HIGH-RISK JAK2 V617F ALLELE BURDEN IN A FIVE-YEAR COHORT OF MYELOPROLIFERATIVE NEOPLASM PATIENTS

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A research report submitted as a "submissible article" to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine (Haematology)

Johannesburg, 2019

DECLARATION

I Moses Chatambudza declare that this Research Report is my own, unaided work. It is being submitted for the degree of Master of Medicine (Haematology) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.


(Signature)

18th day of June 2019 in Johannesburg, South Africa

To my wife Belinda, my son Munesu, my grandmother Sophia and my late mom Lina

PRESENTATIONS FROM THIS STUDY

Oral presentations as follows:

1) M Chatambudza, S Carmona & T Wiggill. Detection and quantification of JAK2 V617F allele burden in myeloproliferative neoplasm patients at a Single Centre in South Africa. Faculty of Health Sciences Research Day, 6th of September 2018, University of the Witwatersrand, Johannesburg.

2) M Chatambudza, S Carmona, V Kana, L Skhosana & T Wiggill. Detection and quantification of JAK2 V617F allele burden in myeloproliferative neoplasm patients in South Africa. PathCape 56th International Federation of South African Societies of Pathology Congress. 16-18 August 2018, Spier Estate, Stellenbosch, South Africa.

3) M Chatambudza, S Carmona, V Kana, L Skhosana & T Wiggill. Myeloproliferative neoplasm-related Janus Kinase 2 V617F mutational status at a single centre in South Africa. 11th Annual Haematology Oncology Symposium. 11-12 May 2018, Sandton, Johannesburg.

PUBLICATIONS FROM THIS STUDY

This article will be submitted to Seminars in Haematology journal.

ABSTRACT

Introduction

JAK2 V617F is the most common somatic mutation associated with essential thrombocythemia (ET), polycythaemia vera (PV) and primary myelofibrosis (PMF). JAK2 V617F allele burden is important for diagnosis, prognosis and disease monitoring. This study aimed to verify detection and quantitation of allele burden using a commercial quantitative allele specific PCR assay (QUASA). Furthermore, the study intended to determine JAK2 V617F allele burden in a five-year retrospective cohort of patients previously diagnosed with ET, PV and PMF.

Materials and Method

QUASA was verified using World Health Organization (WHO) DNA standards, external quality assurance material (EQA) and characterised remnant patient DNA samples. A laboratory database search was performed to identify demographic and laboratory results of all patients who tested positive for JAK2 V617F mutation in the Molecular and Virology Laboratory at Charlotte Maxeke Johannesburg Academic Hospital from 1 January 2012 to 31 December 2016. Allele burden was measured on residual DNA samples from these patients that were previously diagnosed with JAK2 V617F-positive ET, PV and PMF.

Results

QUASA correctly detected JAK2 V617F mutation qualitatively in all samples tested (WHO standards, EQA material and remnant patient DNA samples). It quantified the allele burden from 0,1% to 100% with a bias of 0,288 and correlation coefficient of 0,9999. One thousand two hundred and twenty (1220) screens were performed for JAK2 V617F mutation over the 5-year period. Of these, 205 were found to be positive. Median age at diagnosis of myeloproliferative neoplasms (MPNs) was 65 years with a male: female ratio of 1,1:1. ET, PV and PMF accounted for 11%, 46% and 43% of the MPNs, respectively. Median allele burden for ET, PV and PMF was 47,25%, 72,05% and 62,62%, respectively. Allele burden was significantly lower in ET compared to PV (p=0,011) and PMF (p=0,025) and correlated with leucocytosis, neutrophilia, eosinophilia and a low erythrocyte mean cell volume (p<0,05).

Conclusion

QUASA is sensitive and specific for detecting JAK2 V617F, as well as accurate and precise in quantitating allele burden. JAK2 V617F-positive MPNs occurred in older patients with

approximately equal sex ratio. ET was the least common MPN. Allele burden was overall high for all 3 subtypes of MPNs, which may predispose to a poorer prognosis.

A prospective community-based study would be invaluable to confirm the above findings and to establish an accurate database of MPNs in South Africa. Prospective follow-up of a cohort of patients with known allele burden and correlation with clinical outcome would also be of great value.

ACKNOWLEDGEMENTS

My sincere gratitude goes to:

i. My supervisors Dr Tracey Wiggill and Dr Sergio Carmona who have imparted their expertise and spirit of hardworking to me,
ii. The Beit Trust and Wits Health Faculty Research Centre for funding this research project,
iii. The Beit Trust and University of the Witwatersrand for funding my postgraduate studies,
iv. NHLS PCR medical scientists Vibha Kana and Lindiwe Skhosana for training me on technical issues and troubleshooting in PCR,
v. Dr Tina Kresfelder, Roche®/Molecular Diagnostics and Applied Science Applications Specialist Trainer, for training and reviewing PCR settings on Lightcycler 480 II,
vi. My mentor, Professor Johnny Mahlangu, who has been a pillar of strength and support throughout this project,
vii. Professor Moosa Patel for advice on epidemiology of MPNs in our setting,
viii. NHLS for granting me necessary laboratory resources to carry out this research, especially Academic Affairs department and Central Data Warehouse for allowing me access to patients' information.

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LIST OF ABBREVIATIONS

ARMS	Amplification refractory mutation system
BMA	Bone marrow aspirate
BMT	Bone marrow trephine
CDW	Central Data Warehouse
CHIP	Clonal haemopoiesis of indeterminate potential
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
ddPCR	Digital droplet polymerase chain reaction
ET	Essential thrombocythaemia
EQA	External quality assurance
FAM	Fluorescein amidite
FBC	Full blood count
JAK2	Janus kinase 2
LDH	Lactate dehydrogenase
MDS	Myelodysplastic syndrome
MPN	Myeloproliferative neoplasm
NHLS	National Health Laboratory Service
NGS	Next generation sequencing
PCR	Polymerase chain reaction
PMF	Primary myelofibrosis
PV	Polycythaemia vera
QUASA	Quantitative allele specific PCR assay
RCPA	Royal College of Pathologists of Australasia
STAT	Signal transducer and activator of transcription
WCC	White cell count
WHO	World Health Organisation

SUBMISSIBLE ARTICLE

1 │INTRODUCTION

The World Health Organisation (WHO) identifies polycythaemia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF) as the classical Philadelphia chromosome negative myeloproliferative neoplasms (MPNs)[1]. These are clonal myeloid disorders characterised by autonomous proliferation of haematopoietic precursor cells leading to increased mature blood cells[2]. As a result, patients have significant disease burden including organomegaly, increased thrombotic and bleeding complications, and risk of progression to myelofibrosis and acute leukaemia[1].

JAK2 V617F (hereafter referred to as V617F) is the most common driver mutation implicated in MPNs, reportedly present in over 95% of PV and 50-60% of ET and PMF patients[3]. The mutation results in a nucleotide change from guanine to thymine at base 1849 in exon 14 of the JAK2 gene, resulting in substitution of valine for phenylalanine at codon 171. Consequently, this leads to enhanced and dysregulated cell signalling via JAK/STAT (signal transducers and activators of transcription) pathway[4].

V617F-associated MPNs are biologically related[5,6] with mutant allele burden important in determination of diagnosis, clinical phenotype, prognosis and monitoring of therapy[7-9]. Allele burden may add valuable information on the subtype of MPN and on the prognosis of the patient. Follow-up of allele burden may be useful to predict transformation or more aggressive disease [10]. V617F allele burden is generally low in ET patients at diagnosis and does not frequently exceed 20%[11]. ET patients with allele burden above 25% have high risk of arterial thrombosis and splenomegaly[12,13]. The allele burden usually rises with transformation of ET to either PV, myelofibrosis or acute leukaemia, which are more aggressive disease states[7,14,15].

De novo PV and PMF are associated with higher baseline allele burden than ET, and the higher the allele burden, the worse the clinical phenotype[16,17]. Some of the clinical features that are associated with mutant allele burdens greater than 75% in PV patients include pruritus, three-fold higher risk of thrombosis and higher frequency of clinically detectable and massive splenomegaly[18,19]. PMF patients with allele burden above 50% frequently present with leucocytosis, massive splenomegaly, higher requirements for splenectomy and chemotherapy, and high risk of acute leukemic transformation[17,20]. However, PMF patients with allele burden less than 25% have unexpectedly decreased overall survival[21,22], the cause of which is unclear.

V617F allele burden may also be utilised to monitor therapy. Long term treatment of patients with myelofibrosis using ruxolitinib, a targeted JAK1/JAK2 inhibitor, led to significant reduction of allele burden, with some patients achieving molecular remission, improved clinical symptoms and reduction in spleen size[9,23]. Furthermore, allele burden determines the adequacy of pre-transplant conditioning and disease relapse in transplanted myelofibrosis patients[24]. Desirable engraftment would be reflected by good haematological response and undetectable V617F, among other parameters assessed[25]. A highly sensitive and specific molecular technique such as allele-specific or digital droplet PCR (ddPCR) would be desirable in this instance.

The internationally accepted limit to diagnose a MPN is an allele burden of 1-2%, thus the appropriate diagnostic assay should have a lower sensitivity limit of at least 1%[26]. This is to exclude otherwise unaffected individuals with V617F mutation as a clonal haemopoiesis of indeterminate prognosis (CHIP)[26], in whom the allele burden is usually less than 1% and commonly below 0,1%[27], whilst at the same time not missing the diagnosis in patients with low allele burdens. Despite the cut-off limit described, a latent MPN may still be present even with a low allele burden below 1%, necessitating follow-up of these patients[28,29]. Consequently, low level allele burden detection is desirable and non-quantitative assays may have suboptimal sensitivity in detecting low level positivity. Fluorescence Resonance Energy Transfer (FRET) real-time PCR (PCR) and melting curve assay utilized routinely in our laboratory is not sensitive enough to detect low allele burden positive results. Although highly specific, FRET is semi-quantitative, and has suboptimal analytical sensitivity, in detecting an allele burden ranging between 2% and 10%[30,31]. This is a recognised limitation of the assay. [26,30].

