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RESEARCH ARTICLE

JAK2/STAT5 Pathway Mutation Frequencies in South African BCR/ABL Negative MPN Patients

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Abstract

Background: **Mutations in JAK2/STAT5 proliferation pathway genes are key in the diagnosis of myeloproliferative neoplasms (MPN$^{BCR/ABLneg}$), with *JAK2V617F* being found in 50–97% of MPN$^{BCR/ABLneg}$ subtypes. Low *JAK2V617F* positivity at our facility suggested that our South African MPN$^{BCR/ABLneg}$ population may have a different mutational landscape.**

Objectives: **We aimed to determine the JAK2/STAT5 mutation frequencies associated with our local MPN$^{BCR/ABLneg}$ population, thus determining the relevance of these molecular tests in this group. We also investigated the haemato-pathological relevance of each test request, to assess testing practises.**

Method: **This study involved the retrospective audit of 886 patients for whom *JAK2V617F* mutation testing had been requested for a suspected MPN diagnosis. FBC indices, erythropoietin levels and bone marrow biopsy results were used to classify the patients. *JAK2V617F* ^{negative} patient DNA was tested for calreticulin (*CALR*) exon9, myeloproliferative leukaemia protein (*MPL*) codon515 and *JAK2* exon12 mutations.**

Results: **Only 23% of the patients demonstrated *JAK2V617F* positivity, with an additional 29 cases of *CALR*/*MPL* mutations being detected. Mutations were only detected in patients with abnormal FBC indices, as expected, yet 37% of the test requests were not associated with abnormal parameters at the time of testing. Mutation frequencies were as follows: Polycythaemia Vera: 97% *JAK2V617F*/3% (*JAK2*,*CALR*,*MPL*) triple negative; Essential thrombocythemia: 72% *JAK2V617F*/23%*CALR*/5%triple negative; Primary Myelofibrosis: 78%*JAK2V617F*/16%*CALR*/6%triple negative.**

Conclusion: **Our study demonstrated that our MPN$^{BCR/ABLneg}$ patients have a similar genetic landscape to other MPN populations, with >93% being able to be diagnosed by testing for the *JAK2V617F* and *CALR* exon9 mutations alone. Adoption of the WHO 2016 guidelines is recommended to guide testing practices.**

Keywords: JAK2, MPL, CALR, African, MPN, ET, PV, Myelofibrosis

1. Introduction

Essential Thrombocythemia (ET), Polycythemia Vera (PV) and Primary Myelofibrosis (PMF) are classified as BCR/ABL negative myeloproliferative neoplasms (MPN$^{BCR/ABLneg}$), distinguishing them from the MPN Chronic Myeloid Leukaemia (CML), where the primary pathological event is the t(9;22) translocation and the expression of the oncoprotein BCR/ABL [1,2]. Historically they have been laborious to diagnose, as their haematopathological parameters are initially difficult to discern from reactive conditions such as allergic reactions, infection or even a reaction to severe dehydration [1,3]. With the discovery that a malfunctioning Janus kinase 2/Signal transducer and activator of transcription 5 (JAK2/STAT5) signal transduction pathway is a key pathological event in the majority of these MPN diseases [4–8], diagnosis has become

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considerably easier and quicker. Diagnosis of MPN$^{BCR/ABLneg}$ now includes the detection of clonal mutations in the one of following genes:*JAK2* (exon14 g.1849G>T, referred to as *JAK2V617F* and exon12 mutations), myeloproliferative leukaemia protein (*MPL*) exon10 mutations (both types directly affecting the functioning of the JAK2-associated receptor) [7,8], and calreticulin (*CALR*) exon9 mutations [4,9,10], which affect the downstream STAT5 signalling component [4,11]. It has been reported that 90–97% of patients presenting with MPN$^{BCR/ABLneg}$ diseases have mutations in these genes [12] with defective signalling via the JAK2/STAT5(A and B) pathway, and consequential cell cycle activation [7]. This finding has not only led to improved diagnostics, but also specific JAK2 inhibitors, such as ruxolitinib, that can be used to treat these disorders [13].

The most predominant mutation in this disease group is the *JAK2V617F* mutation, a G-T base change that alters an amino acid in the JH2 pseudokinase domain (valine to phenylalanine), allowing autophosphorylation of tyrosine residues and receptor dimerisation, in the absence of ligand binding [5,6]. This mutation was the first to be associated with these diseases [7,14] and has been shown to have a frequency of 74–100% in PV, 23–57% in ET and 43–50% in PMF patients [5–7,11]. Due to its high positivity rate and clear diagnostic potential, most molecular haematopathology labs around the world rapidly instituted testing for the mutation in suspected MPN cases, with *JAK2V617F* positivity becoming a key part of the World Health Organistaion (WHO) 2008 and 2016 diagnostic guidelines [1,2]. Our laboratory (National Health Laboratory Service/Groote Schuur Hospital (NHLS/GSH), South Africa (SA)) initiated testing in 2007. However, while we received a large number of test requests, our positivity rate appeared low and we were concerned that our MPN$^{BCR/ABLneg}$ patient population may be different genetically from those of other countries, notably European countries and the USA.

The aims of this current study were to audit our *JAK2V617F* test requests over a 7-year period, to primarily assess the clinical relevance (in terms of associated haematopathological parameters) of each request. Secondly, we wanted to describe the mutational landscape of this cohort, by retrospectively testing for additional *CALR*, *MPL* and *JAK2* exon12 mutations in *JAK2V617F*^{negative} patients. This was to determine the relevance of these additional JAK2/STAT5 pathway mutations in our MPN$^{BCR/ABLneg}$ patients and establish if we had a significant triple mutation negative (*JAK2/CALR/MPL*) population that requires further study.

2. Methods

2.1. Ethical considerations

Ethics approval for this retrospective study was obtained from the Health Sciences Human Research Ethics Committee of the University of Cape Town (UCT), reference number: HREC REF 150/2016. All procedures performed in this study were in accordance with the ethical standards of UCT, which are in line with the 1964 Helsinki Declaration and its later amendments. As this was a retrospective analysis, individual informed consent was not acquired.

