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RESEARCH ARTICLE

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Hematological relevance of JAK2 V617F and calreticulin mutations in Tunisian patients with essential thrombocythemia

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Abstract

Background: The genetic investigation of essential thrombocythemia(ET) has highlighted the presence of driver mutations in ET. Janus kinase JAK2V617F and calreticulin(CALR) mutations are the most frequent driver mutations and have significantly improved the molecular diagnosis of ET. The impact of genetic heterogeneity on clinical features has not been fully elucidated.

This is the first study which aimed to determine the frequency of JAK2V617F and CALR exon9 mutations in Tunisian ET patients and to establish the correlation between hematological characteristics and mutational status.

Methods: This study included Tunisian patients suspected with ET and was conducted between September 2017 and March 2021. Genomic DNA of patients was isolated from peripheral blood samples. JAK2V617F was detected by AS-PCR and CALR mutations were detected by PCR/direct sequencing. Clinical and hematological characteristics were also analyzed.

Results: Two hundred and fifty ET patients were enrolled in this study. JAK2V617F mutation was found in 166/250 (66.4%) of patients, whereas CALR mutations were detected in 27/84 (32.1%) patients without JAK2V617F. Compared with JAK2V617F-positive patients, those with CALR mutations showed lower hemoglobin level and lower leucocytes count ($p = 0.007$ and $p = 0.004$,respectively). CALR type 2 was the most frequent mutation of CALR detected in 55.55% of CALR mutated. Six of seven patients with thrombotic events harbored JAK2V617F mutation.

Conclusion: The prevalence of driver mutations JAK2V617F or CALR mutations was 77.2% in Tunisian ET patients. Moreover, patients with JAK2 V617F mutation had a higher risk of thrombosis. The mutational status is necessary to improve the diagnosis and contribute to the therapeutic decisions.

KEYWORDS
calreticulin, driver mutations, essential thrombocythemia, hematological characteristics, Janus kinase (JAK) 2V617F

1 | INTRODUCTION

Essential thrombocythemia (ET) is a Philadelphia-negative myeloproliferative neoplasm (MPN), characterized by the clonal proliferation of the megakaryocytic lineage within the bone marrow causing an elevated platelet count in peripheral blood.[1,2] The annual incidence of ET is estimated at 1.2 to 3 per 10^5 individuals.[3] The median age at diagnosis is 60 years.[4] ET is associated with long overall survival with the a median survival approximately 20 years.[5,6] However, life expectancy of ET patients can be affected by the occurrence of haemorrhagic and thrombotic complications which are the primary cause of morbidity and mortality in ET.[7,8] These events can be identified at diagnosis or during the disease progression.[9] Consequently the treatment of ET aimed at reducing the risk of vascular complications.[10]

In most of ET cases, the overproduction of hematopoietic cells is stimulated by a driver mutation in JAK2 or CALR genes which were detected only in myeloid cell line.[1,11] JAK2V617F consisting of somatic mutation G to T at nucleotide position 1849 in exon 14 of JAK2 gene, results in the substitution of valine to phenylalanine at codon 617 of the protein.[9] The JAK2V617F protein is constitutively active leading to the activation of the transcription JAK/STAT pathway, which ensures the control of hematopoietic cell proliferation and survival, causing ET disease.[1,12] JAK2V617F is found in 50% to 60% of ET patients.[13]

Calreticulin is a highly conserved chaperone protein localized in the endoplasmic reticulum. It plays a crucial role in cellular proliferation, differentiation, and apoptosis.[14,15] Somatic mutations in CALR exon 9 are found between 20% and 30% in ET patients[13] The CALR exon 9 mutations are mostly found in patients who are negative for JAK2 V617F.[16–18] Two mutations of CALR gene seem to be more common than all others: type 1 (c.1092_1143del; L367fs*46) in 55% and type 2 (c.1154_1155insTTGTC; K385fs*47) in 35% of ET. [15] These mutations represent more than 80% of CALR mutations.[17–21] The mutant CALR protein interacts with MPL (Thrombopoietin receptor) through its extracellular domain, activating the downstream JAK–STAT pathway and leading to the cytokine independent growth.[15,22]

These somatic mutations play essential roles in the diagnosis and prognosis of the disease and are included in the 2016 WHO classification criteria of ET.[17,23,24]

The prevalence of JAK2V617F and CALR mutations in ET Tunisian patients remains undetermined.