Various assays are available for measurement of V617F allele burden. These include ddPCR, Taqman and allele-specific quantitative real-time PCR, amplification refractory mutation system (ARMS), pyrosequencing, DNA sequencing and mass spectrometry[32].

Digital droplet PCR is a highly specific, sensitive, precise and accurate technique which has a limit of detection as low as 0,01% for V617F mutation[33] and appears superior to qPCR in sensitivity and precision[24,33]. However, it is currently unavailable in our laboratory.

Given the shortcomings of FRET and melting curve assay, we aimed to verify a commercially available quantitative allele-specific PCR assay (QUASA) to improve detection threshold as well as to quantify V617F allele burden in MPNs. Quantitation of the V617F allele burden would enhance prognostication and monitoring of MPN patients during follow up. Moreover,

unlike ddPCR-based assays, QUASA could be easily set up on existing PCR equipment in our laboratory.

The incidence and type of MPNs is known to vary with age sex and geographic location [1,34,35]. Notably, 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[36,37]. We aimed to determine demographic and laboratory characteristics of a cohort of patients previously 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.

2 | MATERIALS AND METHODS

2.1| Ethics approval

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

2.2| Study setting

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

2.3| QUASA assay

2.3.1| Principles of the QUASA assay

V617F QUASA commercial kit (Primerdesign® Ltd, Chandler's Ford, United Kingdom) employs a modified mastermix, hydrolysis probe and two modified primers: a V617F single nucleotide polymorphism (SNP) detecting primer; and a wild-type (WT) detecting primer. The proportion of mutant amplicons is measured and expressed as a percentage relative to WT amplicons.

2.3.2|QUASA methodology

QUASA was performed as per the manufacturer's instructions (see attached protocol in appendix 7.5). Appropriate PCR quality control measures were employed to avoid contamination. The DNA detection mixes comprising of PCR mastermix, probe/primer mix, and DNase/RNase free water were prepared to analyse both SNP and WT for each sample. Subsequently, 15µl of DNA detection mix and 5µl of 5ng/µl DNA sample were dispensed into respectively labelled wells on the strips. Finally, 5µl of 1% V617 allele burden control were added to positive control well and 5µl of RNase/DNase free water dispensed into the template negative control well (blank). A JAK2 V617F negative control sample was included with each run (patient sample, EQA sample or WHO standard).

Real-time qPCR was performed using Lightcycler 480 (Roche® Molecular Diagnostics, California, USA). Cycling conditions included 2 minutes of enzyme activation followed by 45 cycles of denaturation, annealing and extension. Data was collected through Fluorescein amidite (FAM) channel during the 60°C annealing step.

The proportion of V617F SNP and WT in each sample was corrected against positive control standard, where SNP was present at a known proportion of 1%. This allowed detection WT: mutant ratio, which was used to calculate relative percentage of mutant and WT in each sample using the delta Ct formula.

2.4| Verification of QUASA

Qualitative detection of V617F mutation was verified using three types of material i.e.

a) Patient samples, including 43 positive and 17 negative samples identified with routine FRET and melting curve analysis. These were analysed in duplicate using QUASA. Sample size was determined using raosoft sample size calculator[38] with 99% confidence level, 5% margin of error and an estimated prevalence of V617F mutation in the general population of 0,2%[39].
b) Royal College of Pathologists of Australasia (RCPA) EQA material samples, including five randomly selected cases.
c) Known WHO standards, including seven samples.

Quantitative verification was performed using seven WHO standards with known allele burdens of 0%, 0,1%, 1%, 10%, 50%, 90% and 100%. These commercially available WHO standards were derived from mixing WT and mutant human donor genomic DNA and allele burdens were established using ddPCR[40]. Each standard was measured in this verification in triplicate in a single run to assess for intra-assay variability. To determine inter-assay variability, the standards were re-analysed in duplicate on a different day. Average allele burden was obtained for each of the standards and this was used to determine the accuracy, lower limit of detection and precision of QUASA.

2.5| Study population and data collection

All patients above 18 years old with a histologically confirmed diagnosis of ET, PV or PMF and complete demographic and laboratory results were included in the data analysis. The 2008 or 2016 WHO diagnostic criteria[1,41] for myeloproliferative neoplasms were used to confirm the diagnoses by the responsible haematopathologist depending on when the diagnosis was made

(please see Appendix 7.3 for 2016 WHO criteria for diagnosing ET, PV and PMF). Demographic data and relevant laboratory results were collected on patients that tested positive for V617F mutation using FRET and melting curve analysis assay in the MHV laboratory at CMJAH over a 5-year period (1January 2012 to 31 December 2016). Results were obtained from database searches of NHLS laboratory information system (Trakcare, InterSystems Corporation, Cambridge) and 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. Quality and quantity of DNA samples was assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific®, Wilmington, USA).

2.6 | Data analysis

Data was analysed using STATA software version 13 (College Station, TX: StataCorp LP). 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. Bland-Altman plot was used to assess for accuracy of allele burden as measured by QUASA on WHO standards. Spearman rank order correlation was used to assess relationship 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 or less.

3 | RESULTS

3.1| Qualitative Detection of V617F mutation using QUASA

The results using QUASA concurred with those obtained using FRET and melting curve analysis for 59 out of 60 patient samples (Table 3.1). One DNA sample from a peripheral blood specimen repeatedly tested positive on QUASA with a mean allele burden of 5,78% but was negative on two occasions using FRET and melting curve analysis. However, bone marrow sample from the same patient tested positive for V617F mutation with FRET and melting curve analysis. Furthermore, this patient was histologically confirmed to have a diagnosis of ET.

Table 3.1 Comparison of QUASA and FRET and melting curve analysis for the detection of V617F mutation in patient samples

	V617F positive cases using FRET and melting curve assay	V617F negative cases using FRET and melting curve assay
V617F positive cases using QUASA	43	1
V617F negative cases using QUASA	0	16

Additionally, QUASA correctly identified V617F mutational status (positive or negative) of 5 randomly tested Royal College of Pathologists of Australasia (RCPA) EQA material, one of which had been previously missed by FRET and melting curve assay. The latter sample had an allele burden of 3%. QUASA also correctly detected the mutational status of all seven WHO standards, 2 of which had been incorrectly classified as negative using FRET and melting curve analysis (the 0,1% and the 1% WHO standards).

3.2 | Quantification of V617F allele burden

The mean intra-assay variability was 12,2% (range: 0 – 55,45%) while mean inter-assay variation was 19,2% (range: 0 – 50,49%) when using a threshold of 0,1% allele burden (Table 3.2). This reduced to 6,0% (0,28% – 17,32%) and 16,2% (0,51% – 43,67%) respectively when considering a clinically relevant diagnostic threshold of 1% allele burden[26].

Almost 93% of allele burden measured by QUASA was within 2 standard deviations limits of agreement with a positive bias of 0,288% (95% confidence interval of the difference between the means: -0,126; 0,702) (Figure 3.1).

Linearity between 0% and 100% allele burden was demonstrated, with a correlation coefficient close to 1 (Figure 3.2).

Table 3.2 Intra- and inter-assay variability of V617F allele burden as measured by QUASA

WHO Standard allele burden (%)	Mean Intra-assay allele burden (%)	Intra-assay CV (%)	Mean Inter-assay allele burden (%)	Inter-assay CV%
0,00	0,00	0,00	0,00	0,00
0,10	0,13	55,45	0,10	50,49
1,00	1,70	17,32	1,32	34,67
10,00	14,08	9,86	11,34	28,70
50,00	50,89	1,70	50,89	1,01
90,00	90,66	0,95	90,21	0,94
100,00	99,11	0,28	99,55	0,51

Each standard was measured in triplicate in a single run to assess for intra-assay variability. Each standard was measured in duplicate on a different day to assess inter-assay variability

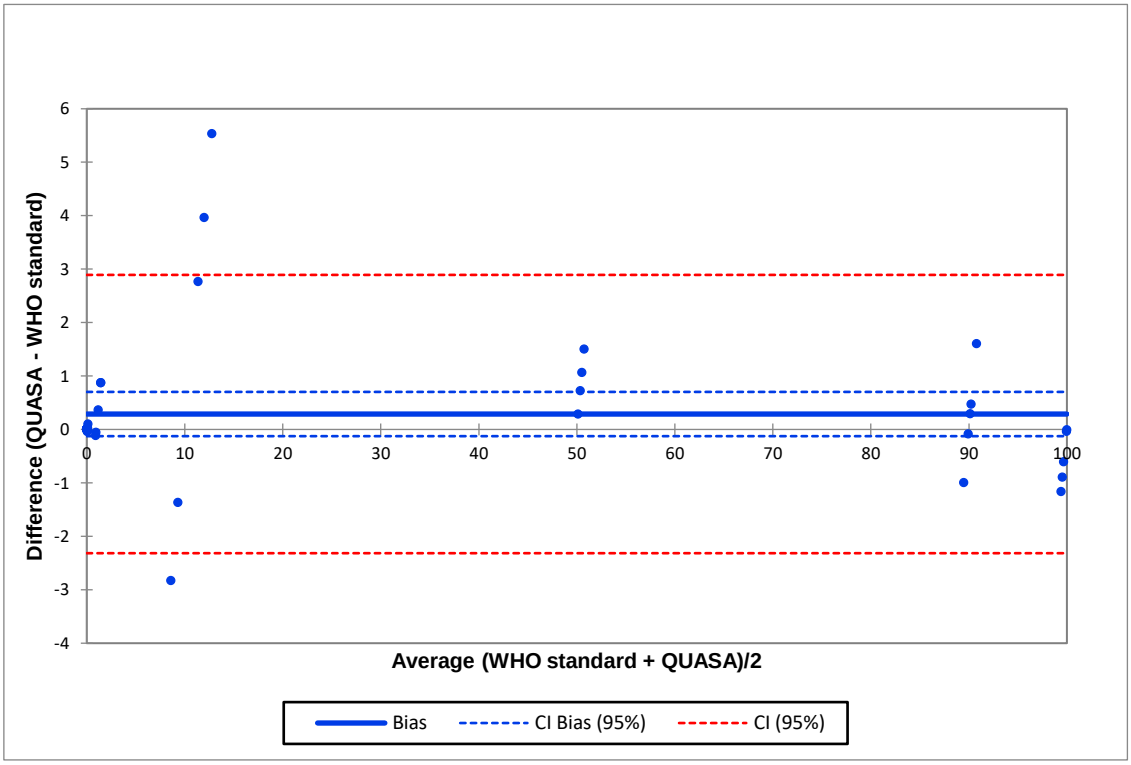

Figure 3.1 Limits of agreement of QUASA and the expected results from the WHO standards (derived by ddPCR). *Bias: 0,288. 95% confidence interval of the difference between the means: -0,126; 0,702*