2.2. Patient cohort and MPN diagnosis

This study involved the analysis of 886 consecutive J*AK2V617F* test requests received at the NHLS/GSH Molecular Haematology laboratory over a 7-year period (2011–2018). These samples were received from GSH, various Western Cape primary state clinics/hospitals, private Western Cape Haematology/Oncology clinics and several Eastern Cape state hospitals (Port Elizabeth, Livingstone, Frere) and the test request was based on a query MPN diagnosis and/or indications of polycythaemia or thrombocytosis. The following data, resulted within 7 days of the genetic request was collected for each patient: evidence of splenomegaly, white cell count (WCC), red cell count (RCC), platelet count (Plt), haemoglobin (Hb), haematocrit (Hct), erythropoietin levels (EPO), lactate dehydrogenase (LDH), peripheral blood (PB) film observations and bone marrow (BM) aspirate/trephine reports. All BM samples were assessed centrally by NHLS/GSH Haematology pathologists. Definitive diagnoses of ET, PMF (overt/pre-fibrotic) and PV were based primarily on the WHO 2016 guidelines [2], with the utilisation of WHO 2008 [1] and BSH 2018 guidelines (for PV) [15] in the absence of a clonal mutation or BM analysis.

2.3. JAK2/STAT5 pathway mutation detection

It must be noted that all DNA extractions and mutation testing were performed centrally at the NHLS/GSH Molecular Haematology laboratory.

2.3.1. JAK2V617F

DNA was previously extracted at the NHLS/GSH complex from EDTA-anticoagulated PB, using the Maxwell 16 nucleic acid extractor (Promega), with samples only being acceptable if extracted within 3

days of collection to preserve the myeloid cellular component and having a concentration of >25 ng/μl and an A260:A280 > 1.70. The presence of the *JAK2V617F* (G-T base change) mutation was tested first using an NHLS/GSH in-house high-resolution melt real-time PCR assay (with previously reported primers/probes [16[), on a ROCHE Lightcycler 2.0 system, to a sensitivity of 2% mutated alleles (details in Appendix A). The *JAK2V617F* homozygous status was only reported when the peak area of the mutant peak was >50% (area of mutant peak/area mutant peak + area WT peak).

2.3.2. Detection of CALR exon9, MPL exon10 and Jak2 exon12 mutations

Stored *JAK2V617F*^{negative} DNA samples were first re-tested for the presence of *CALR* exon9 insertion/deletion (INDEL) and *MPL* g.1544G>T and g.1544TG>AA (codon515) exon10 mutations simultaneously, using a novel multiplex PCR - capillary electrophoresis approach based on the methods of Klampfl et al. [4] and Takei et al. [17] (described in Appendix A). The detection limit of mutant alleles was determined to be 5% for these mutations. DNA samples demonstrating triple negative *JAK2V617F/CALR/MPL* mutation status were tested for the most common *JAK2* exon12 mutations, namely INDEL mutations (covering mutation hotspot codons 534–547) [18], using a PCR-capillary electrophoresis approach (details in Supplementary document A) specifically utilising primers designed to avoid amplification of known non-pathogenic polymorphisms [19]. All INDEL positive DNA samples were re-tested and sequenced via standard Sanger sequencing methodology using the Big Dye terminator cycle sequencing kit (ThermoFisher Scientific). DNA was only viewed as harbouring a *JAK2* exon12 mutation if duplicate analyses correlated.

2.4. Data and statistical analysis

Patients in the cohort were grouped based on *JAK2V617F* mutation status and/or MPN disease subtype. The negative sub-cohort was filtered based on FBC indices at diagnosis and further mutation testing was then performed on the suspected MPN JAK2V617F^{negative} group. The number of each MPN subtype in the cohort, as well as the number of the different types of mutations in these subtypes was graphical presented. The median value of the FBC indices of patients within each mutation positive MPN subtype were also determined. No additional statistical analysis was required for this study.

3. Results

3.1. Audit of JAK2V617F test requests

The results of an audit of the *JA2V617F* test requests during the 7-year period are shown in Fig. 1, revealing a low 23% *JAK2V671F* positivity rate.

The relevant clinical data from the remaining *JAK2V617F*^{negative} patients was collected, to establish if the timing of the test requests was directly linked with MPN-associated diagnostic FBC indices. The most significant finding was the lack of any abnormal haematological parameters, within 7 days of testing, in 37% of the total cohort and 48% of the *JAK2V617F*^{negative} cohort (as shown in Fig. 1). It must be noted that in contrast, all *JAK2V617F*^{positive} patients had at least 1 abnormal parameter (defined in Fig. 1), in line with diagnostic features of MPN$^{BCR/ABLneg}$. Additional testing (e.g.: microbiology and immunology studies) allowed for diagnosis of non-MPN disorders in a further 27% of the *JAK2V617-*^{negative} cohort. The most common of these were reactive conditions (54/183), as expected, including evidence of infection (viral and microbial) or inflammatory conditions such as Rheumatoid Arthritis and Systemic Lupus Erythematosus. A cancer diagnosis, including both solid and haematological cancers (e.g.: breast cancer, CML) was finally established in a further significant proportion (42/183) (see Fig. 1).

The remaining 168 *JAK2V617F*^{negative} patients, all with at least 1 abnormal FBC indices, were combined with the *JAK2V617F*^{positive} cases to give a possible MPN$^{BCR/ABLneg}$ cohort of 373 patients. As indicated in Fig. 1, an additional 29 *JAK2V617F*^{negative} patients demonstrated either *CALR* or *MPL* mutations, but no *JAK2* exon12 mutations were detected. This bought the mutation positive rate in the clinically suspected MPN$^{BCR/ABLneg}$ cohort to 63% (234/373).

To confirm the relevance of abnormal haematological parameters in directing JAK2/STAT5 pathway mutation testing for suspected MPN$^{BCR/ABLneg}$ cases, all 330 patients showing normal FBC indices (including EPO), at the time of *JAK2V617F* testing, were also reanalysed for *CALR/MPL* mutations, with none detected.

