RESEARCH ARTICLE

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Frequency of *JAK2V617F* Mutation Zygosity and Impact on Disease Outcome in MPN Patients

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Abstract

Background: Myeloproliferative Neoplasm (MPN) is a clonal disorder of blood progenitor cells during haematopoiesis. *JAK2V617F* mutation represents one of the major identified genetic reasons of MPN disorder. It has been shown that *JAK2V617F* zygosity is associated with disease phenotype. However, studies showed variable findings regarding individual phenotype parameters and significance of effect. Objectives: the aim of this study was to determine frequency of *JAK2V617F* zygosity and corresponding outcomes in this population. **Methods:** This cross-section study recruited *JAK2V617F*-mutated polycythemia Vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF) patients. DNA samples were extracted from peripheral blood and *JAK2V617F* zygosity was determined using ARMS-PCR method. **Results:** A total of 162 patients (91 males and 71 females) were analysed. Of total, 131 (80.86 %) were heterozygous and 31 (19.14 %) were homozygous for *JAK2V617F*. homozygous characterized with higher age (P= 0.0153) and WBC count (P= < 0.0001). Among total MPN cases, the number of PV, ET, and MF patients was 102 (62.96%), 37 (22.84%), and 23 (14.2%) respectively. The frequency of homozygous cases was 20.6 %, 10.8 %, and 26.1 % for PV, ET, and MF patients respectively. No significant difference seen in age, BMI, and HCT between homozygous and heterozygous cases in all MPN groups. In ET group, homozygous patients showed significant HGB reduction (P= 0.0186) and PLT increase (P= 0.0034) compared with heterozygous patients. WBC count in homozygous patients was significantly higher than heterozygous in PV (P= 0.0071), ET (P= <0.0001), and MF (P= 0.07) patients. **Conclusion:** *JAK2V617F* zygosity is useful marker in determining disease severity and can be used for prediction of prognosis in MPN patients. ARMS-PCR is simple, easy, and useful technique in determining zygosity of mutations and does not need to advanced laboratory resources. We recommend including zygosity in laboratory reports when conducting *JAK2V617F* using ARMS-PCR technique.

Keywords: *JAK2V617F*- ARMS-PCR- zygosity- allele burden- MPN

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Introduction

Myeloproliferative Neoplasm (MPN) is a clonal disorder of blood progenitor cells during haematopoiesis. BCR-ABL1 oncogene-negative MPN patients is mainly classified into three groups on the basis of affected clone and clinical features. These groups are Polycythemia Vera (PV), Essential Thrombocythemia (ET), and Primary myelofibrosis (PMF) [1, 2]. It has been shown that these disorders are developed due to number somatic driver mutations within genes that control pathways responsible for myeloid linage growth and maturation.

JAK2V617F is considered the most prevalent driver mutation and firstly discovered in 2005 [3-6] It has been shown that *JAK2V617F* exists in majority of PV cases and about half of ET and PMF cases [3, 4, 7, 8]. Since its discovery, it has been considered as one of the hallmarks of MPN according to WHO [2, 9]. *JAK2V617F* represents a gain of function mutation with single nucleotide replacement involves G to T nucleotides substitution in exon 14 of JAK2 gene locus. This result in Valine to phenylalanine amino acids substitution in *JAK2* protein and consequently, activation *JAK2*/STAT signalling pathway which triggers excessive production of blood cells and development of MPN [10, 11].

It has been shown that in addition to driver mutations such as *JAK2*, development of MPN is accompanied with various and complex genetic abnormality [12]. It has shown that *JAK2V617F* mutational load is associated with disease phenotype. [13-16] as well as risk factors, progression and survival in MPN patients [17-21]. In addition, measuring of *JAK2V617F* allele burden contributes in prediction of response to therapy [22]. It is common to indicate *JAK2V617F* mutation on the basis of allele burden as heterozygous (allele burden < 50%) or homozygous (allele burden > 50%) [17, 23, 15]. Studies have shown that heterozygous state might also become homozygous due to mitotic recombination [6,

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24]. Various techniques are available to detect zygosity with quantitative Polymerase Chain Reaction (qPCR) considered the most common. However, this method might not be available in all labs due to limited resource. Allele specific PCR (AS-PCR) is rapid, sensitive, and applicable in conventional thermal cycler instruments with detection sensitivity of 1-2 % [3]. In addition, zygosity of mutations can be simply and directly inferred by AS-PCR without the need to further analysis.