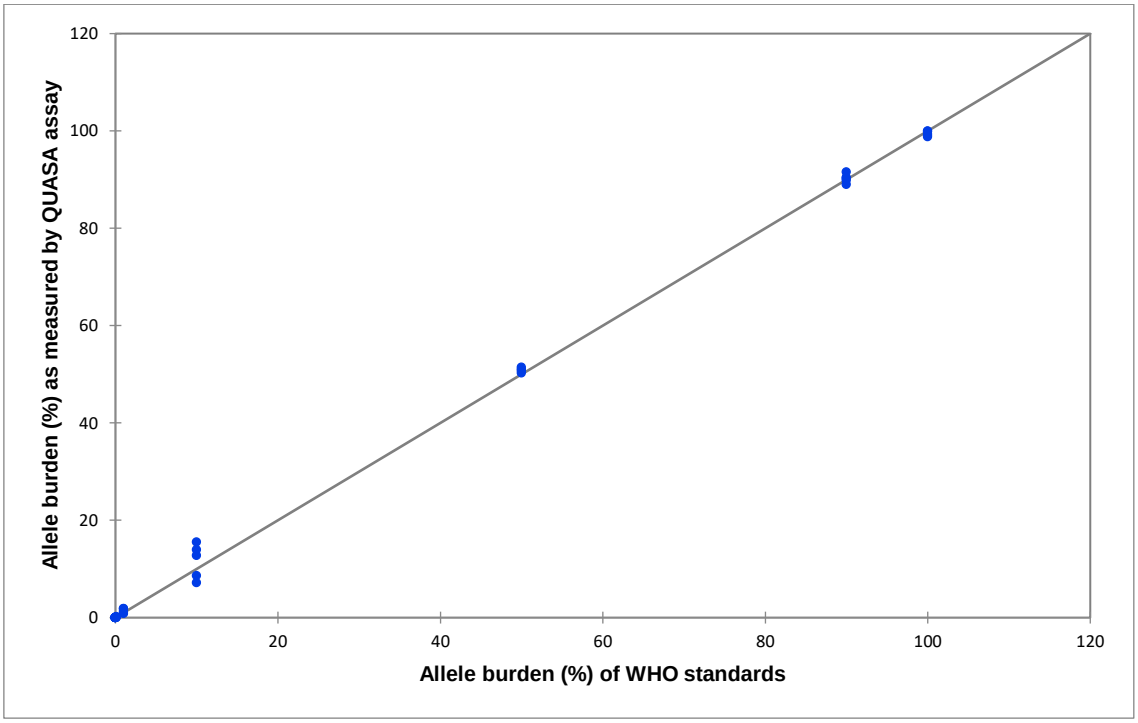

Figure 3.2 Correlation between allele burden measured by QUASA and the expected results as per the WHO standards. *R^2= 0,9999; y=0,4136 + 0,9947x; p-value = 0,0000*

3.3 | V617F mutation analysis in CMJAH MHV laboratory from 2012 to 2016

The MHV laboratory at CMJAH is a referral laboratory and analysed 1120 samples for V617F mutation from 2012 to 2016 using FRET and melting curve assay, with an average of 224 tests per year. There were 206 (18%) positive results over the 5-year period, of which 175 (16%) were not repeat samples. After the exclusion of private sector patients and patients without available results, a definite diagnosis of ET, PV or PMF could be confirmed on a bone marrow aspirate and trephine biopsy in 63 of the patients (36%), and adequate residual DNA material was available for 43 patients (25%) (Figure 3.3).

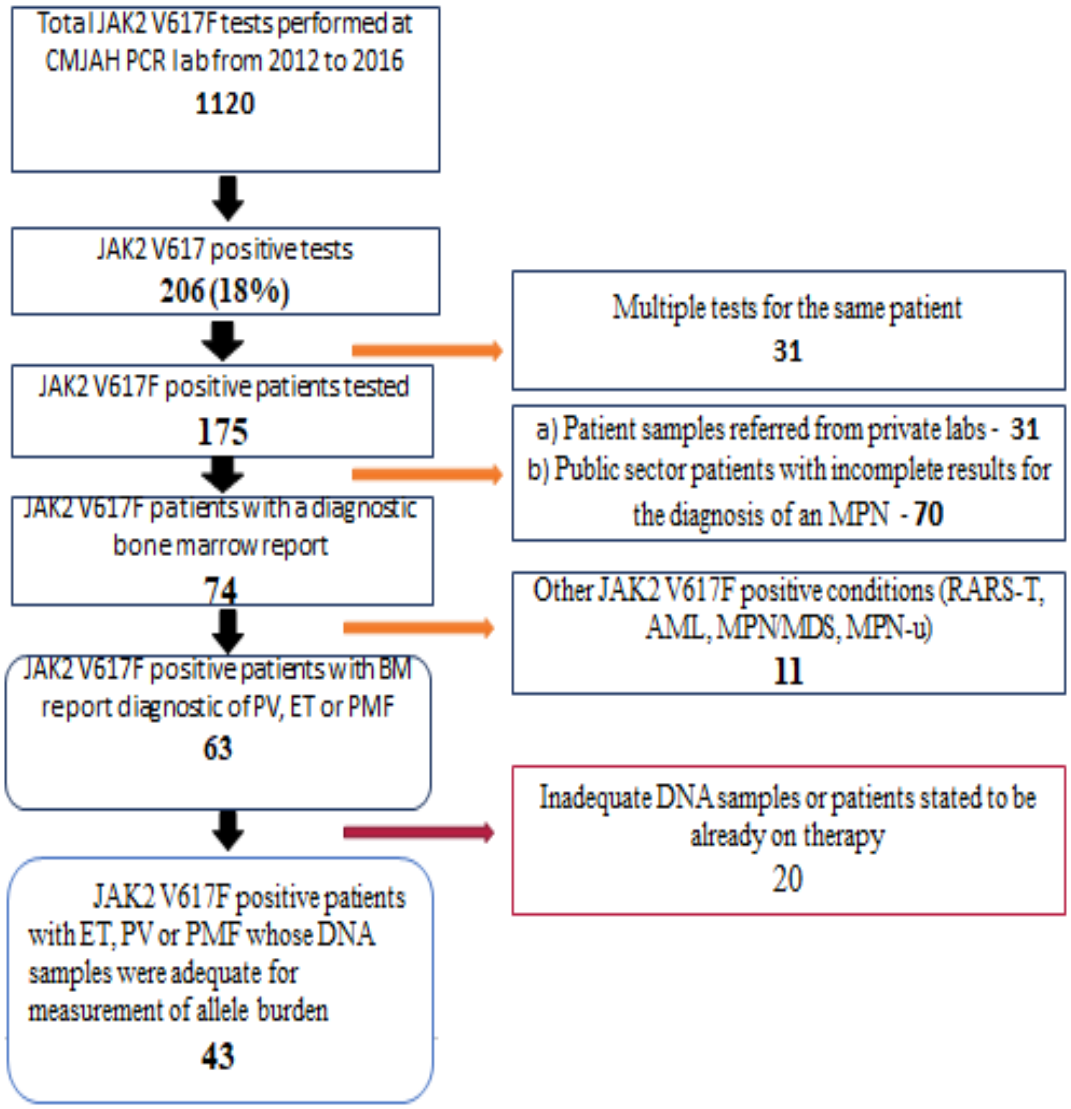

Figure 3.3 Enrolment of patients for the study

FISH for BCR-ABL was negative in all patients.

AML-acute myeloid leukaemia; BM-bone marrow; MPN - myeloproliferative neoplasm; MPN/MDS-myeloproliferative/myelodysplastic syndrome; MPN-u: MPN-unclassifiable; RARS-T - refractory anaemia with ringed sideroblasts with thrombocytosis.

3.4| Demographic features of MPNs

Amongst patients who fulfilled criteria for inclusion in the study, there were 7 with ET, 29 with PV and 27 with PMF. The overall median age of presentation for the MPNs was 65 years (range: 20 - 84 years). Median age at presentation for ET, PV and PMF was 70 years, 59 years and 64 years respectively with no statistical difference among the 3 MPNs (p=0,5734). Patients older than 60 years at presentation accounted for 54%. The overall male to female ratio was 1,1:1 and males accounted for 57,1%, 37,9% and 66,7% of ET, PV and PMF patients, respectively. There were more female patients with PV than PMF (p=0,0331) but no difference between ET and PMF (p=0,6434) or PV and ET (p=0,3615).

3.5| Laboratory results in MPNs

Full blood (FBC) and differential counts are summarised in Table 3.3. The median platelet count for ET patients was significantly higher than in PV and PMF (p=0,0013). None of ET patients showed a leucoerythroblastic reaction. Approximately 83% of PV patients had a haemoglobin level of at least 16 g/dL, with the remaining 17% having iron-deficiency.

On review of our cohort using the more recently published WHO 2016 guidelines[1], the majority of our PMF patients appeared to have overt PMF (78%) with pre-PMF being uncommon. Approximately 43% of patients with PMF, including all with pre-PMF, had thrombocytosis. Clinically significant thrombocytopenia, defined as a platelet count below 100 x 10^9/L, was seen in about 18% of PMF patients. Thrombocytopenia was only observed in overt PMF patients. Moreover, 57,1%, 53,6% and 42% of PMF patients had leucocytosis, leucoerythroblastosis and at least 1% blasts on peripheral blood smear, respectively. Lactate dehydrogenase (LDH) was significantly higher in PMF than in PV (p= 0,0148). Over 70% of ET patients did not have LDH results rendering any comparisons with other MPNs inappropriate.