3.2. Genetic landscape of MPN$^{BCR/ABLneg}$ cohort

3.2.1. Association of JAK2/STAT5 mutations with MPN subtypes

The above audit resulted in the identification of 234 patients with DNA mutations usually associated

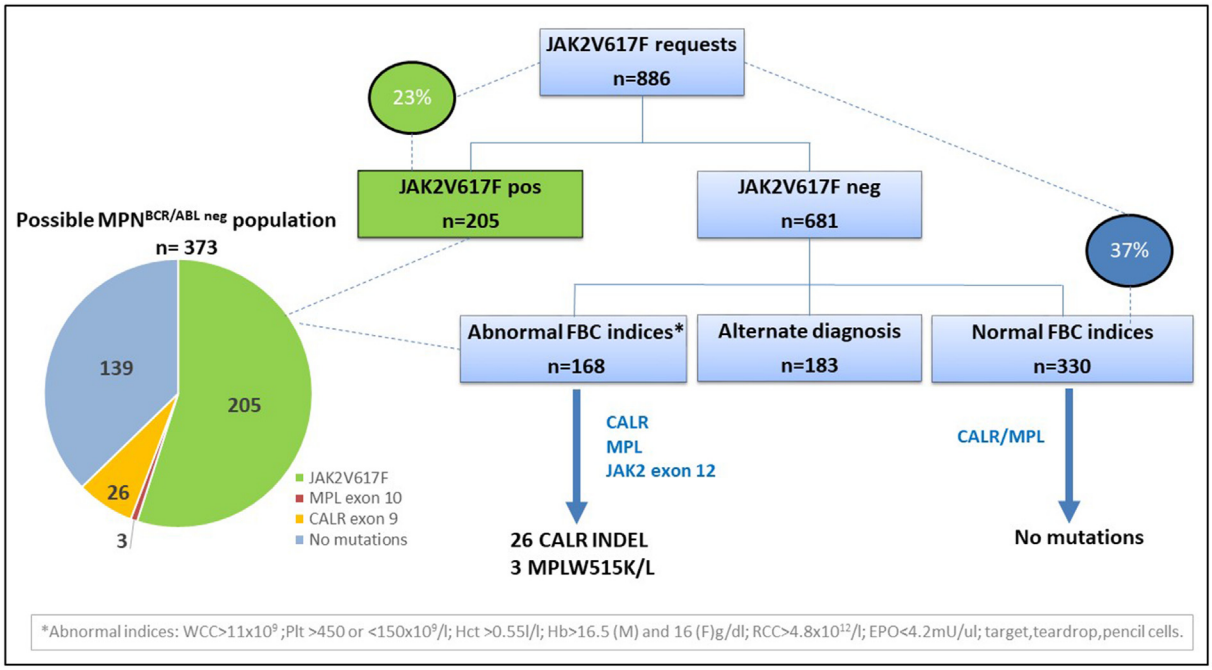

Fig. 1. ***Audit of JAK2V617F test requests and their association with abnormal pathological parameters.*** *A schematic showing the process followed to identify possible MPN$^{BCR/ABLneg}$ cases from those sent for JAK2V617F mutation testing. As only 205 cases were positive for the JAK2V617F mutation, the FBC indices (and serum EPO levels), BM biopsy reports and additional pathology test results were analysed to confirm the presence of MPN-associated indices and excluded alternate diagnoses. Abnormal WCC, Plt and Hb ranges were those defined by WHO[2], while abnormal Hct, RCC and EPO levels were defined using local reference ranges. Groups tested for additional CALR INDEL, MPL codon 515 and JAK2 exon 12 mutations are indicated. A final cohort of possible MPN cases was defined, with the pie-chart showing the mutational landscape of this patient group.*

with a diagnosis of MPN$^{BCR/ABLneg}$. Table 1 shows the distribution of these JAK2/STAT5 pathway mutations across the different MPN subtypes. Unfortunately, as can be seen in Table 1 26 (11%) of these mutation positive cases could not be definitively assigned an MPN classification, predominately due to the lack of a BM assessment record.

JAK2V617F mutations in our cohort were associated with a diagnosis of the 3 classical MPN$^{BCR/ABLneg}$ - ET, PV and PMF; while *CALR* INDEL mutations were only detected in ET and MF cases, as expected [20–22], *MPL* mutations were however unexpectedly not associated with the classical thrombocytosis usually observed in ET and PMF cases [1,2] and these patients could not be assigned a specific MPN diagnosis.

Table 1. Association of JAK2/STAT5 mutations with MPN subtypes.

Disease	JAK2V617F pos n = 205	CALR INDEL pos n = 26	MPL W515 K/L pos n = 3
ET	28	9	
PV	84		
PMF	59	12	
MPN-AML	5	3	
CMML	1		
CML on TKI	1		
Unclassified	21	2	3

JAK2/STAT5 mutations, namely *JAK2V617F* and *CALR* INDEL mutations, were also associated with a diagnosis of secondary AML (MPN-AML) in 8 cases, with patients having progressed from known cases of PMF or PV. In line with what has been previously reported [23], this evidence of MPN transformation was seen predominately in males (7/8).

As suspected CML cases are most commonly tested for *BCR/ABL* expression first, only 6 diagnosed CML cases were present in our patient cohort. While 5 were negative for *JAK2V617F* as expected, 1 CML case was found to possess the *JAK2V617F* mutation, along with the t(9;22) translocation. In this particular case, the patient had been on TKI therapy for several years and had achieved a major molecular response but had developed an elevated WCC with no associated *BCR/ABL* transcript increase. This result signals the development of an additional mutated clone, that is not associated with the *BCR/ABL* abnormality itself and will not be responsive to TKI therapy. This co-expression has been reported as a rare event in other studies [12].