In this study, we investigated the profiles of JAK2V617F and CALR mutations in Tunisian ET patients. The clinical and hematological features were compared according to mutation patterns.

2 | PATIENTS AND METHODS

2.1 | Patients

This was a retrospective study including all ET Patients referred to the hematology department of Pasteur institute of Tunis between September 2017 and March 2021.

The diagnosis of ET was established based on the 2016 World Health Organization (WHO) diagnostic criteria after exclusion of secondary thrombocytosis.

Clinical data including sex, age and a complete blood count were obtained by reviewing the medical records.

The study was approved by the ethics committee of Pasteur institute of Tunis. Written informed consents for mutation analyses were obtained from each patient enrolled in this study.

2.2 | Methods

2.2.1 | DNA extraction

For molecular analysis, DNA was extracted from 5 ml of peripheral blood samples collected on EDTA (ethylene diamine tetraacetic acid).

The genomic DNA was extracted by using blood DNA mini kit (Qiagen) as indicated by manufacturer's protocol.

2.2.2 | JAK2 V617F detection

All samples were assessed for JAK2V617F status using allele-specific polymerase chain reaction (AS-PCR) Kit from Qiagen as indicated by the manufacturer's protocol, on Applied Biosystems 7500 Real-Time PCR System using the protocol described by Sassi et al.[25]

2.2.3 | Detection of CALR exon 9 mutations

JAK2 V617F unmutated Patients were screened for CALR exon 9 mutations. The CALR exon 9 mutations were detected using PCR/direct sequencing. The PCR reaction used one primer pair newly designed and tested by bioinformatic tools and public databases to amplify the CALR exon 9: Forward (5′-GAGTTTGGCAACGAGACGTG-3′) Reverse (5′-CCACCCCAAATCCGAACCAG -3′). For the amplification, each PCR worked efficiently with 50 ng/µl of genomic DNA added to 2.5 µl of buffer (10×), 0.2 mM of DNTP, 0.04 U Ampli Taq polymerase, 1.5 mM $MgCl_2$ and 0.2 µM of each forward and reverse exon 9 primers giving an amplicon of size 444 bp.

The thermal cycling conditions were initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 66°C for 30 s, extension at 72°C for 45 s, and a final extension at 72°C for 7 min. PCR products were checked for size and purity by electrophoresis on a 3% of agarose gel and visualized under UV light using Gel Doc program.

PCR fragments were sequenced in both directions using Big Dye Terminator v3.1 Kit (Applied Biosystems) on ABI 3500 genetic analyser. The Same PCR primers were used for sequencing.

Sequences were aligned to the reference sequence, and mutations were detected and tested by bioinformatic tools and public databases (http://www.ncbi.nlm.nih.gov/SNP/; https://www.ensem bl.org/Homo_sapiens/Transcript/ProtVariations?db=core; http://cancer.sanger.ac.uk/cosmic).

2.2.4 | Statistical analysis

Statistical analysis of the data was conducted using SPSS software (version 22.0; SPSS Inc.). Kolmogorov–Smirnov test was used to verify the distribution normality of continuous variables. These variables were compared using the independent Student *t* test or Mann–Whitney *U* test, as appropriate. Quantitative variables are shown as mean values ± standard deviation (SD) or median (minimum–maximum) as appropriate. The hematological features including sex, age, hemoglobin level, white blood cell and platelet counts were compared according to mutation patterns. Categorical variables were compared using the Pearson chi-square test. Statistical differences were considered as significant from $p < 0.05$.

3 | RESULTS

In this study, the cohort included 250 ET patients with 56.7% were female and 43.3% were male. The mean age at diagnosis was 57.7 years [3–89 years].

