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Impact of JAK2V617F Mutational on Haematologic Features in Sudanese Patients with Essential Thrombocythemia and Thrombotic Risk Assessment

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Abstract

Objective: We correlated selected hematological parameters in Sudanese essential thrombocythemia (ET) patients based on their homozygous/heterozygous JAK2V617F genotype, as well as the application of thrombotic risk assessment using different thrombotic risk scoring models. **Methods:** In this single-center study, we evaluated 60 patients with ET at the time of the diagnosis without any prior treatment. Amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) technique was used to determine JAK2V617F mutation status. Complete blood count was evaluated using the Sysmex analyzer. Furthermore, the thrombotic risk assessment of ET patients using different thrombotic risk scoring models was applied.

Results: The JAK2V617F mutation was detected in 29/60 patients (48.3%), of whom 23 (38.3% of total) were heterozygous and 6 (10.0%) were homozygous. Compeered to JAK2 wild-type or JAK2 heterozygous patients, JAK2 homozygous patients for JAK2V617F mutation were associated with older age($p<0.05$), significantly higher mean leukocytes count ($P=0.001$), significantly lower Hb concentration ($p<0.05$), and splenomegaly ($p<0.05$), while the mean of the platelet counts was slightly higher, although not reached a significant level. We also found two patients who developed thrombotic events throughout follow-up and

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were initially classified as a low-risk category in the traditional classification. One of them with age < 60 years, hypertension, and JAK2 homozygosity but without thrombosis history, was allocated in a high-risk category by IPSET-t and r- IPSET-t scores. The second patient was stratified in a low-risk category by all scoring models with age < 60 years, hypertension, leukocytosis, unmutated JAK2, and without a history of thrombosis.

Conclusions: The JAK2 V617F homozygosity correlated with older age, higher leukocyte count, lower Hb concentration, and a higher risk of thrombosis in Sudanese ET patients. Evaluation of hypertension and identification of JAK2 V617F homozygosity at diagnosis of ET might give the clinician more meaningful prognostic information and so improve the therapeutic management.

Keywords: *Essential Thrombocythemia; JAK2 mutation, JAK2 V617F homozygosity; Thrombotic risk.*

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1 Introduction

Essential thrombocythemia (ET) is a chronic myeloproliferative neoplasm (MPN) characterized by a clonal expansion of megakaryocyte and marked thrombocytosis (Campbell et al., 2006) The JAK2V617F exon 14 mutation in the tyrosine pseudokinase region of the JAK2 gene is the leading mutation for MPNs, occurring in approximately 50-60% of ET patients(Baxter et al., 2005, Cross, 2011, Levine et al., 2005). JAK2V617F mutation is a somatic mutation resulting from the substitution of valine for phenylalanine at codon 617 of the JAK2 protein, causing constitutive activation of JAK2V (Campbell et al., 2006, Levine et al., 2005, James et al., 2005, Kralovics et al., 2005). Activated JAKs prompt the activation of downstream positive signaling pathways, including JAK-STAT, MAPK, and PI3K/AKT (Yao et al., 2006, Yamada and Kawauchi, 2013, Loureiro et al., 2010). These pathways are involved in various cellular processes including differentiation, proliferation survival, and apoptosis with well-established roles in the initiation and progression of hematologic malignancies (Yao et al., 2006, Yamada and Kawauchi, 2013, Loureiro et al., 2010).

The median age at presentation of ET patients is over 60 years (Tefferi and Barbui, 2017, Oliva et al., 2012). Patients with ET are usually asymptomatic but clinical signs and symptoms if present include excessive sweating, fatigue, bruising, and headaches (Mesa et al., 2007, Elbager et al., 2018, Mesa et al., 2016). Thrombotic events in ET patients represent the major clinical complication with a prevalence of 20.7% (Rungjirajittranon and Owattanapanich, 2019)and they are the second most common cause of morbidity and mortality after infections (Casini et al., 2013). Accordingly, many models were established to assess the risk of thrombotic complications.