3.2.2. Identification of possible JAK2/CALR/MPL triple negative MPN$^{BCR/ABLneg}$ cases

While the absence of clonal mutations or BM assessment makes classification of MPN$^{BCR/ABLneg}$

difficult, we used a combination of WHO 2008 and 2016 guidelines [1,2], as well as the BSH 2018 guidelines [15], to try to sub-classify all MPN$^{BCR/ABLneg}$ patients in our cohort, including potential *JAK2/CALR/MPL* triple negative cases. Using these systems, we were able to classify 211 of the 373 clinically suspected MPN$^{BCRs/ABLneg}$ cases, with Fig. 2 showing the distribution of these disease subtypes. Fig. 2 also details the mutation landscape of the classical MPN$^{BCR/ABLneg}$ cases (ET, PV, PMF) in our cohort.

As can be seen in Fig. 2, PV was the most common diagnosis amongst this suspected MPN cohort and demonstrated the expected high *JAK2V617F* positivity (97%) [1–3], with 19% of these having a dominant homozygous clone. No *JAK2* INDEL exon12 mutations were associated with the PV classification. Identification of potential *JAK2*^{negative} PV cases was complicated by the low number of BM assessments for patients with erythrocytosis, however, 3 potential *JAK2*^{negative} PV cases were putatively identified, based on the WHO and/or BSH 2018 criteria [1,2,15].

Defined ET cases demonstrated a high JAK2/STAT5 pathway mutation positivity of 95%, where *JAK2V617F* was the dominant mutation (72%) and a further 23% possessed *CALR* INDEL mutations. No *MPLW515L/K* mutations were associated with this MPN subtype, but 5% demonstrated triple mutation negativity.

A relatively high number of PMF cases were classified, with a ratio of overt fibrosis: pre-fibrotic cases being 3:1, demonstrating advanced disease of the majority of patients at diagnosis. As with the ET cases, a high level of MPN mutation positivity was observed (93%), with the *JAK2V617F* mutation again being dominant in 78% of cases and a slightly lower *CALR* INDEL positivity of 16% compared to ET, as previously reported [21]. No *MPLW515L/K* mutations were definitively associated with this MPN classification and only 7% of MF cases showed *JAK2/CALR/MPL* mutation negativity. Of particular interest was the presence of cases (18%) with a high *JAK2V617F* allelic burden (homozygote clones) in this MPN sub-cohort.

Table 2 shows the haematological and demographic parameters associated with the diagnosis of each MPN subtype. The median ages of diagnosis were in line with those previously reported [1,2], reflecting the association of an MPN$^{BCR/ABLneg}$ diagnosis with older patients. Female predominance was observed in ET diagnoses, as expected [9,11,20,23], but the expected male majority in PV cases [15,23] was not as obvious, with a 1.6:1 ratio observed. Clinical features were in good agreement with classical haematological features of each disease [1,2,15] with median values for RCC, WCC and Plt values in the PV cohort being higher than the normal range and 79% of PMF patients

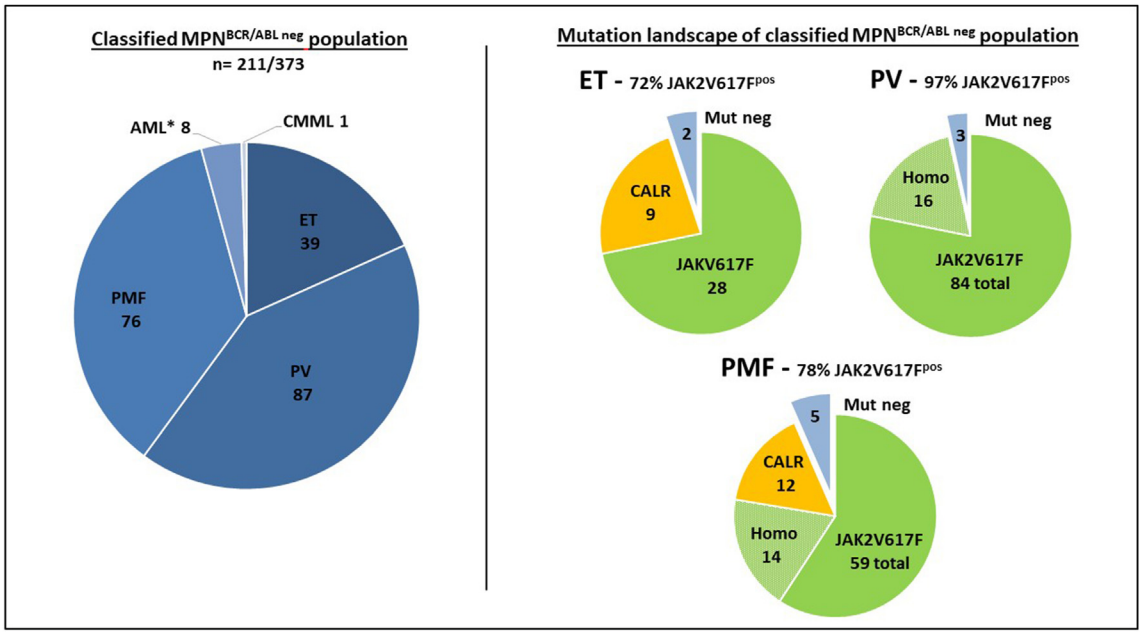

Fig. 2. ***Mutation landscape of classified MPN$^{BCR/ABLneg}$ subtypes.*** *A total of 211 patients in our suspected MPN cohort were sub-classified according primarily to WHO guideline[2]. and the pie-chart shows the proportions of each diagnosis. The mutational landscape of the common subtypes: ET, PV and PMF is also indicated, including the numbers of CALR INDEL (orange), JAK2V617F(green) and JAK2/MPL/CALR triple negative cases (pale blue). No MPL or JAK2 exon 12 mutations were associated with these common subtypes. The relative fraction of cases with a very high allelic JAK2V617F burden, indicative of homozygosity (homo) is also indicated.*

Table 2. Characteristics of mutation positive ET, PV and PMF cases (median values).