Previous studies on frequency of *JAK2*617F zygosity and associated clinical outcomes reported variable data and association between *JAK2V617F* zygosity and characteristic features of disease is not fully clear. In addition, up to our knowledge, none of these studies were conducted on Iraqi MPN patients. Therefore, we aimed to determine frequency of *JAK2V617F* zygosity and corresponding outcomes in this population.

Materials and Methods

Patients

This cross section study involved 162 *JAK2V617F*-mutated MPN patients of various types. Patients admitted to National center of Hematology (NCH), Baghdad from January 2021 to March 2024 were enrolled in this study. Ethical approval was granted from the review ethical committee at the NCH (reference: nch-erc-22-16) and patient's consent was collected from all participants.

Blood samples and DNA extraction

Peripheral blood samples were collected from all patients. DNA was extracted immediately using blood genomic DNA kit (Promega) as described by manufacturer. DNA samples were stored at -20 °C before analysis.

JAK2V617F zygosity analysis

All patients were tested for *JAK2*V617 mutation by amplification refractory mutation system PCR (ARMS-PCR) as previously described [25]. Briefly, two primer pairs were used to amplify a region flanking mutation site and two primer pairs specific for either wild-type or mutant sequence. The former pairs produce an amplicon of (463 bp) regardless of *JAK2V617F* status (as internal control), while the latter pairs should produce an amplicon for either wild-type (229 bp) or mutant allele (279 bp) or both. These Primers are (FO) 5`-TCCTCAGAACGTTGATGGCAG-3` and (RO) 5`-ATTGCTTTCCTTTTTCACAAGAT-3` (internal control), and (Fwt) 5`-GCATTTGGTTTTAAATTATGGAGTATATG-3` (wild-specific), and (Rmt) 5`-GTTTTACTTACTCTCGTCTCCACAAAA-3` (mutant-specific). Samples showed both 229 bp and 279 bp amplicons were considered heterozygous and those showed only 229 bp amplicon were considered homozygous. PCR reaction mixture (25 µl) involved 12.5 µl 2X GoTaq green master mix (Promega, USA), 2 µl DNA template, 1 µl of each of 4 primer stocks (10 µmol), and 6.5 µl nuclease-free water. For PCR reaction, C1000 thermal cycler (Bio-Rad, USA) instrument was used for initial denaturation (94 °C for 5 min) followed by (94 °C for 30 sec; 56 °C for 30 sec; 72 °C for 30 sec) for 38 cycles, then final extension (72 °C for 5 min). Analysis of PCR products were then performed by (2 %) agarose gel electrophoresis followed by band visualization by UV-transillumination.

Statistical analysis

Collected data was analysed and graphs were created using GraphPad Prism (v7.0) software. The probability was calculated using the following tests: unpaired t-test, Mann-Whitney test, and Chi-square test for parametric, non-parametric, and categorical data respectively. Analysis of variance (ANOVA) test was used to calculate difference between group means. P value cut off of ≤ 0.05 was considered significant difference.