The traditional risk factors for thrombosis in ET include advanced age ≥60 years and a prior thrombotic event (Barbui et al., 2011). According to the presence or absence of these factors, patients are stratified into low- or high-risk groups to monitor treatment. In 2012, the International Working Group for Myeloproliferative Neoplasms Research and Treatment (IWG-MRT) have developed a new international prognostic score to identify the prognostic risk for devolving thrombosis in ET named (IPSET-thrombosis) (Barbui et al., 2012). This model takes into account four risk factors: age >60, history of thrombosis, cardiovascular risk factors, and JAK2V617F mutation. IPSET-t divides patients into three risk categories; low-risk, intermediate-risk, and high-risk. In 2016 the IPSET-thrombosis model was revised into three risk factors: age > 60, thrombosis history, and presence of JAK2 V617F. Patients were stratified into very low-risk, low-risk, intermediate-risk, and high-risk categories (Haider et al., 2016). Based on such risk stratification, ET patients at high risk of thrombosis require cytoreductive therapy to preventing thrombotic complications (Tefferi and Barbui, 2017). This study aimed at certain selected hematological parameters in Sudanese ET patients based on the homozygous/heterozygous JAK2V617F genotype. Moreover, we assessed the thrombotic risks using different thrombotic risk scoring models.

2. Materials and Methods

Patients and Samples

A total of 60 newly diagnosed ET patients who attended the Radiation and Isotope Center at Khartoum (RICK) and the general outpatient department of the Military hospital were enrolled in

this study. Diagnosis of ET was performed according to the 2008 revised WHO criteria. There were 42 (70%) females and 18 (30%) males; the age range between 23-80 years old. Six milliliters of venous blood was collected from each patient, 3 ml each for CBC and molecular analysis placed in ethylene diamine tetraacetic acid (EDTA) container.

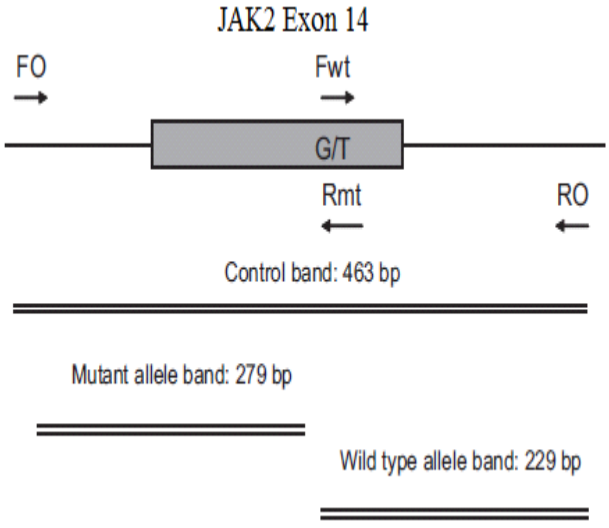
Blood samples were tested for leukocyte count, Hb concentration, and platelet count performed using Sysmex 21 hematological analyzer.

JAK2 V617F genotyping

The human genomic DNA was extracted from peripheral blood using the G-spin™ Total DNA Extraction Mini Kit (iNtRON Biotechnology, Korea) following the manufacturer's instructions. The DNA extracted was dissolved in nuclease-free water, and stored at -20°C until further use.

The JAK2 V617F mutation was genotyped by ARMS-PCR. Different techniques reported in the literature for JAK2 V617F genotypic analysis, such as direct sequencing, allele-specific, polymerase chain reaction (PCR), PCR-restriction fragment length polymorphism, and amplification refractory mutation system (ARMS)-PCR (Didone et al., 2015). ARMS-PCR is a technique initially developed for the analysis of single nucleotide polymorphisms (SNP), and the technique has been effectively applied to evaluate the JAK2 genotypes (Jones et al., 2005). This technique has several advantages; simple, fast, inexpensive, enables immediate amplification of the mutant and normal alleles consensus JAK2 internal control product with just two pairs of primers in a single-tube reaction and does not require any special equipment other than a thermocycler.