Parameter	ET mut pos n = 37	PMF mut pos n = 71	PV mut pos n = 84
Male: Female ratio	1:2 (12M/25F)	1.2:1 (39M/32F)	1.6:1 (50M/34F)
Age (yrs.)	59.6	63.6	59.7
WCC (x 10^9/l)	9.82	18.50	12.75
Plt (x 10^9/l)	1055	810	510
RCC (x 10^{12}/l)	4.28	4.13	6.71
Hb (g/dl)	12.4	10.2	18.2
Hct (l/l)	0.38	0.31	0.57
EPO (mU/μl)	No data	No data	2.5
2+ abnormal PB indicators#	83%	100%	100%
3+ abnormal PB indicators#	33%	77%	85%

= including the presence of teardrops/target cells/pencil cells on PB film morphology.

demonstrating anaemia (Hb < 12 g/dl (M), <13 g/dl (F)) [2], with the median Hb value of this cohort being 10.2 g/dl. As reported in other studies [11,20,24–26], ET patients with *CALR INDEL* mutations were a significantly younger population than ET *JAK2V617F*^{positive} (10 years, p value = 0.05), with significantly lower leukocyte counts and Hb at diagnosis (p value = 0.009 and 0.02, respectively). There was however no significant difference between the Plt counts of these ET groups.

It must be noted that the diagnostic utility of LDH and EPO levels was severely underutilised in the clinical investigations of these patients, with only 31% of classified PV cases having been tested for EPO and 17% of classified MF cases having a reported LDH value (all of which were out of the normal local ranges, as expected). BM assessment in confirmed *JAK2V617F*^{positive} PV cases was only performed in 25 cases (30%), potentially missing key signs of progression, notably fibrosis.

Probably the most important finding of this specific analysis was that 100% of mutation positive cases of PV and PMF showed 2 or more abnormal PB indicators and at least 75% showed 3 or more abnormal parameters (as detailed in Fig. 1), indicating the value of these parameters (including EPO) in guiding MPN mutation testing.

Despite the request for *JAK2V617F* testing and a query MPN diagnosis, 129 *JAK2/CALR/MPL* triple negative cases, all of which had abnormal FBC indices at the time of testing, did not have a follow-up BM. Fig. 3 highlights how a BM assessment may have allowed for an MPN$^{BCR/ABLneg}$ diagnosis in some of these cases despite the triple negative status, especially in the 18 patients showing polycythaemia and confirmed sub-normal EPO levels.

4. Discussion

Genetic analysis of patients is becoming a key pathology tool in both the diagnosis and prognostication of many different diseases, including myeloproliferative disease. Unfortunately, most of the research determining the pathogenesis of mutations is performed in northern hemisphere populations, usually with a Caucasian population bias. It is starting to become apparent that African populations may have different disease-causing mutations in some diseases, possibly leading to underestimation of disease burden, or inappropriate risk stratification and incorrect therapy administration. It is thus very important that African countries, including SA, report on the genetic landscape of their diseased populations to improve the clinical outcome of their patients. In addition, while the technology to do this type of testing is advancing at a rapid rate, education of clinicians and pathologists is lagging behind, leading to confusion over when to test and how to interpret the mutation status of a patient. In light of these potential issues, it was unclear whether the reportedly low *JAK2V617F* positivity rate (23%) in our suspected MPN patients was due to a genetically different MPN population or inappropriate test requests. We therefore performed a retrospective audit of 886 *JAK2V617F testing* requests over a 7-year period, to evaluate the "true" MPN$^{BCR/ABLneg}$ population in this cohort, determine the value of testing for additional *CALR* and *MPL* mutations and thus establish if our MPN population indeed showed a similar genetic landscape to those of European and American studies.

Our analysis unearthed a clear issue regarding when to test for JAK2/STAT5 mutations, specifically *JAK2V617F*, which is only associated with a diagnosis of MPN$^{BCR/ABLneg}$. While all of the patients with JAK2/STAT5 mutations (regardless of the final MPN subtype diagnosis), demonstrated at least 1 abnormal FBC parameter, in line with the WHO guidelines [1,2], none of the patients with a normal parameter profile tested positive for these mutations. This represented more than a third of the patient test requests (37%). While it is acknowledged that a small number of these patients may

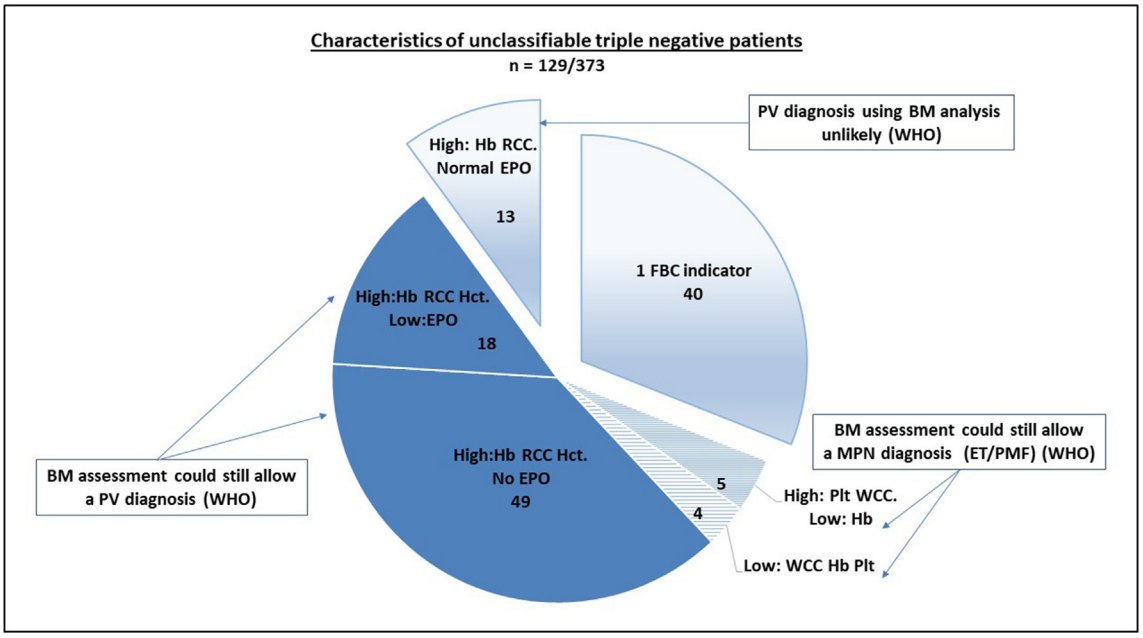

Fig. 3. ***Characterisation of unclassifiable JAK2/CALR/MPL triple negative cases.*** *Schematic showing the MPN-associated features of the remaining JAK2/CALR/MPL mutation negative patients in our patient cohort, all of whom exhibited at least 1 FBC parameter at the time of testing but did not undergo a BM biopsy. The WHO[1,2] allows for MPN$^{BCR/ABLneg}$ diagnoses in the absence of mutations, but BM assessment is required for this. Arrows indicate possible cases that may have benefited from a BM biopsy.*

