two-reaction protocol

with two modified primers and hydrolysis probes: a mutant primer for the JAK2 V617F single nucleotide polymorphism (SNP) and a WT primer. The reaction was performed in duplicate and a 1% JAK2 V617F allele burden positive control, a negative control, and a no template control were included in each run.

Real-time quantitative PCR was performed using a LightCycler 480 (Roche® Molecular Diagnostics, Pleasanton, CA, USA). Cycling conditions included two minutes of enzyme activation (at 95°C) followed by five PCR cycles with denaturation at 95°C, annealing at 50°C, and extension at 72°C to increase specificity. The annealing temperature was increased to 60°C for the remaining 45 PCR cycles. Data was collected through the fluorescein amidite (FAM) channel during the 60°C annealing step.

By comparing the detection levels obtained by both primer/probe sets, the proportion of sequences that contained the mutation was measured and corrected against the positive control, where SNP was present at a known proportion of 1%. This allowed detection of the WT: mutant ratio, which was used to calculate relative percentage of mutant and WT in each sample using the delta cycle threshold (Ct) formula (utilizing a manufacturer supplied software tool).

Data analysis

Data was analyzed using STATA software version 13 (StataCorp LP, College Station, TX, USA). Continuous variables were summarized using medians and ranges or interquartile ranges, while categorical variables were expressed as ratios and percentages. Statistical comparisons of quantitative variables were performed using Kruskal-Wallis or Mann-Whitney U test. Spearman's rank order correlation was used to assess relationships between allele burden and demographic and laboratory variables of V617F-positive MPNs. Statistical significance was set at a two-sided p-value of < 0.05.

RESULTS

V617F mutation analysis in CMJAH MHVL from 2012 to 2018

There were 235 patients who tested positive for JAK2 V167F mutation over the 7-year period using FRET real-time PCR and melting curve analysis. Of these, a new diagnosis of ET, PV or PMF was confirmed on a bone marrow (BM) biopsy in 87 patients. The rest of the patients had incomplete results (no or an inadequate BM biopsy); an alternative diagnosis such as acute myeloid leukemia (AML), myeloproliferative/myelodysplastic syndrome (MPN/MDS) or myeloproliferative neoplasm (MPN) unclassifiable; or the PCR test was performed on behalf of other laboratories as a referral service.

Demographic features of MPNs

There were 10 patients with ET (11.5%), 39 patients with PV (44.8%), and 38 (43.7%) patients with PMF. The overall median age at presentation for the MPNs was 64 years (range: 20 - 85 years). Median age at presentation for ET, PV and PMF was 64, 60, and 66.5 years, respectively, with no statistical difference among the three MPNs in terms of age ($p = 0.71$). Patients older than 60 years at presentation accounted for 54%. The overall male to female ratio was 1.2:1 with no overall gender difference ($p = 0.088$) and males accounted for 50%, 43.6%, and 68.4% of ET, PV and PMF patients, respectively. However, gender distribution differed between PMF and PV ($p = 0.0293$), with more males having PMF and more females affected by PV.

Laboratory results in MPNs

Laboratory results are summarized in Table 1. All patients were negative for t (9;22), but positive for the JAK2 V167F mutation. As expected, the median platelet count for ET patients was significantly higher than in PV and PMF ($p = 0.0001$) and the median hemoglobin level was higher in PV patients ($p = 0.0001$). None of the ET patients showed a leukoerythroblastic reaction. Approximately 77% of PV patients had a hemoglobin level of at least 16 g/dL, with the remaining 23% having masked PV due to iron deficiency. The latter were identified by reviewing trend of laboratory results prior to diagnosis of PV, iron study and bone marrow aspirate iron staining results.

As per WHO 2016 guidelines [1], the majority of PMF patients had overt PMF (87%). Approximately 39% of patients with PMF had thrombocytosis, while thrombocytopenia of $< 100 \times 10^9$/L was seen in about 18.4% of PMF patients. All these latter patients had overt PMF. In addition, 57.9%, 53.6%, and 42% of PMF patients had leukocytosis, leukoerythroblastosis, and at least 1% blasts on peripheral blood smear, respectively. Lactate dehydrogenase (LDH) was significantly higher in PMF than in PV ($p = 0.0045$) and ET ($p = 0.0372$) but not statistically different between PV and ET ($p = 0.2188$).