The ARMS-PCR technique uses 4 primers as follows; a forward outer primer (FO), a reverse outer primer (RO), a forward inner wild type-specific primer (Fwt), and a reverse inner mutant specific primer (Rmt). Primers FO and RO resulting in a band of 463 bp to control for DNA quality and quantity. Primers Fwt and RO generating a band of 229 bp as wild-type allele, and primers FO and Rmt generate a band of 279 bp as a mutant allele. Principles of the ARMS-PCR assay and primers sequences are shown in Figure 1.



Forward outer (FO)
5-TCCTCAGAACGTTGATGGCAG-3
Reverse outer (RO)
5-ATTGCTTTCCTTTTTCACAAGAT-3
Forward wild-type (Fwt)
5-GCATTTGGTTTTAAATTATGGAGTATaTG-3
Reverse-mutant–specific (Rmt)
5-GTTTTACTTACTCTCGTCTCCACAaAA-3

Figure 1. Diagram outline of the ARMS assay and primers Sequence. Primers FO and RO resulting in a band of 463 bp to control for DNA quality and quantity. Primers Fwt and RO generating a band of 229 bp as wild-type allele, and primers FO and Rmt generate a band of 279 bp from the mutant allele.

Amplifications were performed according to the protocol of Jones et al (Jones et al., 2005) with some modifications to prevent the formation of nonspecific bands. Amplifications were performed using 12.5 µl of fusion master mix (Thermo Scientific, Germany), 10 µl of DNA (100 ng), 0.4 µl of FO primer, 0.3 µl of RO primer, 0.5 µl of Fwt primer, 1.0 µl of Rmt primer, and 0.3 µl of nuclease-free water in a total volume of 25 µl. Next, the reaction samples were placed in TaKaRa PCR thermal cycler dice, programmed in the following cycles: initial denaturation at 95°C (5 minutes) and 40 cycles of 94°C (30 seconds), 58°C (45 seconds), 72°C (45 seconds), and a final 72°C extension for 10 minutes.

A total of 10 µl from the PCR product were electrophoresed on 2% standard agarose gels (Thermo Scientific, Germany) with ethidium bromide (0.5

μg/mL) at 80 V for 50 min and visualized using an image analyzer.

Thrombotic risk stratification

Three different thrombotic risk models available in clinical practice (the traditional model, the IPSET-t model, and the r-IPSET-t) were used. The traditional model risk factors include advanced age ≥60 years and a prior thrombotic event are regarded as predictive risk factors for future thrombosis. IPSET-t model takes into account four risk factors: age >60 was scored (1 point), history of thrombosis was scored (2 points), cardiovascular risk factors were scored (1 point), and JAK2V617F mutation was scored (2 points). IPSET-t divides patients into three risk categories (low-risk = 0–1 point, intermediate-risk = 2 points, and high-risk ≥3 points). The revised IPSET-thrombosis model stratifies patients into four risk categories very low-risk (age ≤60 years, no prior history of thrombosis, and no JAK2 mutation); low-risk (age ≤60 years, no prior history of thrombosis, and JAK2 mutation); intermediate risk (age >60 years, no prior history of thrombosis, and no JAK2 mutation); and high-risk (prior history of thrombosis and/or age >60 years with JAK2 mutation).

Statistical analysis

The statistical analysis of laboratory results and patients' data were done using SPSS version 21. Frequency differences of sex, splenomegaly, or thrombotic event correlated to the JAK2 mutational status were determined by descriptive statistical analysis. An independent sample t-test was used to correlate between the JAK2 mutational status and leukocytes count, Hb concentration, or platelet count. Independent-t-tests was used to test correlations between hematological parameters and JAK2 mutational status. Statistical significance was assigned at $p < 0.05$.