Results

The total number of patients involved in this analysis was 162 (91 males and 71 females). Patient's age ranged from 15-87 years (mean= 57.68 ± 15.5). The majority of patients (85.8 %) were older than 40 years. Characteristics of total patients are summarized in (Table 1). All patients were tested for *JAK2V617F* as described in methods section and (Figure 1) shows an example of samples

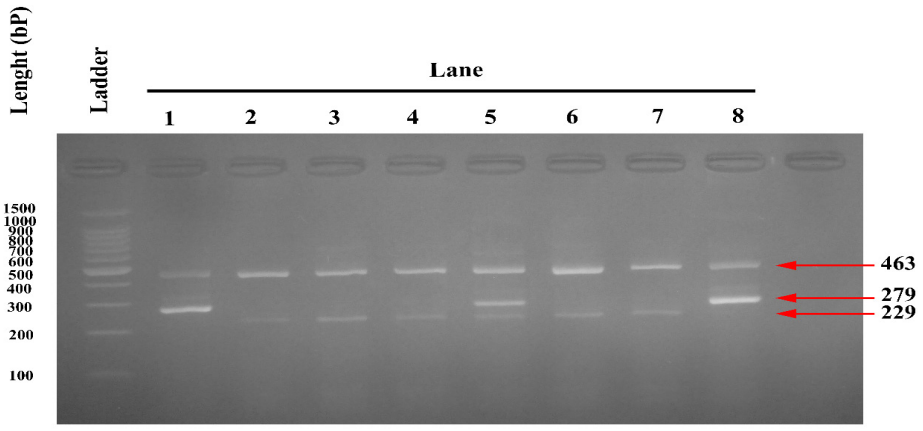

Figure 1. ARMS-PCR for *JAK2V617F* Screening. Samples were Tested for *JAK2V617F* Mutation as Described in Method Section then Analysed by (2%) Agarose Gel Electrophoresis. Red safe stain was used for DNA staining and samples loading volume was 5 µl. Lane number 1 and 8 shows *JAK2V617F* homozygous samples and lane number 5 shows *JAK2V617F* heterozygous sample. The remaining lanes are negative.