At diagnosis the medians of hemoglobin, leucocytes and platelets counts were, respectively, 12.9 g/dl [5–80], $11 \times 10^3/mm^3$ [1.39–111×10^3] and $940 \times 10^3/mm^3$ [515–5223×10^3] (Table 1).

3.1 | Prevalence and pattern of JAK2 V617F mutation

Among 250 patients suspected with ET, 166 (66.4%) harbored JAK2 V617F mutation with sex ratio 0.71 showing a female predominance (57.9%) (Figure 1A).

The hematological and clinical features of patients were compared according to JAK2V617F mutation. JAK2V617F mutated (+) patients representing 66.4% of cases were older (60.26 vs 53.76 years, p = 0.015), had a higher hemoglobin level and higher leucocytes count (13.7 vs 12.050 g/dl, $p < 0.001$; 12 vs $9.345 \times 10^3/mm^3$, $p < 0.001$, respectively) at diagnosis compared to patients without JAK2 V617F (−). Clinical characteristics by mutational groups JAK2 mutated (+)/JAK2 unmutated (−) are shown in Table 1 .

3.2 | Prevalence and pattern of CALR mutations

Eighty-four patients, who tested negative for JAK2V617F, were screened for CALR exon 9 mutations by PCR/direct sequencing method. Only 27 of 84 patients (32.1%) were CALR mutated, the sex ratio was 0.92 with female predominance (51.9%).

TABLE 1 Clinical and laboratory features of Tunisian ET patients with JAK2 V617F and CALR mutations

	ET					*p* value			
			JAK2 V617F(−)[2] (n = 84) 33.6%						
	Total ET (*n* = 250)	JAK2 V617F(+)[1] (*n* = 166 (66.4%))	Total CALR (*n* = 84)	CALR (−)[3] (*n* = 57 [67.9%])	CALR mutated (+)[4] (*n* = 27 (32.1%))	[1] vs [2]	[3] vs [4]	[1] vs [4]	[1] vs [3]
Sex									
Female (%)	56.9	57.9	54.8	56.1	51.9	0.634	0.712	0.555	0.814
Male (%)	43.1	42.1	45.2	43.9	48.1				
Age (years)	57.72 ± 17.82	60.26 ± 16.01	53.76 ± 19.79	50.95 ± 20	58.73 ± 18.27	**0.015**	0.110	0.670	**0.002**
Hemoglobin (g/dl)	12.9 [5–19]	13.7 [7–19]	12.050 [5–16.1]	11.25 [5–16.1]	12.4 [6.9–16]	**<0.001**	0.114	**0.007**	**<0.001**
Leucocytes ($10^3/mm^3$)	11 [1.39–111]	12 [6.3–35]	9.345 [1.39–111]	9.550 [1.39–96]	9 [3.340–111]	**<0.001**	0.731	**0.004**	**0.002**
Platelets ($10^3/mm^3$)	945 [427–5223]	931 [515–2400]	1000 [427–5223]	950 [427–2419]	1041 [531–5223]	0.308	0.353	0.147	0.655

Note: JAK2 V617F(+)/JAK2 V617F(-): ET patients with JAK2 V617F mutated/ JAK2 V617F unmutated; CALR (-): ET patients with CALR unmutated.