have been treated with venesection or hydroxyurea prior to testing (undisclosed information), these results strongly support *JAK2/STAT5* mutation testing for an MPN diagnosis only in the presence of persistent clinical indicators, as recommended by the WHO [1,2].

Following the exclusion of patients with alternate diagnoses, as well as those with normal FBC profiles, 373/886 test requests potentially represented our MPN$^{BCR/ABLneg}$ patient cohort. After testing for the additional *CALR* exon9 INDEL, *MPL* codon 515 and *JAK2 exon12* mutations in the *JAK2V617F*^{negative} sub-cohort, a total of 234 of these patients possessed MPN-associated mutations, with diseases including CMML, secondary AML, ET, PV and MF identified in this mutation-positive group. To identify potential *JAK2/CALR/MPL* triple negative cases, all 373 patients in this potential MPN cohort were classified according to the WHO 2008 and/or 2016 guidelines [1,2] and the genetic landscape of each subtype determined. While the *JAK2V617F* mutation testing alone allowed for the identification of more than 70% of WHO-classified ET/PMF and 97% of PV cases, the detection of additional *CALR* mutations allowed a clear MPN diagnosis in 95% of ET and 93% of PMF cases, demonstrating the utility of testing for this additional MPN-associated mutation type. Analysis of the relevant clinical and haematological parameters of our classified MPN patients also demonstrated the classical features of these disease types, including the significant differences between *CALR* positive and *JAK2V617F* ^{positive} ET patients (i.e.: younger age group) [11,20,24–26].

JAK2 exon12 mutations are rare mutations (0–5%) in most cohorts [8,11,12,27], although occasional studies have noted higher levels (13–16%) [20,28], indicating a possible testing bias. Over 37 different mutations have been reported in the region of exon 12 encoding aa534-547 [18], including point mutations and INDEL mutations and different methodologies are used to try to detect these complex mutations [19,29], although highly sensitive methods are recommended due to the small, mutated cell populations involved [8,18,28,29]. Most studies used isolated granulocytes for genetic testing, often from BM rather than PB, thereby offering a better detection sensitivity [8,19,28–30]. This isolation step is however neither practical nor cost-effective in a standard diagnostic environment, especially considering the low positivity rate. As a PV diagnosis can still be made in the absence of *JAK2* mutations, following a BM assessment [1,2,15], and *JAK2* negativity in PV does not yet appear to hold any prognostic significance, the technical issues surrounding this assay do not necessarily support its routine use. We did not detect any *JAK2 exon12* INDEL mutations in the DNA samples, although the above testing issues may be responsible for this.

The most common *MPL* mutations associated with MPN pathogenesis are those that alter codon 515 (exon10), namely W515 K/L mutations (90% of detected mutations) [31,32], although rarer mutations including W515A and S505N have been reported [31–33]. *MPL* mutations in MPN cases are also a relatively rare event, with frequencies usually reported in the 1–8% range in both ET and PMF [9,17,26,27]. Our study only detected 3 MPL mutations, but as these cases did not show classical thrombocytosis features, they could not be classified into a specific MPN subtype. It is possible that our ET/PMF patients express some of the rarer *MPL* variants and this aspect should therefore be investigated. As shown in Fig. 4, the MPL codon515 mutation status, in addition to the other JAK2/STAT5 mutations, not only allows an MPN diagnosis, but also carries prognostic value, thus *MPL* mutation testing should be performed in suspected ET/PMF *JAK2V617F/CALR* negative cases, despite the rarity.

As can be seen in Fig. 4, defining MPN patients as triple negative cases is important, as it carries some prognostic value, especially in ET and PMF, potentially leading to better management of these patients. In many studies *JAK2/CALR/MPL* triple negativity has been reported at a rate of about 13–20% in ET and PMF, with PV cases being in the 0–5% range [11,26,27]. Our analysis showed slightly lower rates in ET and PMF cases, with 5% and 6% respectively and an expected 3% of PV cases. It is possible that our low BM biopsy rates in suspected MPN cases may be leading to an underestimation of this triple negative MPN group. This was highlighted in our audit, where a large number of triple negative cases could not be classified or an MPN diagnosis excluded fundamentally due to the lack of BM assessment and/or EPO levels. WHO guidelines [1,2] provide for MPN$^{BCR/ABLneg}$ diagnoses in the absence of *JAK2/STAT5* pathway mutations, yet there appears to be a definite bias against this additional testing, especially in potential PV cases.