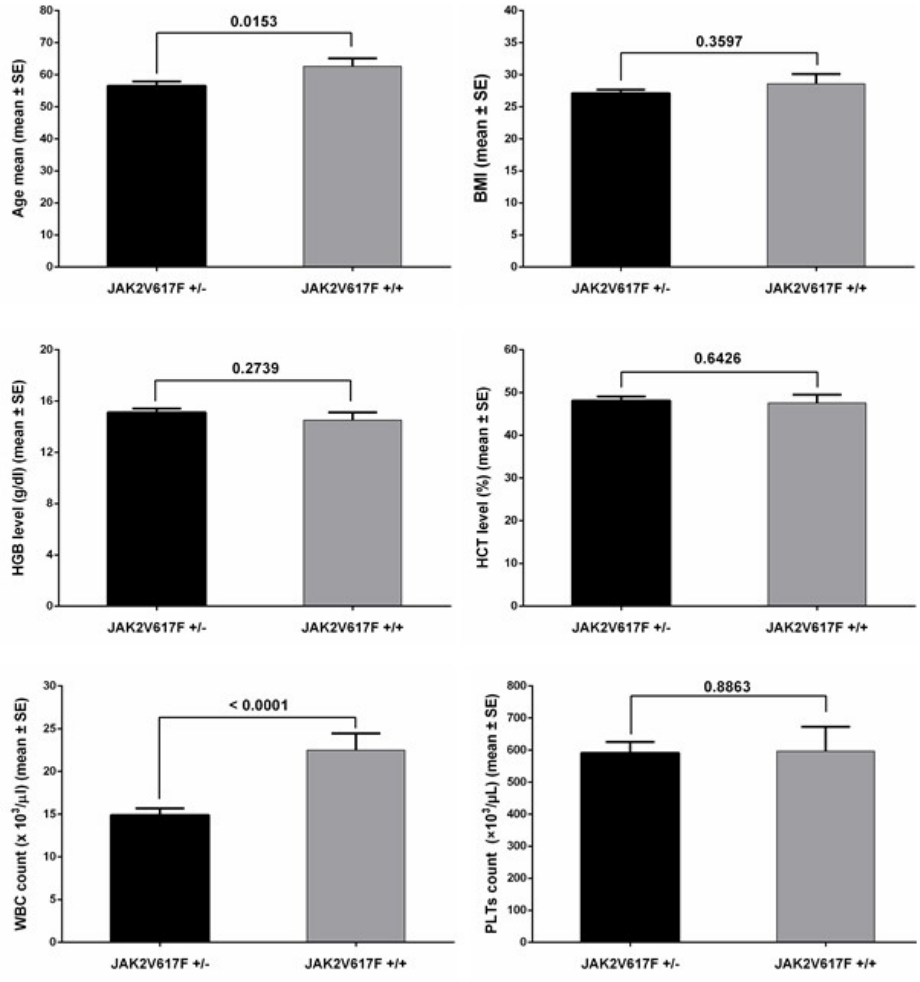

Figure 2. Comparison between Heterozygous and Homozygous MPN patients *JAK2V617F* +/- (heterozygous), *JAK2V617F* +/+ (homozygous).

Table 1. Phenotypic Characteristics (All Patients)

Characteristics		Analysed patients (n=162)
Age, median (range)		60 (15-87)
Age group (n) (%)	≤ 40	23 (14.2)
	> 40	139 (85.8)
Sex, n (%)	Male	91 (56.17)
	Female	71 (43.83)
BMI, mean ± SE		27.32 ± 0.509
Smoking, n (%)	Yes	20 (12.35)
	No	142 (87.65)
HGB median (g/dl) (range)		15.35 (5.3 – 23.1)
HCT median (%) (range)		49.7 (12.1 – 72.1)
WBC median (×10³/µL) (range)		13.45 (3.0 – 64.4)
PLT median (×10³/µL) (range)		473.5 (7.9-2484)

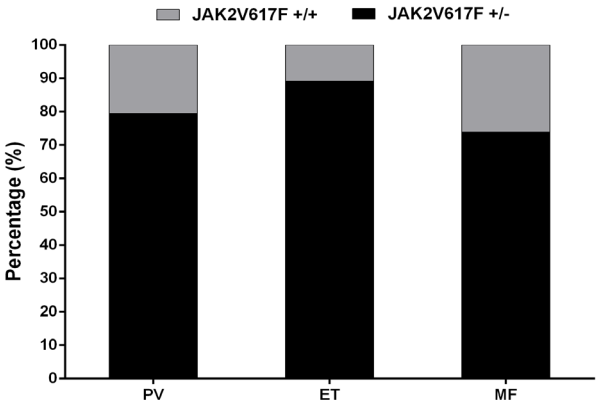

Figure 3. Frequency Distribution of *JAK2V617F* Zygosity in MPN Groups. *JAK2V617F* +/- (heterozygous), *JAK2V617F* +/+ (homozygous).

testing.

Among total patients, 131 (80.86 %) were heterozygous and 31 (19.14 %) were homozygous for *JAK2V617F* mutation (Table 2). Analysis showed that homozygous patients were significantly older than heterozygous patients (P= 0.0153) (Table 2 and Figure 2). No significant difference seen in sex distribution between the two groups (P= 0.868). Interestingly, results showed remarkable difference between the two groups in total WBC count (P= < 0.0001), while no difference seen in other blood parameters (Table 2 and Figure 2).

Among total MPN cases, the number of PV, ET, and MF patients was 102 (62.96%), 37 (22.84%), and 23 (14.2%) respectively (Table 3). No significant difference was seen between MPN groups in terms of age and smoking status, sex distribution, and BMI. In addition, the

Table 2. Phenotypic Characteristics (by *JAK2V617F* Zygosity)

Characteristics		*JAK2V617F*- Heterozygous Patients	*JAK2V617F*- Homozygous Patients	P value
Number, (%)		131 (80.86)	31 (19.14)	
Age, median (range)		57 (15-87)	65 (17-82)	0.0153
Age group (n)	≤ 40	21	2	0.169
	> 40	110	29	
Sex (n)	Male	74	17	0.868
	Female	57	14	
BMI, median (range)		25.95 (15.9-44.4)	29.38 (18.3-37.3)	0.36
Smoking (n)	Yes	18	2	0.267
	No	113	29	
HGB median (g/dl) (range)		15.5 (5.3 – 23.1)	14.9 (8.3-22.2)	0.274
HCT median (%) (range)		49.75 (12.1 – 72.1)	49.1 (27.2 - 70)	0.643
WBC median ($\times 10^3/\mu L$) (range)		12.17 (3.0 – 64.4)	22.2 (6.4-50.8)	< 0.0001
PLT median ($\times 10^3/\mu L$) (range)		478 (7.9- 2484)	402 (98.5-1798)	0.886