Table 3.3 Laboratory results for V617F positive MPNs

	ET (N=7)	PV (N=29)	PMF (N=27)	P-VALUE
Median WCC $x10^9$/L (IQR)	9,53 (8,56 - 19,10)	13,95 (9,70 - 27,00)	17,89 (8,50 - 40,97)	0,4113
Median Hb g/dL (IQR)	13,9 (10,8 - 14,6)	17,6 (16,2 - 19,90)	9,7 (6,9 - 11,9)	**0,0000**
Median MCV fL (IQR)	78,7 (78,7 - 89,2)	78,7 (75,6 - 78,7)	78,7 (78,7 - 88,0) N=26	0,5778
Median Platelet count $x10^9$/L (IQR)	1175 (790 - 1457)	682 (572 - 1061)	358 (177 - 725)	**0,0013**
Median Neutrophils $x10^9$/L (IQR)	7,14 (5,43 - 14,25)	11,01 (7,11 - 24,63) N=28	10,84 (5,84 – 29,50)	0,4041
Median Lymphocytes $x10^9$/L (IQR)	2,13 (1,76 - 3,21)	2,00 (1,62 - 2,56) N=28	1,82 (1,02 - 3,26)	0,7686
Median Monocytes $x10^9$/L (IQR)	0,69 (0,55 - 1,26)	0,65 (0,35 - 1,10) N=28	0,68 (0,21 - 1,27)	0,7388
Median Eosinophils $x10^9$/L (IQR)	0,28 (0,17 - 1,36)	0,40 (0,16 - 0,80) N=28	0,48 (0,09 - 1,00)	0,7940
Median Basophils $x10^9$/L (IQR)	0,06 (0,00 - 0,12)	0,10 (0,04 - 0,26) N=28	0,07 (0,00 - 0,67)	0,5778
Median LDH U/L (IQR)	332,5 (274 – 391) N=2	416 (337 – 639) N=11	821,5 (483 – 1524) N=14	**0,0328**
Median Blasts % (IQR)	0,0 (0,0-0,0)	0,0 (0,0 - 0,0)	0,0 (0,0 - 3,0)	**0,0018**

The statistically significant p-values are emboldened. Hb: Haemoglobin; IQR: interquartile range; LDH: lactate dehydrogenase; MCV: mean corpuscular volume; N: Number; Plts: Platelets; WCC: white blood cell count.

3.6 | V617F allele burden in MPNs

Allele burden was measured on DNA samples of 43 patients with a diagnosis of ET, PV or PMF (Figure 3.3). The median allele burdens were 47,25% (range: 5,78% – 48,84%), 72,05% (range: 17,36% - 94,01%) and 62,62% (range: 13,84% - 95,90%) in ET, PV and PMF respectively. The allele burden was significantly lower in ET than in both PV (p=0,011) and PMF (p=0,025) but showed no statistically significant difference between PV and PMF (p=0,405) (Figure 3.4). All ET patients had allele burden below 50%. About 87,5% of PV and 75% of PMF patients had allele burden over 50%, while at least 40% of both PV and PMF patients had allele burdens above 75% (Figure 3.5).

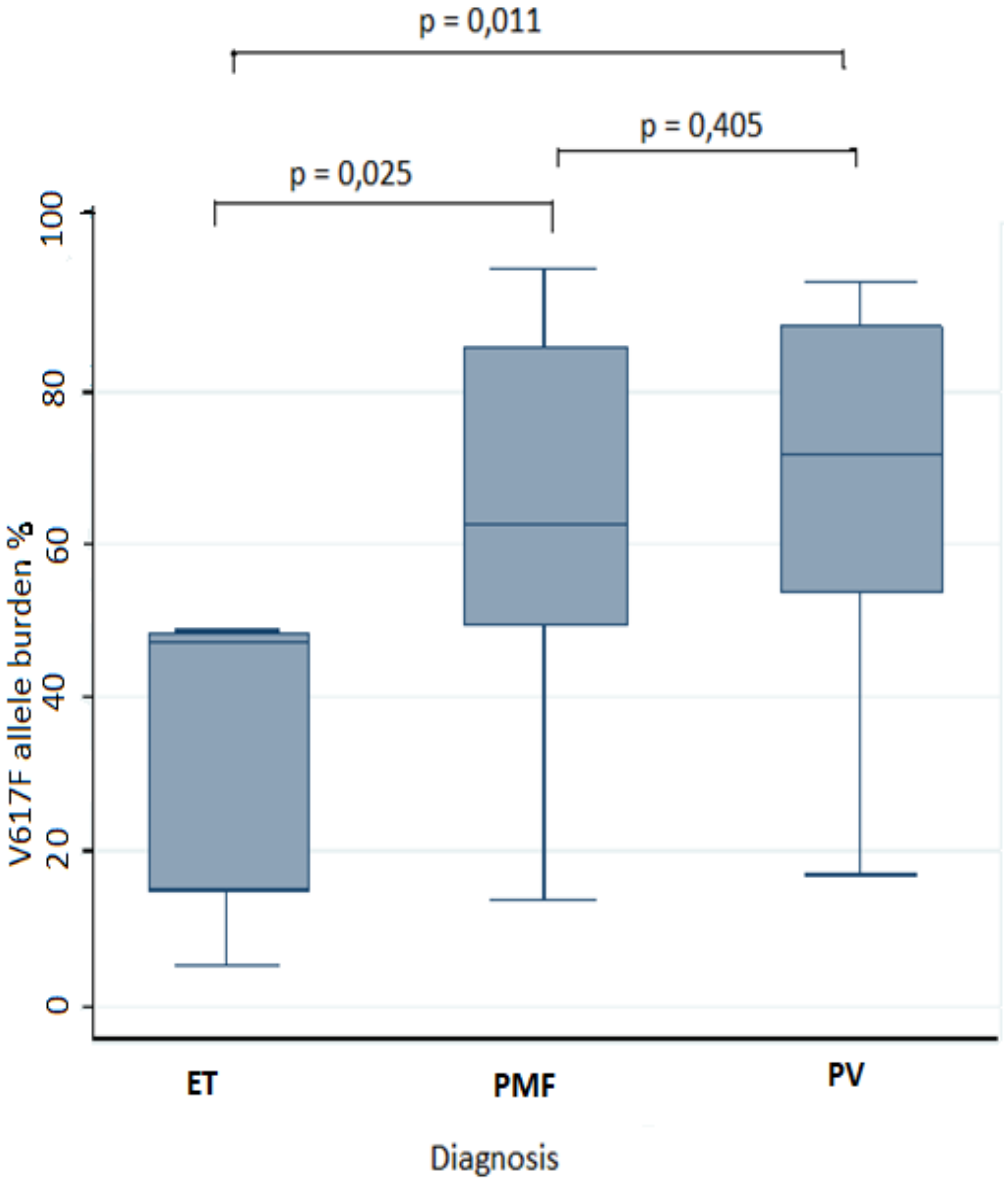

Figure 3.4 *Comparison of V617F allele burden among ET, PMF and PV patients.*

The median allele burdens were 47,25% (range: 5,78% – 48,84%), 72,05% (range: 17,36% - 94,01%) and 62,62% (range: 13,84% - 95,90%) in ET, PV and PMF respectively. The allele burden is significantly lower in ET than in both PV and PMF, but not significantly different between PV and PMF

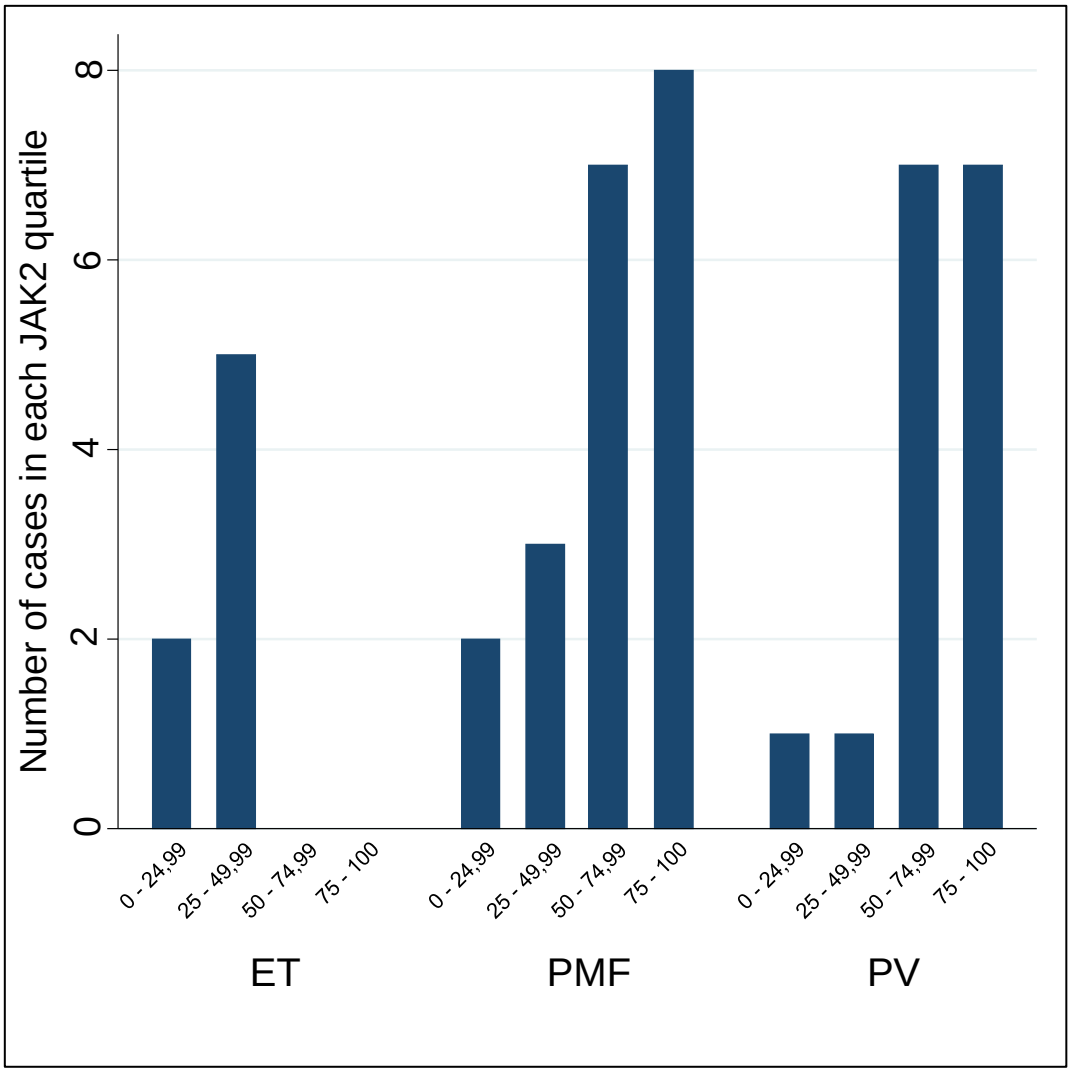

Figure 3.5 Distribution of V617F allele burden in different quartiles (reported in %) for ET, PV and PMF patients