V617F allele burden in MPNs

Allele burden was measured on DNA samples of 62 patients with a diagnosis of ET, PV or PMF. The median allele burdens were 24.9% (range: 5.8% - 48.8%), 71.1% (range: 8.5% - 94.0%), and 55.8% (range: 4.6% - 95.9%) in ET, PV, and PMF patients, respectively. The allele burden was significantly lower in ET than in PV ($p = 0.0003$) and PMF ($p = 0.0023$) but showed no statistically significant difference between PV and PMF (Figure 1). All ET patients had an allele burden below 50%. Moreover, 84% of PV and 68% of PMF patients had an allele burden over 50%, while 44% of PV and 32% of PMF patients had an allele burden above 75% (Figure 2).

Table 1. Laboratory results for V617F positive MPNs.

	ET (n = 10)	PV (n = 39)	PMF (n = 38)	p-value
White cell count (x 10^9/L) (IQR)	10.55 (8.56 - 19.10)	15.56 (9.83 - 26.48)	13.89 (8.60 - 32.94)	0.3615
Hemoglobin (g/dL) (IQR)	14.55 (13.3 - 15.8)	17.4 (16.2 - 19.60)	9.65 (6.9 - 11.9)	**0.0001**
Hematocrit (IQR)	0.45 (0.41 - 0.48)	0.57 (0.48 - 0.63)	0.32 (0.22 - 0.37)	**0.0001**
Mean corpuscular volume (fL) (IQR)	87.8 (77.8 - 89.2)	74.8 (72.3 - 86.0)	86.7 (77.7 - 92.2)	**0.0033**
Platelet count (x 10^9/L) (IQR)	1,153 (907 - 1,188)	696 (572 - 1,036)	343 (122 - 678)	**0.0001**
Neutrophils (x 10^9/L) (IQR)	7.32 (5.43 - 14.25)	13.07 (7.20 - 21.76)	10.44 (5.84 - 21.03)	0.2992
Lymphocytes (x 10^9/L) (IQR)	2.12 (1.76 - 3.21)	1.90 (1.38 - 2.31)	1.96 (1.25 - 3.01)	0.5967
Monocytes (x 10^9/L) (IQR)	0.67 (0.55 - 1.26)	0.79 (0.36 - 1.23)	0.62 (0.24 - 1.09)	0.4557
Eosinophils (x 10^9/L) (IQR)	0.30 (0.23 - 0.62)	0.37 (0.18 - 0.68)	0.46 (0.06 - 1.00)	0.9086
Basophils (x 10^9/L) (IQR)	0.08 (0.05 - 0.15)	0.12 (0.07 - 0.28)	0.06 (0.00 - 0.24)	0.2159
Lactate dehydrogenase (U/L) (IQR)	274 (226 - 391)	367 (301 - 542)	690 (464 - 1,232)	**0.0044**
Blasts (%) (IQR)	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	0.0 (0.0 - 3.0)	**0.0002**

The statistically significant p-values are underlined line. Median values and interquartile ranges (IQR) are reported.
ET - essential thrombocythemia, IQR - interquartile range, MPNs - myeloproliferative neoplasms, N - number, PMF - primary myelofibrosis, PV - polycythemia vera.

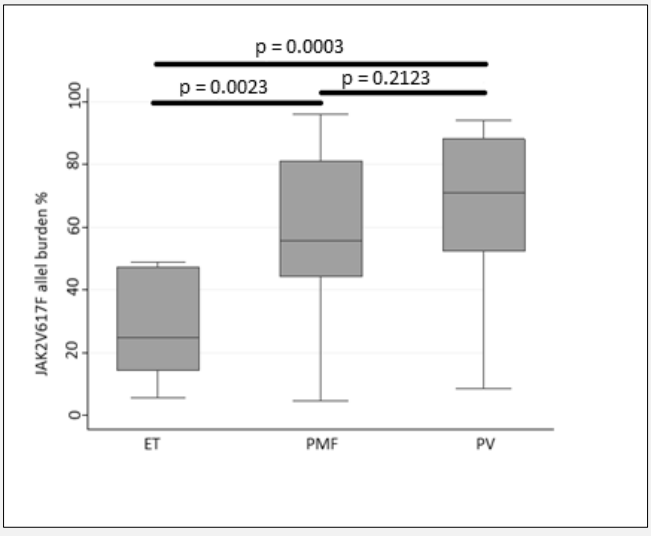

Figure 1. Comparison of V617F allele burden among ET, PMF, and PV patients.