(BMI), Body Mass index; (HGB), Hemoglobin; (HCT), Hematocrit; (WBC), white blood cells, (PLT) Platelet

majority of MPN patients of all groups were older than 40 years old. Expectedly, analysis showed that Hemoglobin (HGB) and Hematocrit (HCT) levels were higher in PV group than ET and MF groups. On the other hand, MF group showed the highest white blood cells (WBC) count, while Platelet (PLT) count in ET group was higher than PV and MF groups (Table 3).

Our results showed the frequency of *JAK2V617F* mutation zygosity in MPN groups. The majority of cases were heterozygous with the number of heterozygous versus homozygous cases was (81 vs. 21) for PV, (33 vs. 4) for ET, and (17 vs. 6) for MF (Table 3). The frequency of homozygous cases was 20.6 %, 10.8 %, and 26.1 % for PV, ET, and MF patients respectively (Figure 3).

To elucidate effects of *JAK2V617F* zygosity on individual MPN types, we compared some parameters of the heterozygous and homozygous patients of PV, ET, and MF (Figure 4). Analysis showed no significant difference in age, BMI, and HCT between homozygous and heterozygous cases in all MPN groups. In ET group, patients showed significant HGB reduction in homozygous cases compared with heterozygous (P= 0.0186). Also homozygous ET patients showed significant increase in PLT count compared with heterozygous patients (P= 0.0034). Notable finding was the difference in WBC count between homozygous and heterozygous patients of all MPN groups. Results showed that homozygous outweigh heterozygous patients in WBC count in PV (19.7 ± 2.05 vs. 14.7 ± 1.09) (P= 0.0071), ET (25.7 ± 4.81 vs. 14.7 ± 1.09) (P= <0.0001), and MF (30.2 ± 5.48 vs. 19.8 ± 2.62) (P= 0.07) patients (Figure 4).

Discussion

Current study showed frequency of homozygous/ heterozygous status of *JAK2V617F* mutation in MPN patients and associated clinical outcomes. Overall, this study confirmed previous findings of high frequency

Table 3. Phenotypic Characteristics (by MPN disorder type).

Characteristics		PV	ET	MF	P value
Number, (%)		102 (62.96)	37 (22.84)	23 (14.2)	-
Age, mean ± SE		58.12 ± 1.5	54.68 ± 3.2	60.57 ± 1.83	0.324
Age group (n)	≤ 40	11	11	1	0.0063
	> 40	91	26	22	
Sex (n)	Male	60	16	15	0.168
	Female	42	21	8	
BMI, mean ± SE		27.80 ± 0.65	27.23 ± 1.1	25.64 ± 1.3	0.338
Smoking (n)	Yes	12	6	2	0.661
	No	90	31	21	
Zygosity (n), (%)	homo	21 (20.6)	4 (10.8)	6 (26.1)	0.284
	hetero	81 (79.4)	33 (89.2)	17 (73.9)	
HGB (g/dl), mean ± SE		16.8 ± 0.24	13.19 ± 0.33	9.87 ± 0.55	< 0.0001
HCT (%), mean ± SE		53.83 ± 0.71	42.72 ± 1.11	30.9 ± 1.84	< 0.0001
WBC ($\times 10^3/\mu L$), mean ± SE		15.73 ± 0.98	14.22 ± 1.07	22.5 ± 2.5	0.0035
PLT ($\times 10^3/\mu L$), mean ± SE		475.3 ± 27.15	927.4 ± 55.27	569.9 ± 130.5	< 0.0001

(BMI), Body Mass index; (HGB), Hemoglobin; (HCT), Hematocrit; (WBC), white blood cells; (PLT), Platelet.