3.7 │Relationship between V617F allele burden, demographic and laboratory results

Significant correlation ($p<0,05$) was observed between an increase in allele burden with an increase in white cell count ($r=0,58$), leucocytosis ($r=0,55$), an increase in neutrophil count ($r=0,59$), an increase in eosinophil count ($r=0,36$) and a decrease in mean erythrocyte corpuscular volume (MCV) ($r = -0,31$) (Figure 3.6). No correlation between allele burden and other laboratory and demographic results was noted.

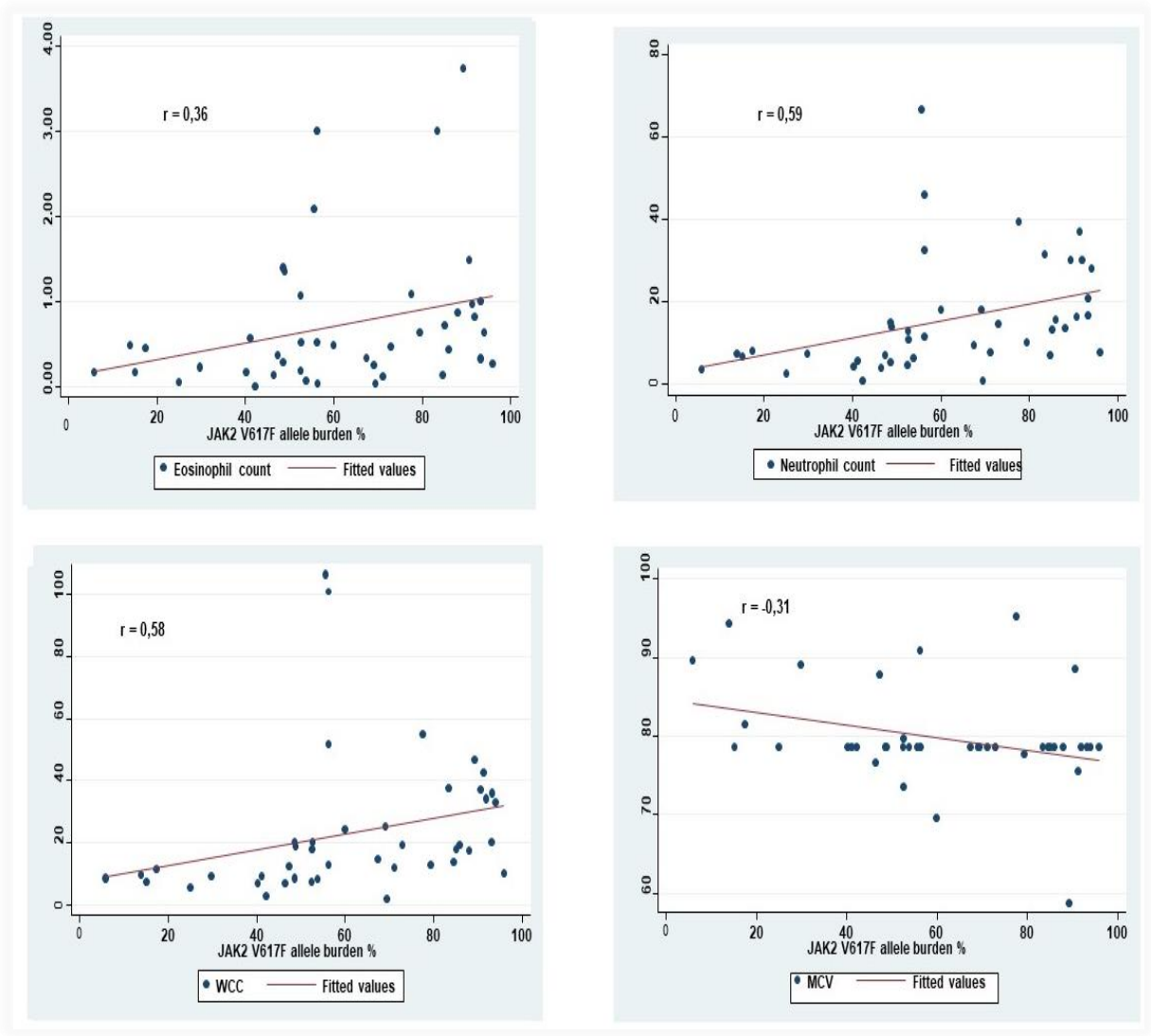

Figure 3.6 Correlation between V617F allele burden and eosinophil count, neutrophil count, total white cell count (WCC) and mean corpuscular volume (MCV)

4| DISCUSSION

4.1 | Verification of the detection and quantitation of V617F allele burden

The verification of analytical performance of QUASA aimed to establish a diagnostic assay with a detection threshold of at least 1% and capable of accurately quantitating V617F allele burden. This study verified that QUASA has an analytical sensitivity of 0,1% and accurately quantitates V617F allele burden with a bias of 0,288%. QUASA detected all tested RCPA EQA materials, WHO standards and a patient sample which were previously missed using FRET and melting curve analysis assay owing to low allele burden below 6%.

Despite the potential for false positive diagnosis of MPNs on detection of V617F mutation in patients with CHIP[42] as a result of high sensitivity of QUASA, a cut-off of 1% allele burden for diagnostic purposes would minimise this occurrence[26]. The 0,1% threshold may, however, be useful during monitoring of therapeutic interventions for minimal residual disease assessment.

QUASA showed comparable specificity to FRET assay in identifying negative clinical samples. Additionally, QUASA correctly identified all EQA and WHO standards which were negative for V617F mutation. The findings concur with reported high specificity of quantitative allele-specific PCR assays[24,26].

Quantitation of allele burden using QUASA yielded comparable results to those of the WHO standards. External validity of QUASA was confirmed as QUASA results were comparable and within consensus to those obtained in the WHO collaborative study which used the same WHO standards[40]. (Table 4.1)

QUASA's precision deteriorated at allele burdens at and below 10%. This concurs with the published data and is a recognised limitation of quantitative allele specific PCR assays in comparison to ddPCR[24,43]. A follow-up validation analysis to determine the precision and accuracy using serial dilutions between 0% and 0,1% allele burden may be necessary to establish suitability of this assay for deep minimal residual disease assessment.

Table 4.1 Comparison of results obtained by QUASA and all methods⁎ used in the WHO collaborative study

WHO standard allele burden (%)	QUASA Mean, (SD)	All methods⁎ Mean, (SD)	QUASA CV (%)	All methods⁎ CV (%)	Consensus between QUASA and All methods⁎
0	0 (0)	0,01 (0,02)	0	228	Within consensus
0,10	0,10 (0,05)	0,04 (0,03)	50,49	80	Within consensus
1,00	1,32 (0,46)	1,05 (0,76)	34,67	72	Within consensus
10,00	11,34 (3,25)	11,71 (5,05)	28,7	43	Within consensus
90,00	90,21 (0,85)	84,81 (12,10)	0,94	14	Within consensus
100,00	99,55 (0,51)	96,27 (14,72)	0,57	15	Within consensus

All methods⁎: these are 34 different quantitative laboratory techniques used in the collaborative WHO study to determine V617F allele burden.

4.2 | Diagnosis of MPNs

The laboratory analysed 1120 samples for V617F mutation from 2012 to 2016 using FRET and melting curve assay, with 206 (18%) positive cases detected over the 5-year period. All cases with available material analysed for JAK2 allele burden (n=43) had a bone marrow aspirate and trephine biopsy performed and were classified as per WHO diagnostic criteria which includes , histology as mandatory to establish the diagnosis and subtype of MPNs[1]. However, in the total cohort of patients with JAK2 positivity (144 state hospital patients), bone marrow histological subtyping was performed or available in only 51,4% of patients. This suggests an over-reliance in the diagnosis of MPNs based on clinical findings, FBC results and a marker of clonality (in this case, V617F positive result), without performing confirmatory diagnostic bone marrow

procedure. Bone marrow investigation should be actively promoted to accurately subtype the MPNs in our setting.

4.3 | Demographic characteristics of MPNs with JAK2 V617F mutations

Almost equal proportions of male and female patients tested positive for V617F mutation with an overall median age at presentation of 65 years. Median age for ET, PV and PMF patients with a V617F mutation was 70 years, 59 years and 63 years respectively, all of which concur with reported occurrence of MPNs between the fifth and seventh decades of life[1]. These results also concur with data available from American and European centres[34,35]. No significant difference in age at presentation was noted for the different MPNs.