Box and whisker plots demonstrating that the allele burden was significantly lower in ET than in PV (p = 0.0003) and PMF (p = 0.0023) but showed no statistically significant difference between PV and PMF (p = 0.2123).
ET - essential thrombocythemia, PMF - primary myelofibrosis, PV - polycythemia vera.

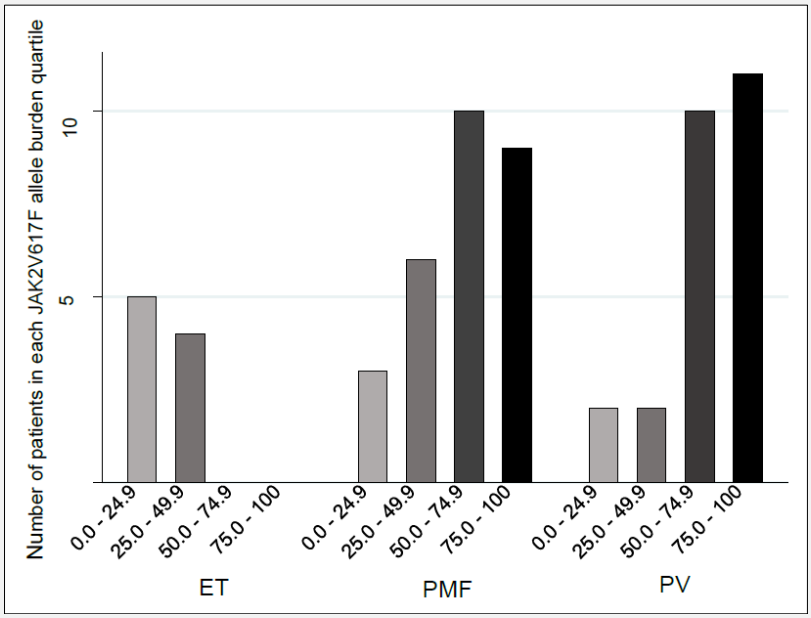

Figure 2. Distribution of V617F allele burden in different quartiles for ET, PV, and PMF patients.

Allele burden reported as percentages.
ET - essential thrombocythemia, PMF - primary myelofibrosis, PV - polycythemia vera.

Relationship between V617F allele burden, demographic, and laboratory results

Significant correlation ($p < 0.05$) was observed between increasing V617F allele burden and leukocytosis ($r = 0.58$), neutrophilia ($r = 0.59$), eosinophilia ($r = 0.29$), and decreasing erythrocyte mean corpuscular volume ($r = -0.31$). No correlation between allele burden and other laboratory and demographic results was noted.

DISCUSSION

The MHVL tested 235 patients as positive for V617F mutation over the 7-year period. Of these, 87 patients had clinical and laboratory data confirmatory of a newly diagnosed V617F-mutated MPN.

The median age at presentation in our cohort was 64 years and median age for ET, PV, and PMF patients with a V617F mutation was 64 years, 60 years, and 66.5 years, respectively. Though no statistical difference in gender of all patients with MPNs was seen, a gender difference was noted between PMF and PV ($p = 0.0293$), with relatively more males having PMF than PV and more females affected by PV than PMF. These results generally concur with data available from American and European centers [21,22].

PMF is the most aggressive Ph negative MPN and was prevalent in our study, accounting for 43.7% of cases. Although our study was specific for V617F positive patients, a 25-year retrospective review of clinical data on all MPN patients from a large academic hospital in our region confirmed this finding, with 45% of patients having a diagnosis of PMF [24]. This latter study predominantly reviewed patients diagnosed prior to the discovery of the V617F mutation and the mutational status of the bulk of the patients was unknown. This differs from studies in America, Europe, and Asia where PMF is the least commonly diagnosed MPN, comprising approximately 11 - 25% of all Ph negative MPNs and approximately 12% of V617F positive cases [21,22,27,28]. In addition, the majority of PMF patients in our current study had overt PMF (87% of cases), contrasting with the reported trend to earlier presentation of PMF patients in the pre-PMF stage in other centers, where only approximately 30 - 50% of patients present with overt PMF [28,29]. This may be due to late clinical presentation and/or biologically more aggressive disease in our patients.