A relatively large number of patients presented with overt PMF, and several with secondary AML, both indicating the progressive nature of these diseases in our population. Risk stratification of MPN, to identify patients at high risk of thrombotic/cardiovascular events, fibrotic transformation, leukaemia development and low OS is currently based on a multitude of parameters (i.e.: age, leukocyte count, anaemia) [22,34]. However, genetics are starting to play a more dominant role, including the JAK2/STAT5 pathway mutations (Fig. 4), with *JAK2V617F* homozygosity holding the most value at this point. Homozygote clones have been detected in PV, ET and PMF cases, but they are predominately associated with PV and postPV-MF [10,11,24,25,35–38], thus the identification of a homozygote *JAK2V617F* clone warrants a BM biopsy, to establish the level of fibrosis and BM failure. Understanding the rates of disease progression in our MPN patients may help develop earlier intervention

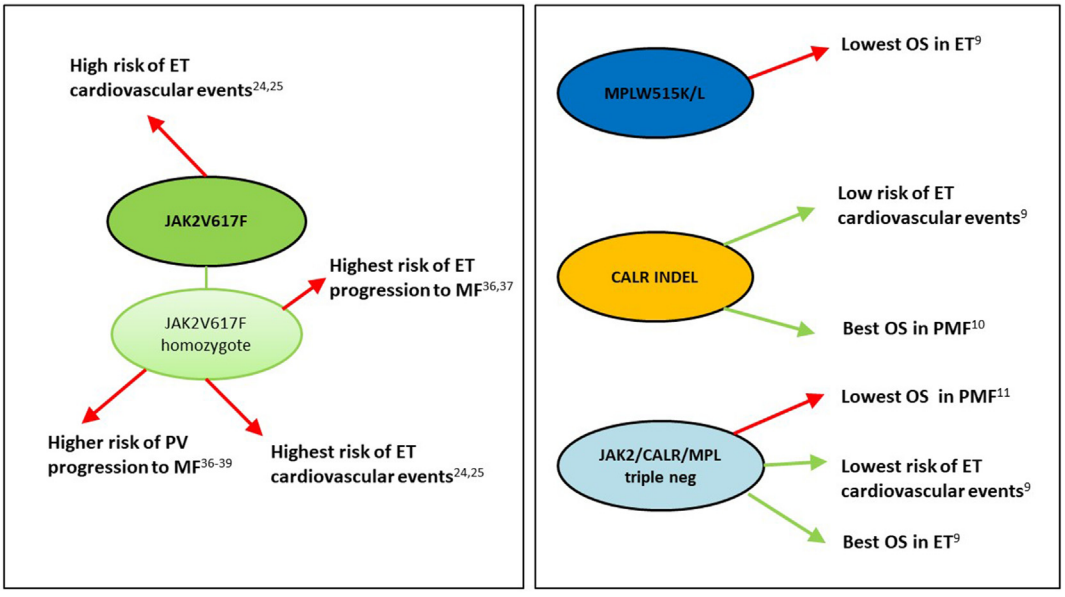

*Fig. 4. **Prognostic significance of JAK2/STAT5 pathway mutations in MPN$^{BCR/ABLneg}$.** A schematic showing the current consensus findings on the effects of JAK2V617F, MPL codon 515 and CALR INDEL mutations on the OS of the patient, risk of thrombotic events and risk of fibrotic transformation. Contested findings have not been included.*

strategies and improve patient outcomes and will thus be the subject of future studies.

In light of our findings, we can conclude that *JAK2V617FF* in addition to *CALR* exon9 INDEL mutation testing is sufficient to diagnose the vast majority (>93%) of the SA $MPN^{BCR/ABLneg}$ patient population and it appears that our patients share common pathological JAK2/STAT5 pathway drivers described in European/North American studies. Adoption of the WHO 2016 MPN diagnostic criteria is strongly recommended to guide the testing criteria for these mutations and thus reduce unnecessary expensive genetic testing. In line with these guidelines, regular testing of EPO levels in PV cases, and BM examination in suspected triple mutation negative MPN cases, will ensure a correct MPN diagnosis and prognostication.

Author contributions

K.S designed and directed the project and supervised students R.H and R.G. R.H, R.G, A.R and J.C designed and performed experiments and analysed raw experimental data; K.S and R.G analysed patient clinical and pathology data. K.S analysed complete data set and wrote article. All authors contributed to the critical analysis of the article.

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Conflict of interest

None declared.

Appendix A

DNA mutation analysis methodology

JAK2V617F (exon 14 g.1849G > T) mutation detection

The *JAK2* exon 14 g.1849G > T SNP was detected using a ROCHE Lightcycler 2.0, with HRM employed to identify the base change. Probes designed by Lay et al. [16] were combined with the following PCR primers to generate a 168 bp amplicon: JAK2F: AAGCAGCAAGTATGATGAG; JAK2R: CACCTAGCTGTGATCCTG; Target probe: 5′LCred640-CGTCTCCACAGACACATACTC-3′pho; Anchor probe:5′AAAGGCATTAGAAAGCCTGTAGTTTTACTTACTCT-6-FAM 3'. The following reaction mix was used (10 μl final reaction volume)": 5 pmol each primer, 2 pmol each probe, 1X LightCycler FastStart DNA MasterHybprobe mix (ROCHE, 1.5 mM additional $MgCl_2$ (4.5 mM in total), and 25 ng genomic DNA. Amplification and HRM were performed as follows: 95 °C/10 min; 40 cycles of 95 °C/5 s- 55 °C/5 seconds−72 °C/10 s, followed by 95 °C/10 s- 45 °C/60 s - 72 °C at 0.3 °C/s with continuous acquisition at 640 nm.

Detection of the CALR exon 9 and MPL exon 10 mutations (novel assay design)