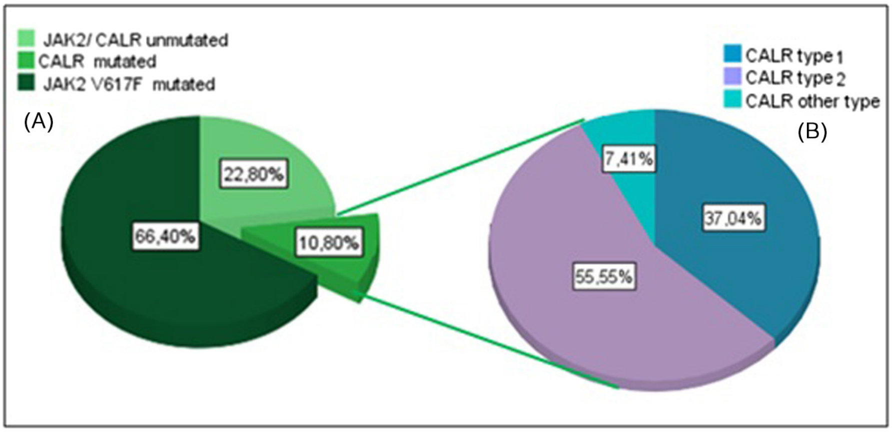

FIGURE 1 Distribution of JAK2 V617F and CALR mutation in 250 Tunisian patients with essential thrombocythemia. (A) Frequencies of JAK2 V617F and CALR exon 9 mutations, (B) Frequencies of categorized CALR mutations

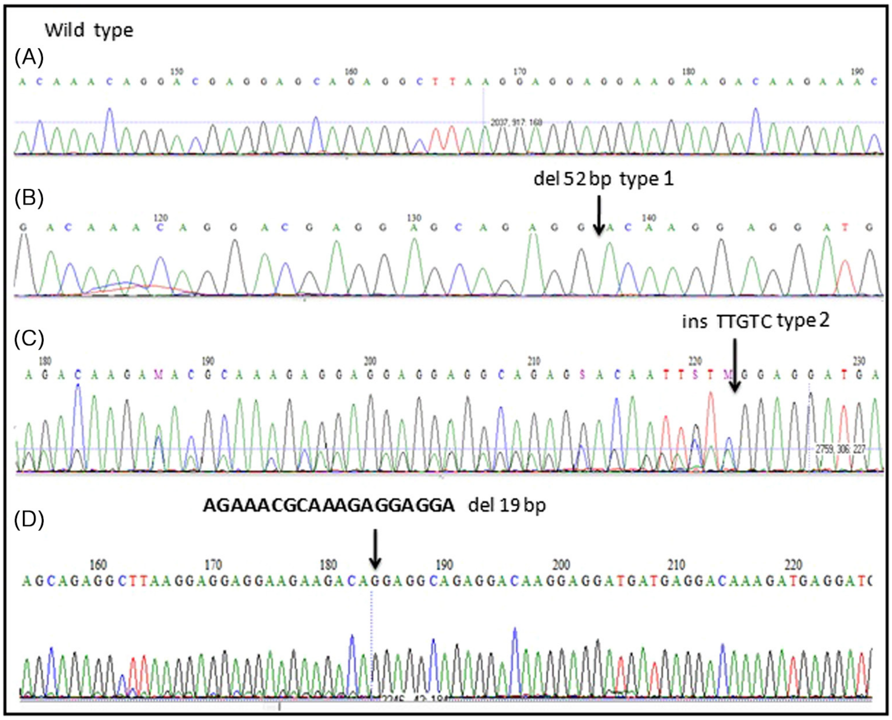

FIGURE 2 Direct sequencing results of exon 9 in the CALR gene. (A) Sequencing result of the wild type of exon 9 CALR. (B) Sequencing result of the mutant type 1 of exon 9 CALR: (c.1092_1143del) deletion of 52 bp between codons 1092 and 1143. (C) Sequencing result of the mutant type 2 of exon 9 CALR: (c.1154_1155insTTGTC) insertion of 5 bp between codons 1154 and 1155, heteroduplex chromatogram. (D) Sequencing result of mutant type of exon 9 CALR; (c.1121_1139del) deletion of 19 bp between codons 1121 and 1139 (AGAAACGCAAAGAGGAGGA). A: Adenine; T: Thymine; C: Cytosine; G: Guanine

The CALR mutations group were older (58.73 vs 50.95 years, $p = 0.110$) at diagnosis with higher hemoglobin levels than the CALR unmutated group without statistically significant differences (12.4 vs 11.25 g/dl, $p = 0.114$) (Table 1).

Of 27 CALR exon 9 mutated patients, 15 CALR type 2 patients (55.55%); a mutation (c.1154_1155insTTGTC) consisting on 5 bp insertion between codons 1154 and 1155 was the most common. Ten patients (37.05%) carried CALR type 1, a 52 bp deletion between codons 1092 and 1143 (c.1092_1143del) and 2 patients (7.41%) had deletion of 19 bp between codons 1121 and 1139 (c.1121_1139del) (Figure 1B, Figure 2) were recorded.