In contrast to studies in Europe where V617F-positive ET is the most common V617F-positive MPN and also the most common of all Ph negative MPNs comprising approximately 30 - 45% of cases [21,27,28,30], ET was surprisingly lower than anticipated in this cohort and was the least represented MPN (11.5%). An attempt

was made to identify possible ET patients from V617F-positive patients who had been excluded due to absence of diagnostic bone marrow biopsy. Based on FBC results (i.e., patients with isolated thrombocytosis), this attempt did not yield significantly more patients with suspected ET. The reason behind the under-representation of ET is uncertain but could reflect a different disease spectrum of MPNs in our local population. ET may also be underdiagnosed due to a milder clinical course [31].

Leukocytosis was noted in more than 50% of patients with PV and PMF at presentation and was predominantly due to neutrophilia. Leukocytosis is known to predispose to thrombotic complications [32] and is associated with inferior survival in MPNs [33]. Over 40% of overt PMF and all pre-PMF patients had thrombocytosis with few or no peripheral blood morphologic features which accurately distinguished them from ET. This has been described in the literature [31] and emphasizes the importance of BM biopsy to accurately subtype MPNs, especially in differentiating ET and pre-PMF. In comparison to ET, pre-PMF and overt PMF have worse prognosis and reduced overall survival [30], thus accurate diagnosis is important for optimal patient care.

In concordance with other studies [3,34-36], significantly lower allele burden was observed in ET than in PV and PMF in this study. However, V617F allele burden was notably higher than in many studies performed in Europe and Asia where allele burden in ET rarely exceeds 25% and the majority of PV and PMF patients have allele burdens less than 50% [3,15,34,37]. Although all ET patients in this study had V617F allele burden below 50%, the majority had an allele burden over 25%. Allele burdens above 25% predispose to increased thrombotic events in ET patients and are a marker for more aggressive disease [15]. The majority of PV and PMF patients in this study had V617F allele burden above 50%, and this has been associated with a poorer clinical course [17] and higher risk of transforming to secondary myelofibrosis [38].

A higher V617F allele burden was associated with leukocytosis above 11 x 10^9/L, neutrophilia, eosinophilia, and a decrease in mean cell volume (MCV). However, contrary to other studies [16,38,39], no association was observed with an increase in hemoglobin, platelet count or LDH level.

Some of the limitations of this study include the inherent constraints of retrospective studies, absence of adequate archived DNA samples, and the unavailability of bone marrow biopsies in all patients. For the latter patients, some may have been investigated for reasons other than MPNs such as unprovoked veno-occlusive disease. However, the final cohort had complete results and displayed important demographic and laboratory features of MPNs in a South African, and indeed African setting.

CONCLUSION

This study suggests that MPNs in our setting show features which differ from the published international literature. ET is the least commonly diagnosed V617-associated MPN in this study and PMF comprises more than 40% of cases diagnosed. This differs from many studies where ET or PV are much more commonly diagnosed than PMF which is a much rarer diagnosis. In addition, the majority of PMF patients in this study have overt PMF rather than prefibrotic disease which suggests poorer prognosis at presentation. This contrasts with other studies which show earlier presentation with a higher proportion of patients in the prefibrotic PMF stage. In agreement with published data, V617F allele burden is significantly lower in ET than in both PV and PMF. However, the three MPNs reveal relatively high V617F allele burdens when compared to other published studies, which is known to be associated with higher risk disease. This may reflect a more aggressive disease phenotype in the study population; however, a prospective follow-up of these patients with known allele burden to assess their clinical disease progression would be valuable. Furthermore, prospective community-based, multicenter studies would be invaluable to confirm the above findings and to establish an accurate database of MPNs in South Africa. This may assist in recognizing patients who may benefit from earlier interventions to mitigate complications of MPNs such as hepatosplenomegaly, secondary myelofibrosis, thrombotic and bleeding events as well as blastic transformation.

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The authors declare no conflicts of interest.

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