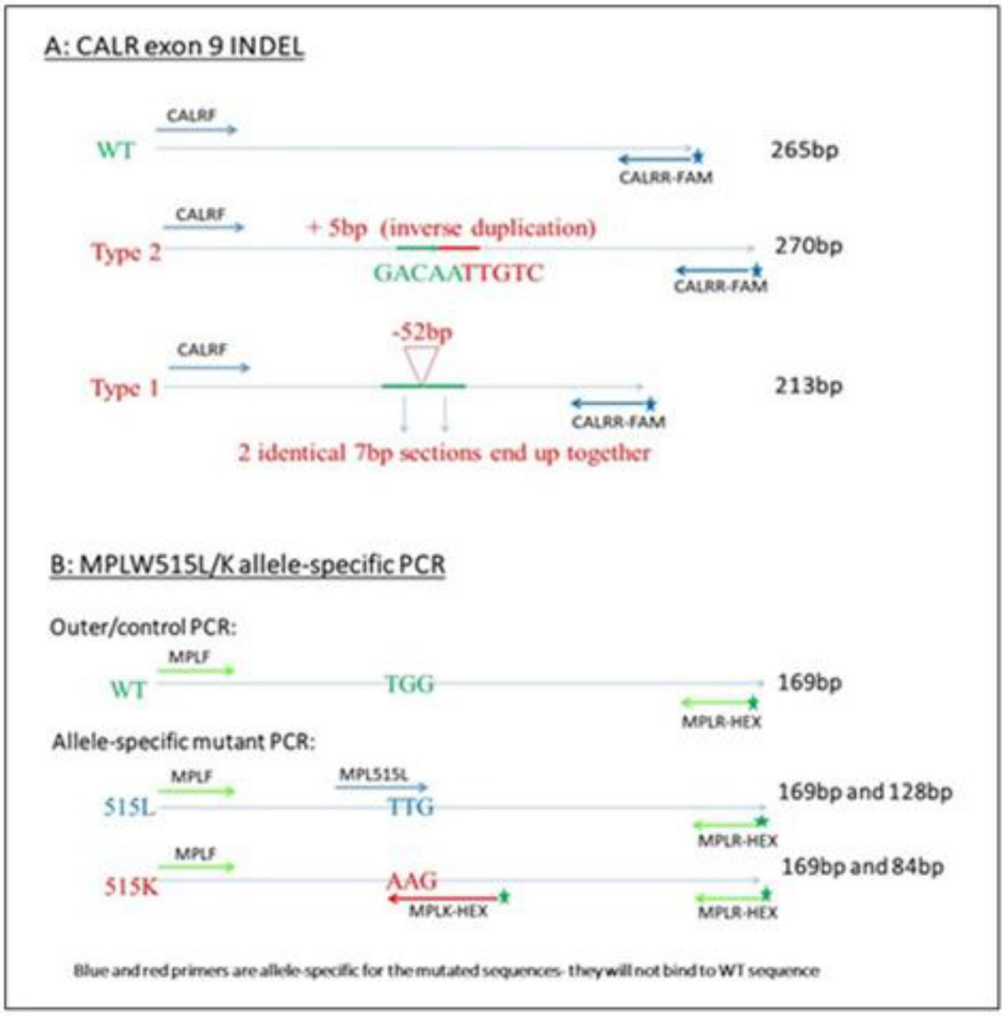
CALR exon 9 insertion/deletion (INDEL) and *MPL* g.1544G > T and g.1544 TG > AA (codon515) exon 10 mutations were assessed using a novel multiplex PCR assay with amplicon separation via capillary electrophoresis. *CALR* mutations were designed for identification through size anomalies (65 bp deletion to 25 bp insertions), while the *MPL* mutations were detected using allele-specific PCR (see Appendix Figure A). The following primers (HPLC-purified) were combined in a pre-prepared primer mix: CALRF: GGCAAGGCCCTGAGGTGT; CALRR: 5′FAM-CTCAGGCCTCAGTCCAGC (modified from Klampfl et al. [4]); MPLF: TGACCGCTCTGCATCTAGTG; MPLR: 5′HEX−GTCACAGAGCGAACCAAGAAT; MPL515L: GCCTGCTGCTGCTGAAGTT; MPL515K: 5′HEX-CTGTAGTGTGCAGGAAACTGCTT (modified from Takei et al. [17]). The following 30 μl PCR reaction mix was used: 2.5 mM $MgCl_2$, GoTaq clear reaction buffer (Promega), 0.3 mM each dNTP, 1.5U GoTaq Hotstart (Promega), 25 ng DNA, 25 pmol MPLF and MPLR-Hex, 10 pmol CALRF and CALRR, and 20 pmol MPL515K and MPL515L. The following cycling conditions were used on an ABI Proflex 96 well PCR system: 95 °C/5 min; 30 cycles of 96 °C/30 seconds−62 °C/30 seconds−72 °C/45 s, 72 °C/10 min. All amplicons were diluted 1:10 using Hi-Di formamide (ThermoFisher scientific) and separated on an ABI3500 analyzer (POP7, 36 cm column). The analysis was performed using Genemapper ID-X version 1.5, with details of the fragment analysis provided in Appendix Figure B.

Bins are pre-defined according to the expected fragment sizes for the WT PCR products and the expected sizes of the amplicons containing the mutations. Of note, the indicated sizes of the MPL products are 8 bp smaller than expected due to sizing inaccuracies of the ILS600 at these sizes.

Detection of the JAK2 exon 12 mutations

Only the most common *JAK2* exon 12 mutations, namely INDEL mutations (covering mutation hotspot codon 534-547) [18], where investigated. A 126bp amplicon was amplified using primers

designed by Jones et a [19], to specifically avoid amplification of known clinically irrelevant SNPs, with the amplicons separated by capillary electrophoresis to discriminate the INDEL mutations. JAK12F: 5'HEX-AATGGTGTTTCTGATGTACC; JAK12R: AGACAGTAATGAGTATCTAATGAC. The following 25μl PCR reaction mix was used: 1.5mM $MgCl_2$, GoTaq clear reaction buffer (Promega), 0.3mM each dNTP, 1.5U GoTaq Hotstart (Promega); 50ng DNA; 25pmol each primer. The following cycling conditions were used, using an ABI Proflex 96 well PCR system: 95°C/5 mins; 35 cycles of 95°C/15sec - 58°C/30sec - 72°C/30sec, 72°C/10 mins. All amplicons were diluted 1:10 into Hi-Di formamide (ThermoFisher Scientific) and separated on an ABI3500 analyser (POP7, 36cm column). Analysis was performed using Genemapper ID-X version 1.5, and allowed for identification of INDEL mutations as small as 1bp. All INDEL positive DNA samples were re-tested and sequenced via standard Sanger sequencing methodology using the BigDye terminator cycle sequencing kit (ThermoFisher Scientific). DNA was only viewed as harbouring a *JAK2* exon 12 mutation if duplicate analysis correlated.



Appendix Figure B: Separation of the CALR and MPL amplicons

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