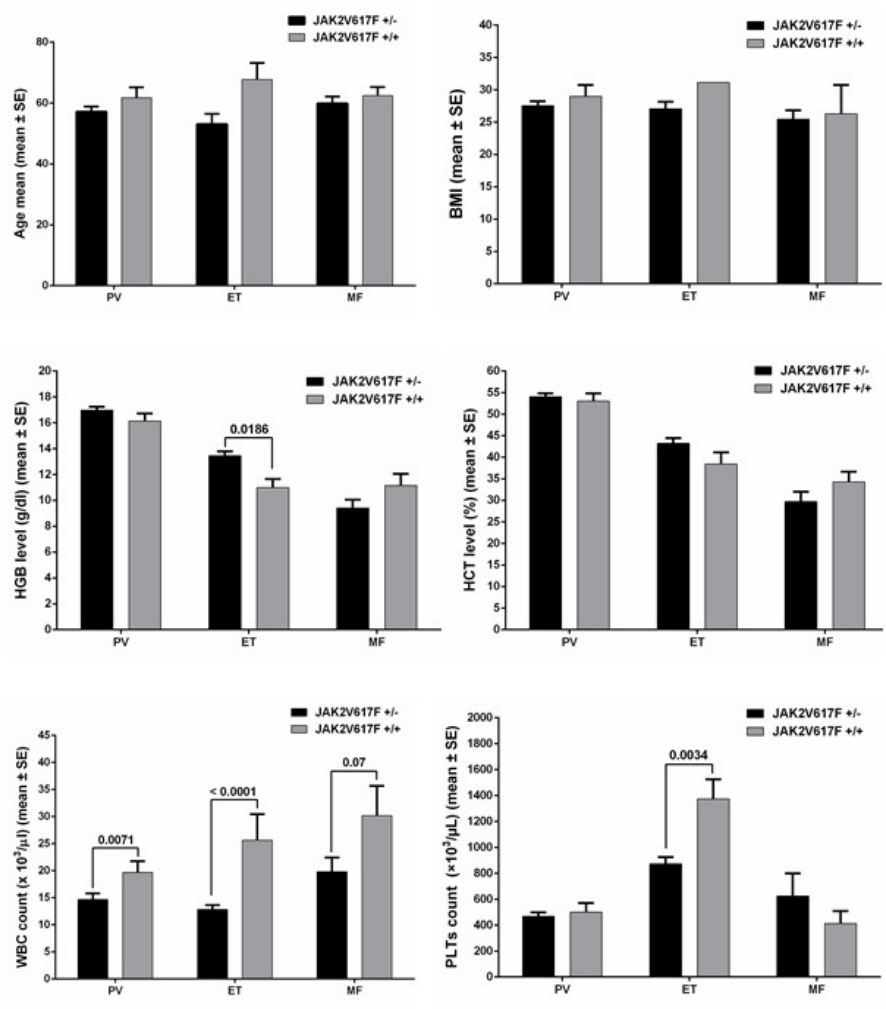

Figure 4. Comparison between Heterozygous and Homozygous MPN Groups *JAK2V617F* +/- (heterozygous), *JAK2V617F* +/+ (homozygous).

of heterozygous status of *JAK2V617F* mutation and association between zygosity and number of parameters in MPN patients. We used ARMS-PCR technique to genotype *JAK2V617F* mutation. This technique is precise, easy, cost-effective, and useful for qualitative detection of zygosity [26], and has been used frequently in previous studies [3, 25, 27]. Patients aged > 40 years represented the majority of population, a characteristic feature of MPN population as shown previously [28]. Analysis showed that the majority of screened patients irrespective of MPN disease type were heterozygous. Similar zygosity trend has been shown from local area studies involved Egyptian [27] and Sudanese patients [29]. Data available from other studies reported frequency of zygosity for individual MPN type which collectively indicated predominance of heterozygous status [30, 27, 6, 16].