In contrast to many studies in America and Europe where myelofibrosis is the least commonly diagnosed MPN[34,35], myelofibrosis was a common diagnosis in our setting (43% of patients who fulfilled criteria for inclusion in the study). Although our data was specific for JAK2V617F positive patients, a 25-year review of data from a large academic hospital in our region confirmed this finding in MPN patients with unknown mutational status (45% patients diagnosed with PMF)[37]. These findings suggest the more aggressive forms of MPN may be more predominant in our patient cohorts.

In contrast to studies in Europe where V617F-positive ET is the most common MPN[44,45], ET was surprisingly lower than anticipated in this cohort and was the least represented MPN. It represented only 11% (7/63) of the MPN patients eligible for inclusion and 18% (7/43) of patients in whom allele burden was tested. An attempt was made to identify possible ET patients from V617F-positive patients who had been excluded due to absence of diagnostic histology. Based on FBC results (i.e. patients with isolated thrombocytosis, normal white cell count and normal haemoglobin level), this did not yield additional possible ET patients. The reason behind the under-representation of ET is uncertain but could reflect a different disease spectrum of MPN in our local population. ET may also be underdiagnosed due to a milder clinical course[46] and/or late presentation with complications such as fibrotic transformation, especially given the reported poor health seeking behaviour in South Africa[47,48].

It is not certain to what extent the less sensitive FRET assay contributed to the under-diagnosis of MPNs in our laboratory, especially ET patients who reportedly may present with allele

burden as low as 1%[11]. The current FRET assay may also not have detected patients with subclinical or latent MPNs who reportedly may have extremely low allele burdens of 1% and below[28,29]. A prospective, community-based study utilising a highly sensitive and specific molecular detection technique such as QUASA would be invaluable to better understand the prevalence and demographic features of MPNs in South Africa.

4.4 | Laboratory findings in MPNs

Leucocytosis was noted in more than 50% of patients with PV and PMF at presentation and was predominantly due to neutrophilia. Leucocytosis is known to predispose to thrombotic complications[49] and is associated with inferior survival in MPNs[50].

Over 40% of overt PMF patients and all pre-PMF patients had thrombocytosis with few or no peripheral blood morphologic features to distinguish them from ET. This has been described in literature[46] and emphasizes the importance of bone marrow histology to accurately subtype MPNs, especially differentiating ET from pre-PMF. In comparison to ET, pre-PMF and overt PMF have worse prognosis and reduced overall survival[45], thus accurate diagnosis is mandatory for optimal patient care.

The majority of patients in our cohort with a diagnosis of PMF had overt fibrosis, contrasting with the reported trend to earlier presentation of PMF patients in pre-PMF stage[51]. This may be due to late presentation of patients with PMF in the South African setting or more aggressive disease in our patients, however bias in performance of bone marrow procedures cannot be excluded. Patients with overt PMF may have been more extensively investigated than the less symptomatic patients with pre-PMF.

4.5 | V617F allele burden in MPNs

In concordance with other studies[52,53], significantly lower allele burden was observed in ET patients than in both PV and PMF patients (Figure 3.4). The median allele burden of 47,25% in ET and 72,05% in PV closely resembled those found in a study in Iran where allele burdens for ET and PV were 49% and 72%, respectively[54]. Further similarity in allele burden for MPNs was observed in a study from Japan[55]. However, V617F allele burden was notably higher than in studies performed in Europe[52] and China[53] where allele burden in ET rarely exceeded 25% and the majority of PV and PMF patients had allele burden less than 50%. This may reflect biological difference in disease severity and/or late clinical presentation of the study population.

Although all ET patients in this study had V617F allele burden below 50%, the majority had an allele burden over 25%, resulting in a median allele burden of 47,25%. This is in contrast with published data where the median V617F allele burden in ET is typically below 25%[12,52,53]. Allele burdens above 25% predispose to increased thrombotic events in ET patients and are a marker for more aggressive disease[12].

The allele burden in 87,5% of the PV patients was above 50%, which concurs with that described in some studies[54,55]. Almost 44% of PV patients had allele burden above 75%; these have been associated with a worse clinical course[18] and would be at high risk of transforming to secondary myelofibrosis[56].

The median allele burden of 62,6% in PMF patients compared well with some studies[52,55] but was much higher than in other studies where a median allele burden of about 30% to 45% was reported[57,58]. Although the higher allele burden observed in this study would correspond to more severe clinical features[17], a longer survival was described in PMF patients with higher allele burden than those with allele burden less than 25%[21]. The reason for this seemingly contradictory finding is not clear, however it is hypothesized that as PMF advances in clinical severity, there is disease evolution, acquisition of non-driver mutations and simultaneous decrease in V617F allele burden. A prospective study would be needed to confirm this hypothesis.

4.6 | Allele burden and laboratory results

A higher allele burden was associated with an increase in white cell, neutrophil and eosinophil counts, leucocytosis above 11 x 10^9/L and a decrease in MCV. However, in contrast to the literature, no association with an increase in haemoglobin, platelet count or LDH was observed[13,56,59]. Therapeutic interventions that reduce leucocytosis, neutrophilia and eosinophilia may suppress the V617F allele burden and disease progression. A prospective cohort would be important to prove this hypothesis.

4.7 | Study challenges and limitations

The diagnoses for 48,6% of patients who tested positive for V617F mutation could not be established due to lack of diagnostic bone marrow histology. Due to the retrospective nature of the study, there were missing data and residual DNA specimens in almost one-third of eligible MPN patients, resulting in exclusion of these 20 patients from quantitation of allele burden. Moreover, as discussed above, patients with ET appeared to be under-represented in this cohort.

However, the final cohort had complete results and displayed important demographic and laboratory features of MPNs in a South African setting.

5 | CONCLUSION

QUASA is sensitive and specific for the detection of V617F mutation. Moreover, QUASA is accurate and precise in quantitating V617F allele burden ranging from 0,1% to 100%.

ET is the least common V617-associated MPN in this study, which differs from many published international studies. In agreement with published data, V617F allele burden is significantly lower in ET than in both PV and PMF. However, the three MPNs reveal high allele burdens known to be associated with high risk disease. This may reflect worse disease phenotype in the South African setting. A prospective community-based study would be invaluable to confirm the above findings and to establish an accurate database of MPNs in South Africa. Prospective follow-up of a cohort of patients with known allele burden and correlation with clinical outcome would also be of great value.

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7 | APPENDICES

7.1 | Ethics Clearance certificate

R14/49 Dr Moses Chatambudza and Dr Sergio Carmona

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M170589

NAME: (Principal Investigator)	Dr Moses Chatambudza and Dr Sergio Carmona
DEPARTMENT:	Molecular Medicine and Haematology Charlotte Maxeke Johannesburg Academic Hospital National Health Laboratory Service University of the Witwatersrand
PROJECT TITLE:	Myeloproliferative Neoplasm-Related Janus Kinase 2 V617F Somatic Mutational Status at a Single Centre in South africa
DATE CONSIDERED:	Adhoc
DECISION:	Approved unconditionally
CONDITIONS:	Sub- Study under Primary Study (M150160)
SUPERVISOR:	Dr Tracy Wiggill
APPROVED BY:	Professor P. Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	09/06/2017

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004,10th floor, Senate House/3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. in this case, the study was initially review May and will therefore be due in the month of May each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

7.2 | Turn-it in Report

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7.3 | WHO 2016 diagnostic criteria of ET, PV and PMF (adopted from reference 1 with minor modifications)

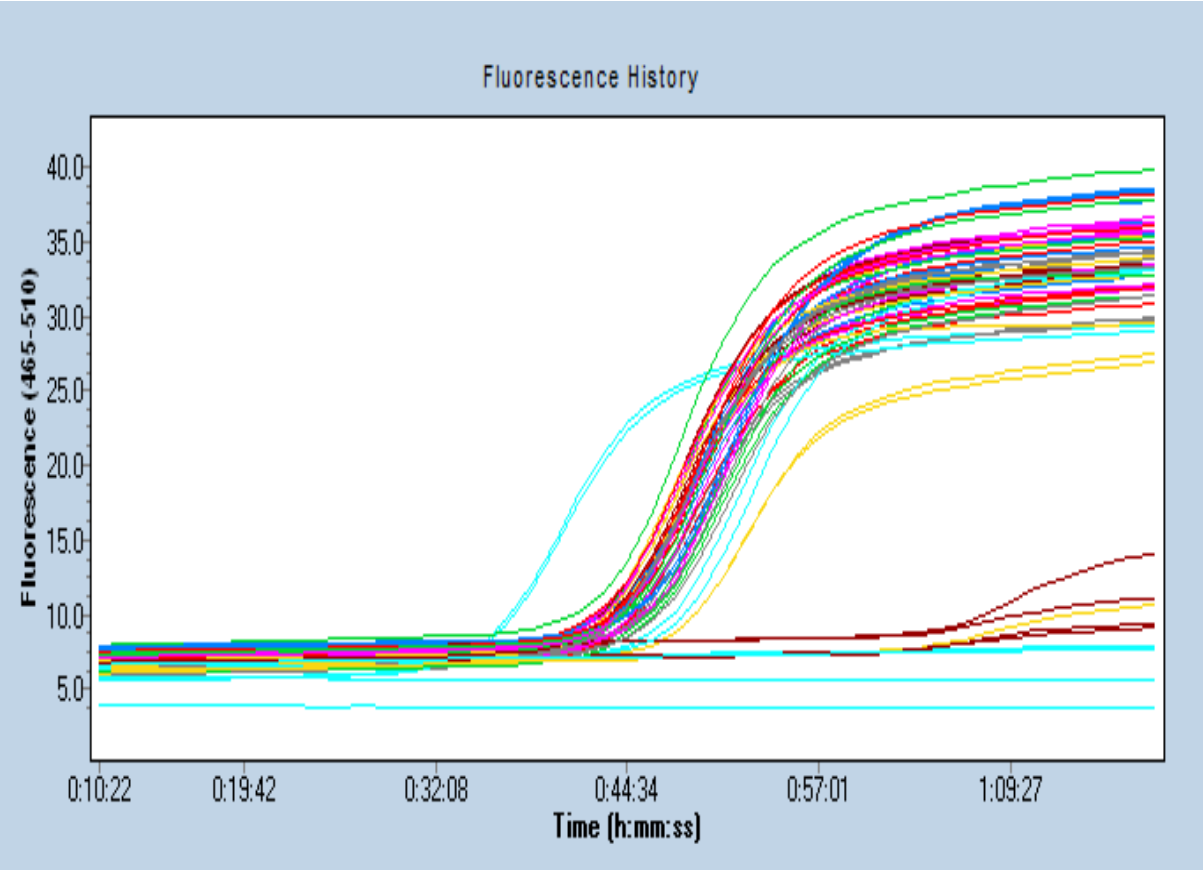
DIAGNOSIS	CRITERIA FOR DIAGNOSIS
ESSENTIAL THROMBOCYTHAEMIA **Diagnosis requires all major or the first 3 major and 1 minor criteria.**	**MAJOR:** 1) Sustained thrombocytosis of at least 450 x 10^9/L 2) Proliferation of enlarged, hyperlobulated and mature megakaryocytes with no significant accompanying granulocytic or erythrocytic hyperplasia on bone marrow investigation. 3) Does not meet the diagnostic criteria for PV, BCR-ABL positive CML, PMF, or any other myeloid neoplasm 4) Mutation of JAK2, CALR or MPL **MINOR:** Identification of a marker of clonality or absence of identifiable reactive cause of the thrombocytosis
POLYCYTHAEMIA VERA **Diagnosis requires either all 3 major OR the first 2 major and the minor criterion**	**MAJOR:** 1) Elevated haemoglobin (>16.5g/dl in males and >16 g/dl in females) or elevated haematocrit (>49% in males and >48% in females) 2) Hypercellular bone marrow biopsy with hyperplastic trilineage haemopoiesis (panmyelosis) with mature megakaryocytes displaying pleomorphism 3) JAK2 V617F or JAK2 exon 12 mutation positivity **MINOR:** Serum erythropoietin level less than normal
PREFIBROTIC PMF **Diagnosis requires at least 1 minor and all 3 major criteria**	**MAJOR:** 1) Proliferation and atypia of megakaryocytes without reticulin fibrosis grade >1, accompanied by hypercellularity, granulocytic hyperplasia and usually suppressed erythropoiesis 2) Does not meet the diagnostic criteria for PV, BCR-ABL positive CML, ET, or any other myeloid neoplasm 3) Presence of JAK2 V617F, CALR or MPL mutation, or presence of another clonal marker***, or absence of a reactive cause of fibrosis