3. Results

Prevalence of JAK2–V617F mutation

The JAK2V617F mutation was identified detected in 29 /60 (48.3%) patients, of whom 23 (38.3% of total) were heterozygous and 6 (10.0%) were homozygous. Fig. 2 represents ARMS assay to detect the JAK2V617F in genomic DNA.

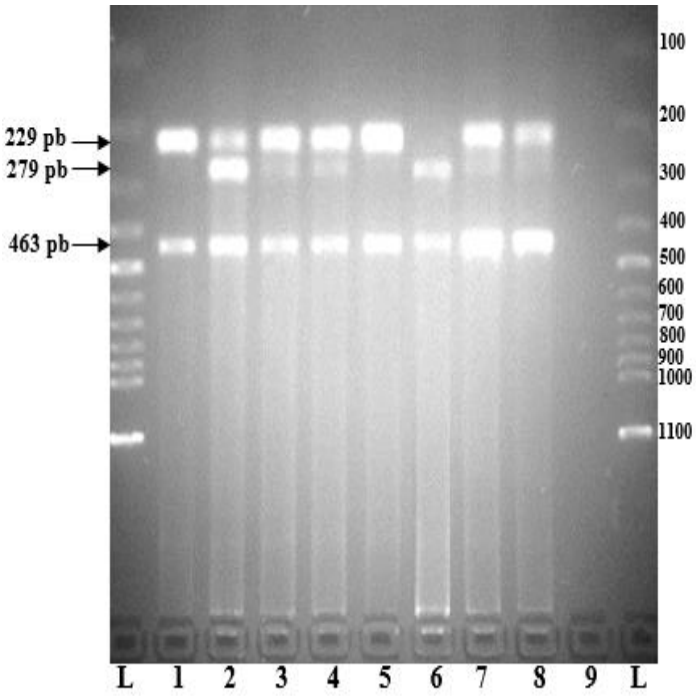


Figure 2. A representative figure of ARMS assay to detect the JAK2V617F in genomic DNA. Track 1 is a negative control show a normal genotype. Track 2 is a positive heterozygous control. Tracks 3,4,7 and 8 show a mutant band and normal band, therefore, counted as heterozygous for the JAK2V617F mutation. Track 5 shows a normal genotype therefore counted as negative for the JAK2V617F mutation. Track 6 shows only mutant band therefore counted as homozygous for the JAK2V617F mutation. Track 9 blank and L is the 100bp DNA ladder

Correlations of JAK2–V617F mutation with clinical and laboratory findings

Table 1 summarized the clinical and laboratory characteristics of the ET patients according to JAK2V617F mutational status. The median age of JAK2 wild-type patients was 42 years (range: 24-74 years), 50 years (range: 22-75 years) in JAK2V617F heterozygous patients, and 57.5 years (range: 43-80 years) in JAK2V617F homozygous patients with predominant female gender in all groups. The mean age of patients with JAK2V617F mutation (heterozygous or homozygous) was significantly higher than the JAK2 wild-type patients ($P < 0.05$). No significant differences were detected when comparing mean Hb concentration between JAK2 wild-type patients and JAK2 V617F heterozygous patients. In contrast, JAK2V617F homozygous patients presented with significantly lower Hb concentration than the

other groups. Moreover, JAK2V617F homozygous patients displayed a significantly higher mean leukocyte count compared to JAK2 wild-type patients and JAK2 V617F heterozygous patients (P<0.05). The mean platelet counts in JAK2 V617F mutated patients (heterozygous or homozygous) were slightly higher compared to JAK2 wild-type patients although not statistically significant (P>0.05). One patient of JAK2 wild-type (3.2%) had a prior thrombotic event at diagnosis. During follow-up, thrombosis occurred in one patient of JAK2 V617F homozygous (16.7%). Four patients had hypertension, 1 (3.2%) JAK2 wild-type, 1(4.3%) JAK2 V617F heterozygous, and 2 (33.3%) JAK2 V617F homozygous. 2 patients were smokers, 1 (3.2%) JAK2 wild-type, 1(4.3%) JAK2 V617F heterozygous as summarized in Table 1.