It has been shown that gain of allele homozygosity is positively associated with mutational load [31]. Previous studies suggested that *JAK2V617F* allele burden is associated with MPN patient's prognosis factors and laboratory parameters. However which factors or parameters associated with allele burden is a debateable question [32, 23]. In agreement with previous findings [33, 17, 34, 32], mean age of homozygous patients was significantly higher than heterozygous. This is consistent with previous findings suggesting accumulation of *JAK2V617F* mutational load over time [18, 35, 36]. However, other studies showed no significant association of allele burden with age [37-39]. Data available from literature about association between *JAK2V617F* zygosity and sex distribution among MPNs is variable, whereby association was shown by groups [40, 15, 41, 42] but not by others [38, 33]. Our results showed a remarkable difference between homozygous and heterozygous groups in terms of WBC count. Previous evidence suggested positive correlation between Leukocytosis and *JAK2V617F* mutation load [18, 38]. We then perform analysis on the basis of individual MPN disease type. Despite insignificant difference in sex distribution between PV, ET, and MF (Table 3), there was a trend toward prevalence of male sex in PV and female sex in ET. This trend was shown by previous studies [42, 41, 36].

MF patients showed the highest frequency of homozygous cases followed by PV then ET (Figure 3). This is consistent with previous study by Antonioli et al. showed that the highest *JAK2V617F* allele burden was in MF patients followed by PV then ET patients [16]. In addition, previous data reported homozygosity in 25-30

% of MF patients [4, 31, 6]. Previous reports also showed higher *JAK2V617F* allele burden in PV than ET patients [38, 34, 36]. Moreover, it has been shown that growing of colonies derived from PV and ET patients expand homozygous subclones mainly from PV patients [24].

Results showed a trend of increased age in homozygous over heterozygous group in all MPN entities but this did not reach significance. Previous groups showed correlation between increased mutational burden and ageing in PV [33, 42, 36], while others, like us, did not [38]. Vannucchi et al. reviewed studies involved association between *JAK2V617F* allele burden and older age and showed positive correlation in 7 of 9 studies [17]. In ET group, analysis showed significant HGB reduction and PLT increasing in homozygous cases compared with heterozygous. This result is disagree with previous findings that *JAK2V617F* allele burden is positively associated with HGB and negatively with PLT levels [17], As well as disagree with studies showed that *JAK2V617F*-positive ET patients were manifested with higher HGB and lower PLT levels compared with unmutated ET group [44, 45, 41]. However, Vannucchi et al showed that homozygous ET patients had higher PLT count and there were no significant difference between homozygous and heterozygous patients in HCT level [32]. *JAK2V617F* allele burden is positively associated with WBC in 7 of 12 previous studies involved ET patients [17]. In PV patients, association between allele burden and Leukocytosis is common and frequently shown in majority of previous studies [37, 38, 13]. Increased WBC count is one of the minor diagnostic criteria of MF and has been shown to be useful in prediction of risk of leukemic transformation in MF patients [18]. This study had number of limitations including: loss of follow up of patients so we could not study effect of zygosity on prognosis and disease complications, unavailability of quantitative estimation of allele burden to do correlation analysis, zygosity screening was performed at different time point from disease onset which might affect precise frequency of homozygosity, and MF patients' data represented all patients with myelofibrosis whether they were primary or post PV/ET MF.

Determining zygosity of *JAK2V617F* mutation is useful in determining disease severity and can be used for prediction of prognosis in MPN patients. ARMS-PCR is simple, easy, and useful technique in determining zygosity of mutations and does not need to advanced laboratory resources. We recommend including zygosity in laboratory reports when conducting *JAK2V617F* using ARMS-PCR technique.

Author Contribution Statement

experimental work, analysis and writing of the manuscript was conducted by Mushtaq M. Khazeem.

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Ethics

this work was approved by the review ethical committee at the NCH (reference: nch-erc-22-16).

Conflict of interest

The author declare no conflict of interests.

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