	MINOR: At least one of the following confirmed on 2 consecutive occasions: 4) Anaemia not secondary to any comorbidity 5) Leucocytosis above 11 x 10^9/L 6) Palpable enlarged spleen 7) LDH above the normal range for the institution
OVERT PMF **Diagnosis requires at least 1 minor and all 3 major criteria**	**MAJOR:** 1) Proliferation and atypia of megakaryocytes with reticulin and/or collagen fibrosis of at least grade 2** 2) Does not meet the diagnostic criteria for PV, BCR-ABL positive CML, ET, or any other myeloid neoplasm 3) Presence of JAK2 V617F, CALR or MPL mutation, or presence of another clonal marker***, or absence of a reactive cause of fibrosis **MINOR:** As for Prefibrotic PMF and in addition: 4) Leucoerythroblastic reaction

ET: Essential thrombocythaemia; **PV:** Polycythaemia vera; **PMF:** Primary myelofibrosis; **JAK2:** Janus kinase 2; **CALR:** Calreticulin; **MPL:** thrombopoietin receptor; **CML**: Chronic myeloid leukaemia; **LDH:** Lactate dehydrogenase

***Grade 1 fibrosis:** loose intersecting reticulin network
****Grade 2 fibrosis:** Extensively intersecting, diffuse and dense reticulin network
***** Other clonal markers include ASXL1, EZH2, TET2, IDH1, IDH2, SRSF2 and SF3B1 mutations**

7.4 | Amplification curves of real-time PCR using QUASA on Lightcycler



7.5 | Approved Research Protocol

MYELOPROLIFERATIVE NEOPLASM-RELATED JANUS KINASE 2 V617F SOMATIC MUTATIONAL STATUS AT A SINGLE CENTRE IN SOUTH AFRICA

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1. INTRODUCTION

The World Health Organisation (WHO) identifies polycythaemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) as the classical Philadelphia negative (Ph-negative) myeloproliferative neoplasms (1). These are clonal myeloid disorders characterised by autonomous proliferation of haemopoietic precursor cells leading to increased mature blood cells. The increased mature blood cells comprising erythrocytes, platelets and leucocytes lead to significant disease burden in the affected patients, including organomegaly, increased thrombotic and bleeding tendencies, and risk of progression to myelofibrosis (in the case of PV and ET) and acute leukaemia (2).

The discovery of Janus kinase (JAK) 2 V617F mutation in 2005 was a breakthrough in the field of Ph-negative myeloproliferative neoplasms (3). This mutation leads to valine being substituted by phenylalanine on codon 617 of the enzyme Janus kinase 2, leading to enhanced, dysregulated function of this enzyme which is involved in cell signalling via the Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway (4). This results in increased proliferation of myeloid stem cells and their progenitors as seen in the myeloproliferative neoplasms. About 95% of patients with PV and 50-60% of patients with ET and PMF have the JAK 2 V617F somatic mutation (5).

The JAK2 V617F somatic mutation, together with somatic mutations of JAK 2 exon 12, calreticulin and the thrombopoietin receptor (MPL), is a founding driver mutation which determines the phenotype of MPNs (5). It is believed that JAK 2 V617F mutation-associated MPNs are biologically related(6, 7) with the mutant allele burden playing a major role in the determination of the phenotypic expression(8). The allele burden of the mutant JAK 2V617F has been shown to be very important in determining clinical phenotype at presentation, progression and prognosis in JAK 2 V617F mutant MPNs (8-10). While being lower in de novo primary myelofibrosis and essential thrombocytosis, the allelic burden is elevated in polycythaemia vera, PMF arising from PV or ET and in the NRNs evolving to acute leukaemia(8, 11, 12).

Various assays are available for the measurement of the JAK 2 V617F mutant allelic burden. These include Taqman quantitative PCR (qPCR), Fluorescence Resonance Energy Transfer (FRET)qPCR, pyrosequencing, allele-specific qPCR and DNA sequencing and peak height analysis (13). The Taqman qPCR and the allele-specific qPCR have been shown to be highly sensitive and reproducible if reference standards are set, while the sequencing assays displayed significant variability and low sensitivity (13).

Although earlier short-term studies did not show effect of therapy on JAK 2 V617F allele burden (14, 15), long-term treatment of MF, post-PV MF and post-ET MF patients with a targeted JAK-inhibiting drug ruloxitinib for a median duration of 22.2 months to 27.5 months was associated with significant reduction of the JAK2 V617F allelic burden, with some patients achieving partial or complete molecular remission (10). Interestingly, the decrease in the allelic burden was associated with improved clinical symptoms and reduction in splenomegaly (10, 16). This shows the potential value of measuring and monitoring JAK 2 V617F mutant allelic burden in minimal residual disease monitoring (17, 18), particularly in the era of personalized medicine.

The incidence of MPNs vary with age, gender, type of MPN, population under survey and different regions of the world (19). MPNs increase with age, mostly occurring in patients above the age of 50 years. PV has a male:female predominance of about 1 to 2: 1, occurring in

0.7 to 2.6 per 100000 people in Europe and North America with a lower incidence in Japan. Overt fibrotic PMF is reported in 0.5 to 1.5 per 100000 persons per year with no gender predisposition while the incidence of ET is unknown, being estimated at 0.6 to 2.5 per 100000 persons per year (20). Notably very limited data is available for Africa regarding demographics of MPNs even though these diseases are commonly seen in our haematology clinics. A single-centre retrospective study at Chris Hani Baragwanath Hospital haematology clinic documented 116 patients with newly diagnosed MPNs (21). Unfortunately, the JAK2 mutational status of about two-thirds of these patients was unknown since they were mainly seen before the discovery of the somatic mutations in MPNs.

2. RESEARCH AIMS AND OBJECTIVES

This study aims to introduce and validate a quantitative PCR technique for measurement of JAK 2 V617F mutant allelic burden in the NHLS PCR referral laboratory at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) and to determine the relationship between JAK 2 V617F mutant allelic burden at presentation and the type of MPN and laboratory findings at presentation. Once successfully validated, this test will be used routinely in initial diagnosis and monitoring of patients with JAK 2 V617F-mutant MPNs.

The specific research objectives are as follows:

l) To review the PCR requests for JAK2 V617F mutational analysis submitted to the NHLS Haematology PCR laboratory at CMJAH over the past five years (2012 to 2016) to

ascertain the JAK2 mutational status of the referred samples and to describe the associated demographic information, laboratory data and type of MPN (PV, ET or PMF). This will be done via screening of PCR requests received in the laboratory and data available from the Central Data Warehouse (CDW) storage repository of the NHLS and the laboratory information system (LIS) of the NHLS.

2) To validate JAK 2 V617F mutation detection and quantification of allelic burden using quantitative allele specific amplification (QUASA) of remnant external quality assurance (EQA) material and routinely refe1Ted samples documented to be positive for the JAK 2 V617F mutation.
3) To evaluate the relationship between JAK 2 V617F mutant allelic burden, the type of MPN and laboratory results at presentation.

3. METHOD

3.1 Study design:

Retrospective cohort study

3.2 Study setting

Laboratory-based quantification of JAK 2 V617F allelic burden using quantitative allele specific amplification (quasa) will be performed in the Haematology PCR Laboratory, Department of Molecular Medicine and Haematology, NHLS, Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) and University of Witwatersrand.