Table 1: Clinical and laboratory characteristics of the ET patients at diagnosis

Variables	**JAK2 wild-type (WT)**	**JAK2 V617F heterozygous**	**JAK2 V617F homozygous**
Patients, no. (%)	31(51.7%)	23(38.3%)	6 (10.0%)
Male/ Female	1:2.4	1:2.3	1:2
Age years, median (range)	42 (24-74)	50(22-75)	57.5 (43-80) * P <0.05
Hb (g/dl) median (range)	12 (8.9-16.1)	12.3 (9.6-15.9)	8.8 (4.2-11.2)** P =0.001
leukocytes count (x 103/mm3), median (range)	9.5 (4.2- 89.6)	10.1(5.5-53.9)	14.4 (8.2-47)**, P=0.001
Platelets count (x 103/mm3), median (range)	720 (469-2838)	1012 (470-1288)	1114.5 (981-2322) P >0.05
Splenomegaly, no. (%)	1 (3.2%)	6 (26.1%)	4 (66.7%) P <0.05
Prior history of thrombosis	1 (3.2%)	0 (0 %)	0 (0 %)
Thrombosis during follow-up	1 (3.2%)	0 (0 %)	1 (16.7%)
CVS risk factors			
Hypertension	1 (3.2%)	1(4.3%)	2 (33.3%)
Smoking	1 (3.2%)	1(4.3%)	0 (0 %)

*p-value is considered statistically significant when p<0.05 in the comparison of homozygous patients to either wild-type or the heterozygous groups.

** p-value is statistical significance when p< 0.001 in the comparison of homozygous patients to either wild-type or the heterozygous groups.

Thrombotic risk classification

According to traditional risk classification score, 70% of the patients (42 patients; 25 JAK2 wild-type, 16 JAK2 V617F heterozygous, and 1 JAK2 V617F homozygous) were classified as low-risk, and 30% (18 patients; 6 JAK2 wild-type, 7 JAK2 V617F heterozygous and 5 JAK2 V617F homozygous) as a high-risk.

According to the IPSET-t, 50% (30 JAK2 wild-type patients) were classified as a low-risk group, 28.3% (one JAK2 wild-type patients and 16 JAK2 V617F heterozygous patients) were classified as intermediate-risk and 21.7% (13 patients; 7 heterozygous patients and 6 homozygous patients) were classified as a high-risk category.

According to the r-IPSET-t 41.7% (25 JAK2 wild-type patients) were classified as a very low-risk group, 26.7% (16 JAK2 V617F heterozygous patients) were classified as a low-risk category, 8.3% (5 JAK2 wild-type patients) as intermediate risk, and 23.3% (14 patients; one JAK2 wild-type, 7 heterozygous and 6 homozygous patients) as a high-risk (Table 2).

Table 2. Classification of ET patients according to different risk scoring

Risk group	Traditional	IPSET-t	r-IPSET-t
Very low	-	-	25 (41.7%)
Low	42 (70%)	30(50%)	16 (26.7%)
Intermediate	-	17 (28.3%)	5 (8.3%)
High	18 (30%)	13 (21.7%)	14 (23.3%)

When patients were reclassified using IPSET-t, of the 42 traditionally low-risk patients, 59.5%, (25 JAK2 wild-type patients) were reclassified as a low-risk, while 38.1% (16 patients heterozygous) were newly classified as intermediate risk and one JAK2 V617F homozygous patient (2.4%) classified as a high-risk. From the 18 traditional high-risk category patients, 27.8% (5 patients JAK2 wild-type) were reclassified as a low-risk, 5.5 % (one patient JAK2 wild-type) were classified as intermediate risk and 66.7% (7 heterozygous patients and 5 homozygous patients) classified as a high-risk (Table 3).