The subgroup of patients with CALR type 2 had a significant predominance for the female and a higher platelet count (73.3 vs 30%; $p = 0.032$, 1146 vs $921 \times 10^3/mm^3$, $p = 0.039$, repectively) at diagnosis compared to patients with CALR type 1. There was no significant difference between CALR other type mutant and the other subgroups (Table 2).

3.3 | Clinical and laboratory features stratified by JAK2 and CALR mutations subtypes

We compared groups with driver mutations, JAK2V617F and CALR mutations; Among 193 patients with driver mutations, 86% had JAK2 V617F mutation and 14% had CALR mutations. Besides, JAK2 V617F mutated patients were found to have a higher leucocytes count (12 vs $9 \times 10^3/mm^3$; $p = 0.004$) and higher hemoglobin level, compared to patients with a CALR mutations (13.7 vs 12.4 g/dl, $p = 0.007$) (Table 1).

In Table 2, we compared groups with JAK2V617F mutation and each type of CALR mutations (type 1, type 2, and other type). We found that the group containing the JAK2 V617F mutation had a higher hemoglobin level at diagnosis than those with CALR type 1 and type 2 (13.7 vs 12.35 g/dl, $p = 0.037$; 13.7 vs 12.25 g/dl, $p = 0.012$, respectively) and had a higher leucocyte counts (12 vs $10.1 \times 10^3/mm^3$, $p = 0.036$) and a lower platelet counts (931 vs

1146x10^3^/ mm^3^, $p = 0.013$) at diagnosis compared to patients with CALR type 2 (Table 2).

Table 3 showed a result of a complementary study which was done to investigate the incidence of thrombotic events in ET patients. The mean age of ET patients with thrombotic complications was 60 years. We found that 6/7 patients experienced thrombotic events carried JAK2V617F mutation. The hematological and clinical features of ET patients according to thrombotic events showed at diagnosis, the medians of hemoglobin, leucocytes and platelets counts were respectively 13.3 g/dl [12.5–15.6], $10.44\times10^3/mm^3$ [$7.71–16.6\times10^3$] and $839\times10^3/mm^3$ [$516–1065\times10^3$] (Table 3).

4 | DISCUSSION

This is the first comprehensive study to describe the molecular profile of Tunisian patients with ET. Using AS-PCR and conventional Sanger sequencing method, we found that 77.2% (193/250) patients carried JAK2V617F or CALR mutations underscoring the importance of combined genetic tests for diagnosis of ET patients.

We found that 66.4% of ET patients carried JAK2V617F mutation which is the most frequent mutation reported in the literature, with a frequency ranging between 54 and 66%.[5,26–28]

CALR mutations caused the change of at least 36 amino acids, which resulted in a transformation of negative charge (glutamic acid) to positive charge (Arginine) of the c-terminus KDEL domain (Lys-Asp-Glu-Leu) in the protein.[5,17,28] This alteration may cause the disruption of normal cellular physiological and pathological processes including chaperone activity and modulation of calcium hemeostasis in the endoplasmic reticulum (ER), in return, outside the ER, it is associated with nuclear transport, regulation of cell adhesion and gene expression. Moreover, the CALR mutated protein allows the constitutive activation of the thrombopoietin receptor (MPL) by binding to its extracellular domain.[29]

We found CALR mutations in 32.1% of patients without JAK2 V617F. Previous studies reported a higher incidence of CALR mutations in the JAK2V617F negative group which can reach 67%.[17]

Three different types of CALR mutations including deletion/insertion were found in our study; type 2 with 55.55%, type 1 with 37.04% followed by another type with 7.41% of CALR mutant patients. However, these finding were in disagreement with previous reports which CALR type 1 was the most frequent CALR exon9 mutations.[17,18,30] In the other hand, Haunstrup et al indicated that CALR type 2 presented 70% of patient CALR mutated, which were in accordance with our study.[31]