3.3 Study samples

Measurement of the allelic burden by QUASA will be performed on remnant EQA material of known allelic burden (1 st World Health Organization International Genomic Reference Panel for JAK2 V617F) and stored DNA material of cases that tested positive for the JAK2 V617Fmutation on the standard non-quantitative PCR methodology in routine use in the Haematology PCR laboratory. This will be performed using a quantitative allele specific amplification (QUASA) JAK 2 V617F mutation kit (Primerdesign@ Ltd, Chandler's Ford, United Kingdom)

Fifty consecutive DNA samples (working retrospectively from the newest samples first) which fulfil the inclusion criteria ofthe study will be selected for inclusion.

Inclusion criteria

All adult patients (>18 years of age) with a presentation diagnosis of an MPN who are positive for the JAK 2 V617F mutation will be eligible for inclusion in the study, provided that:

1) The DNA samples selected for testing meet the standard operating procedure (SOP)specified requirements (NJHHOI 88v6) and must have been stored in the DNA sample storage freezer at -20^0 C in the NHLS CMJAH Haematology PCR laboratory.
2) The stored DNA sample should be sufficient in quality and quantity to run a quantitative JAK2 V617F PCR test. This will be assessed using a nanodrop. Appropriate DNA concentration will be between 5-10 ng/ul.
3) The associated demographic data, full blood count and diagnostic bone marrow aspirate or trephine report should be retrievable on the NHLS databases.

3.4 Study methodology

Principles of quantitative allele specific amplification (QUASA) test

Quantitative allele specific amplification (QUASA) is a sensitive method which allows detection of mutations present at low levels in PCR samples. The QUASA JAK2 V617F mutation kit (Primerdesign@ Ltd, Chandler's Ford, United Kingdom) detects and amplifies both the wildtype JAK 2 and mutant JAK 2 V617F by polymerase chain reaction and quantifies the proportion of the mutant to the wild type JAK2 gene with a detection limit of 0.1 The kit employs two modified primers which have been designed for maximum sensitivity and specificity: a mutant detecting primer to detect the V617F single nucleotide polymorphism (SNP), and a wild-type (WT) detecting primer to detect the unmutated JAK2. A two- reaction protocol is used with one reaction detecting the level of wild-type sequence present and at the same time another reaction detecting the level of mutant sequence present. By comparing the detection levels obtained by both primer/probe sets, the proportion of sequences that contain the mutation can be measured and expressed as a percentage relative to the wild type sequences. Reactions are run in duplicate to improve the accuracy of the results.

PCR methodology

The test will be conducted as per manufacturer's guidelines (22), using all appropriate PCR quality control measures to avoid contamination and including a positive control (1 % mutant positive control included with the kit) and a negative control (RNase/DNase free water). The kit also includes fluorescein amidite (FAM) labelled JAK2 V617F Genotyping primer/probe mixes, FAM labelled JAK2 WT Genotyping primer/probe mixes and QUASA A PCR Mastermix (see table l).

Table 1. Contents of the QUASA JAK 2 V617F test kit (modified from 22).

Component
JAK2 V617F Genotyping pHmer/probe mixes FAM labelled
JAK2 WT Genotyping primer/probe mixes FAM labelled
QUASA A real time PCR mastermix
Positive Control Template
RNase/DNase free water

The DNA master mix for the mutant and wild type (WT) primer will be prepared according to table 2 below in the reagent preparation room. Sufficient mastermix will be prepared to analyse both WT and mutants for each sample, perform each sample in duplicate and to include positive and negative (no template) controls (NTC).

Table 2. The Mastermix preparation (modified from 22).

Component	
2X Precision QUASA A Mastermix	10µl
Genotyping primer/probe mix (BROWN)	1µl
RNase/DNase free water (WHITE)	4µl
Final volume	15µl

15µl of the mastermix will then be dispensed into the plates as shown below in Figure 1.

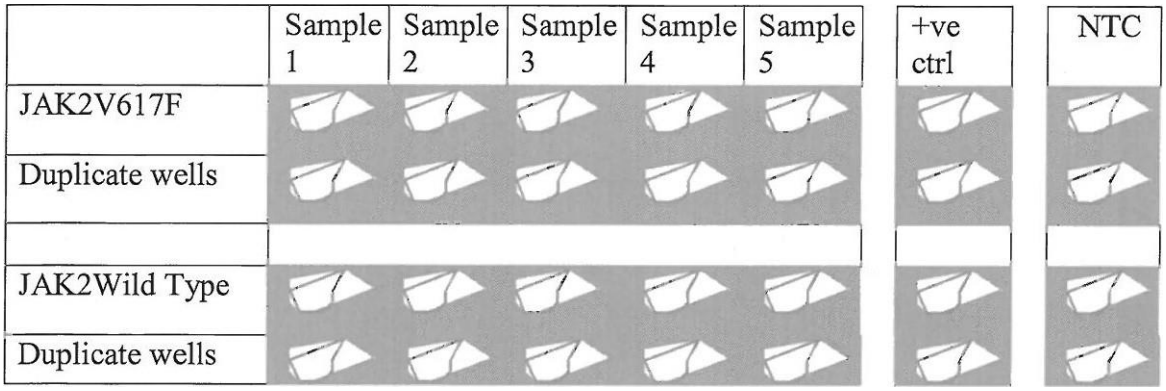

Figure 1. Set-up of wells for QUASA (adapted from reference 22).
+ve ctrl: positive control, NTC: no template negative control

5µl of DNA sample from JAK2 V617F mutant cases will then be dispensed into the respectively labelled wells as per plate setup (Figure 1) in the sample preparation room.5ul of diluted positive control will be added to the positive control wells and Sul of RNase/DNase free water to the negative control wells.
PCR will be performed utilising the Lightcycler 480 (Roche Molecular Diagnostics, California, USA). Cycling conditions will be as per manufacturer's guidelines (see Table 3 below) and data will be collected through the FAM channel during the 60^0C annealing step.

Table 3. PCR cycling conditions (22).

Step	**Time**	**Temperature**
PCR Enzyme Activation	2 minutes	95^0 C
First 5 cycles		
Denaturation	10 seconds	95^0 C
Annealing	15 seconds	50^0 C
Extension	15 seconds	72^0 C
Subsequent 40 cycles		
Denaturation	10 seconds	95^0 C
Annealing	30 seconds	60^0C
Extension	15 seconds	72^0 C

The proportions of the V617F SNP and WT in the sample will be corrected by reference to the positive control standard where the SNP is present at a known proportion of 1 %. This will allow detection of the WT:mutant ratio when calibrated against the 1 % control. The ratio will be used to calculate the relative percentage of mutant and wild type in the sample.

Data collection:

Demographic data and laboratory results will be collected on each patient included in the study via a database search of the laboratory information system of the NHLS (Trakcare, InterSystems Corporation, Cambridge) and extraction of data from the CDW. Data to be obtained where available include age, gender, JAK 2 V617F mutational status, any other MPN-related mutations tested, full blood count (FBC) and differential count before or at the time of testing for JAK2 V617F mutation, bone marrow aspirate and trephine biopsy reports at presentation and other laboratory results (serum erythropoietin level, haematinic studies results, C-reactive protein, HIV test, D-Dimers, thrombophilia screen and connective tissue screen). Please refer to appendix 1.

3.5 Data collection and analysis

Data capture

Data will be presented in a Microsoft excel sheet (see appendix 1).

Data analysis / statistics

Data will be analysed using STATA software and GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). Numerical continuous variables will be summarized using medians and ranges while ratios, percentages and relative frequency will be used for categorical variables. Statistical comparisons of quantitative variables will be performed using non-parametric Mann-Whitney U test or Wilcoxon rank sum test. Linear regression analysis will be used to assess relationships between variables. Statistical significance will be set at a two-sided P value of 0.05 or less.

Access to data

Only the principal investigator and the supervisors will have access to the data.

Confidentiality

All patients will be allocated a study number and the identity of patients whose DNA samples are going to be analysed will be anonymous and unlinked. Patient identifying

information will only be used for confirmation of the identity of the stored DNA samples and to retrieve the associated laboratory results and will be password protected. No results will be communicated to referring clinicians.

3.6 Ethics approval

An application has been submitted to the Wits Human Research Ethics Committee and an application for data access will be sent to the NHLS Central Data Warehouse Manager.

3.7 Budget

EXPENSE	APPROXIMATE COST
Quantitative allele specific amplification kit for JAK2V617F (50 reactions) x 3 kits	R31300
Pipette tips (10ul and 200 ul tips)	R2500
Gloves x 2 boxes	R100
Strip tubes and plates	R5500
Stationery and printing	R1000
TOTAL	R40400

3.8 Funding

Applications for funding will be made to:

1) Beit Trust (the sponsor for my current postgraduate studies)
2) NHLS research trust development grant.
3) University of the Witwatersrand Medical Faculty Research Endowment Fund.

Proposed timetable

2017-2018	*Mar*	*Apr*	*May*	*Jun*	*Jul*	*Aug*	*Sep*	*Oct*	*Nov*	*Dec*	*Jan*	*Feb*
Proposal writing												
Ethics application												
HREC application												
Data collection												
Data analysis												
Thesis write up												
Writing up paper												

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APPENDIX 1

Study Number	DOB	Age	Gender	Clinical diagnosis	WCC	HB	MCV	PLT	Neutrophils	Lymphocytes
KEY										
DOB: date of birth										
WCC: white cell count										
HB : haemoglobin										
PLT: platelet count										
MCV: mean cell volum										
EPO: erythropoietin										
HIV: human immunodeficiency virus										
CD: cluster of differentiation										
JAK 2: Janus kinase 2										
FISH: flourescence in situhybridisation										
CRP: C-reactiveprotein										
ANA: antinuclear antibodies										
anti-dsDNA: anti- double stranded DNA antibodies										
RhF: rheumatoidfactor										

Continuation..

Monocytes	Eosinophils	Basophils	Serum EPO	Clinical history	Peripheral smear	Bone marrow aspirate features

Continuation..

Bone marrow trephine features	Cytogenetic findings	FISH results	Serum folate	Vitamin B12	Ferritin	CRP	ANA

Continuation..

anti-dsDNA	RhF	HIV status	CD4 count	Viral Load	JAK 2 V617F mutation	Calreticulin mutation	Other