Table 3. Distribution of patients according to thrombotic risk calculated with the traditional classification and IPSET-t score

	IPSET-t score			
Traditional classification	Low	Intermediate	High	Total
Low	25 (59.5%)	16 (38.1%)	1 (2.4%)	42 (100%)
High	5 (27.8%)	1 (5.5%)	12 (66.7%)	18 (100%)
Total	30	17	13	60

Furthermore, by comparing the IPSET-t to r-IPSET-t stratification, from 30 low-risk patients according to IPSET-t, 80.6% (25 JAK2 wild-type patients) were classified as very low risk and 19.4% (5 JAK2 wild-type patients) classified as an intermediate-risk category. For IPSET-t intermediate-risk patients, 88.9% (16 heterozygous) patients were classified as low-risk, and 11.1% (one JAK2 wild-type patients) classified as a high-risk group. The IPSET-t high-risk category was the most conserved, with 100% of the patients classified in the same risk category when using the r-IPSET-t (Table 4).

Table 4. Distribution of the patients according to the thrombotic risk calculated with the IPSET-t score and the r-IPSET-t score

	r-IPSET-t score				
IPSET-t score	Very low	Low	Intermediate	High	Total
Low	25 (83.3%)	0 (0%)	5 (16.7%)	0 (0%)	30 (100%)
Intermediate	0 (0%)	16 (94%)	0 (0%)	1 (6%)	17 (100%)
High	0 (0%)	0 (0%)	0 (0%)	13 (100%)	13 (100%)
Total	25	16	5	14	60

Discussion

This study aimed to evaluate selected hematological parameters in ET patients based on the homozygous/heterozygous JAK2V617F genotype, as well as the application of the thrombotic risk assessment using different thrombotic risk scoring models.

Approximately 50% of ET patients were reported to have JAK2V617F mutation, either heterozygous or homozygous (Cetin et al., 2014, Karkucak et al., 2012, Ho et al., 2012, Ferdowsi et al., 2016, Yasin EB et al., 2019). Homozygosity of JAK2V617F results from a duplication of the mutant allele (Godfrey et al., 2012, Scott et al., 2006). Currently greater than 50% JAK2V617F allele burden within a granulocytic cell population indicating JAK2V617F homozygosity(Larsen et al., 2007, Campbell et al., 2006, Tefferi et al., 2011). Studies have reported that homozygosity for the JAK2V617F mutation is displayed by 2% to 5% of ET patients (Levine et al., 2005, Kralovics et al., 2005) (Vannucchi et al., 2007, Rumi et al., 2014, Antonioli et al., 2005, Antonioli et al., 2008). In agreement with previous, JAK2V617F mutation in this study was positive in 48% of ET cases, while JAK2V617F homozygosity was reported only in 10% of cases which is high compared to others studies but comparable to the result of Yasin EB et al (Yasin EB et al., 2019) at 14.3%.

The impact of JAK2V617F mutation burden on leukocyte counts, hemoglobin concentrations, platelet counts, spleen size in ET patients has been addressed by a limited number of studies (Antonioli et al., 2008, Malysz and Crisan, 2009, Popova-Labachevska et al., 2019). In our study, JAK2V617F homozygous ET patients presented with older age at diagnosis compared to JAK2 wild-type or heterozygous patients is consistent with previous reports (Ho et al., 2012, Antonioli et al., 2008, Lin et al., 2013). Moreover, it is in agreement with previous reports, as our results displayed that JAK2V617F homozygous ET patients presented with a higher leukocyte count and splenomegaly at diagnosis compared to other groups (Ho et al., 2012, Antonioli et al., 2008, Lin et al., 2013). In contrast to a previous report (Vannucchi et al., 2007, Rumi et al., 2014, Popova-Labachevska et al., 2019, Rumi et al., 2013), yet consistent with the study of (Yonal-hindilerden et al., 2015) and (Pich et al., 2012) our homozygous ET presented with lower hemoglobin concentrations at diagnosis compared to wild-type or heterozygous patients. In contrast to some earlier studies (Vannucchi et al., 2007, Rumi et al., 2014, Pich et al., 2012), but agreement with the study of (Popova-Labachevska et al., 2019) no statistically significant association were found between JAK2 mutational status and the mean platelet at diagnosis in ET patients. The differing data of the impact of JAK2V617F mutation on laboratory parameters in ET may relatively result from different ethnic backgrounds or low number of subjects evaluated.

Thrombotic complications are very frequent in ET. Therefore, the evaluation of the thrombotic risk is vital to make the optimal therapeutic decision. Compared with the traditional model, IPSET-t outlined a clear definition of 'intermediate-risk group' and provided a well-defined specification to the general cardiovascular risk factors and JAK2V617F mutation. When performing a comparison between the traditional classification and the IPSET-t score as well as the r-IPSET-t, there is a subset of patients that move off from the low to intermediate/high-risk group and from the high to low/intermediate-risk group. One patient (1.7%) was moved from the low-risk category of traditional classification into the high-risk category according to IPSET-t and r-IPSET-t scores. From the high-risk category of traditional classification, 5 (10.0%) patients with age >60 years, no thrombosis history, and JAK2-unmutated were moved into the low-risk category and intermediate low-risk category according to IPSET-t and r-IPSET-t scores respectively. The application of IPSET-t or r-IPSET-t scores has altered the risk stratification of 6 (11.7%) patients which consequently could change the therapeutic management. These results agree with those of Barbui et al (Barbui et al., 2012, Navarro et al., 2016, Accurso et al., 2020), who similarly indicated that IPSET-t seems to be better than the traditional model.

In our study, two patients who developed thrombotic events during follow-up were classified as traditionally low-risk patients and none of them receiving cytoreductive therapy. One patient was age < 60 years, with hypertension and JAK2 homozygosity but without thrombosis history. This patient was moved from the low-risk category of traditional classification into the high-risk category according to IPSET-t and r-IPSET-t scores. The second patient was stratified as low-risk by all scoring models with age < 60 years, hypertension, leukocytosis, unmutated JAK2, and without thrombosis history. These results highlighted the possible impact of cardiovascular risk factors on

the thrombotic risk for ET patients of less than 60 years and with cardiovascular risk factors. This in agreement with other authors who recommended evaluating the opportunity of initiating a cytoreductive therapy in ET patients with cardiovascular risk factors including hypertension, dyslipidemia, diabetes, cigarette smoke, obesity, even if classified into low/ intermediate-risk category according to other scoring models (Barbui et al., 2012, Accurso et al., 2020).

Interestingly, most of the JAK2-negative (wild type) patients were still correlated more frequently to the low-risk group. Most of the JAK2V617F heterozygous patients are associated with low/intermediate thrombotic risk categories, while JAK2V617F homozygosity was found to be associated with intermediate to high-thrombotic risk groups.

Conclusions:

The JAK2 V617F homozygosity correlates with older age, hematologic features including (higher leucocyte count and lower Hb concentration) and a higher risk of thrombosis in Sudanese ET patients. Evaluation of the cardiovascular risk factors and the identification of JAK2 V617F homozygosity at diagnosis of ET might provide the clinician with meaningful prognostic information and consequently improve the therapeutic management.

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Ethics

Ethics Committee Approval: This study was approved by the ethical committee of the Khartoum State Ministry of Health, Research Department.

Informed Consent: Informed consent was obtained from all participants before their inclusion in the study.

Authorship Contributions

Concept: S.G.E.; Perform the Laboratory Work and Interpretation of Findings: S.G.E.; Design and Supervision the Research Project: M.A.B., A.A.D.; Supervision of the Molecular Analysis of JAK2 Mutation: E.R.M.T., M.I.L.; Writing the Manuscript; S.G.E.; revising the manuscript: M.A.B., A.A.D., E.R.M.T., M.I.L.

Conflict of Interests

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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