These differences of frequencies may be due to the poor sensitivity of Sanger sequencing with limit of detection of CALR mutations is between 10 and 20% compared with other techniques such as PCR and fragment analysis, High-resolution melting, real-time PCR, digital PCR and NGS which their limit of detection ranging between 0.01 and 10%.[15,30]

In addition, patients with CALR type 1 mutation had a predominance of male sex and lower platelet count compared with CALR

TABLE 2 Clinical and laboratory features of Tunisian ET patients with JAK2 V617F and different types of CALR mutations

	Total ($n = 193$)	JAK2 V617F(+)^(1)^ ($n = 166$ [86%])	CALR mutated (+) ($n = 27$ [14%]): CALR type 1^(2)^ ($n = 10$ [5.2%])	CALR type 2^(3)^ ($n = 15$ [7.8%])	CALR other type^(4)^ ($n = 2$ [1%])	*p* value: ^(1)^ vs ^(2)^	^(1)^ vs ^(3)^	^(1)^ vs ^(4)^	^(2)^ vs ^(3)^	^(2)^ vs ^(4)^	^(3)^ vs ^(4)^
Sex											
Female (%)	57.1	57.9	30	73.3	0	0.084	0.245	0.1	**0.032**	0.371	**0.041**
Male (%)	42.9	42.1	70	26.7	100						
Age (years)	59.97 ± 16.49	60.26 ± 16.01	57.8 ± 19.22	57.42 ± 19.38	72.5 ± 12.02	0.647	0.543	0.286	0.963	0.332	0.311
Hemoglobin (g/dl)	13 [6.9–19]	13.7 [7–19]	12.35 [8.9–14.4]	12.25 [6.9–14]	14.75 [13.5–16]	**0.037**	**0.012**	0.454	0.787	0.116	0.082
Leucocytes ($10^3/mm^3$)	11.620 [3.340–111]	12 [6.3–35]	7.98 [3.34–111]	10.1 [4.7–12.670]	9.015 [9–9.030]	0.079	**0.036**	0.154	0.741	0.602	0.766
Platelets ($10^3/mm^3$)	940 [515–5223]	931 [515–2400]	921 [531–1238]	1146 [770–5223]	9565 [890–1023]	0.584	**0.013**	0.862	**0.039**	1.000	0.144

Note: JAK2 V617F(+): ET patients with JAK2 V617F mutated; CALR (-): ET patients with CALR unmutated.

TABLE 3 Clinical and molecular features of Tunisian ET patients with thrombotic events

Patients	Sex	Age (years)	Hemoglobin (g/dl)	Leucocytes (10^3/mm^3)	Platelets (10^3/mm^3)	Mutation status
1	Male	57	15.6	7.71	776	JAK2 V617 (+)
2	Male	44	13.5	10.13	516	JAK2 V617 (+)
3	Female	68	15.2	10.44	1400	JAK2V617F (+)
4	Male	71	13	11	1065	JAK2V617F (+)
5	Female	45	13.3	15.7	839	JAK2V617F (+)
6	Female	55	12.6	16.6	550	JAK2V617F (+)
7	Male	80	12.5	9.93	1065	JAK2V617F (−) CALR (−)

Note: JAK2 V617F(+): ET patients with JAK2 V617F mutated; CALR (−): ET patients with CALR unmutated.

type 2 patients, which were similar to our study.[21,32] The clinical phenotype significance of type 1 versus type 2 CALR mutations remain unclear.

JAK2 V617F and calreticulin mutations are considered as drivers in ET and are mutually exclusive in most cases. These mutations are associated with distinct clinical characteristics and may modulate the patient's clinical course, the risk of thrombosis and survival.[17]

The comparison of ET patients with JAK2V617F or CALR mutations revealed that patients with JAK2V617F were older (the mean age >60 years) than patients with CALR mutations which is compatible with previous studies.[33]

JAK2V617F ET patients presented a higher hemoglobin level and leucocytes count compared to patients with CALR mutations. We also noticed that JAK2V617F patients had a lower platelet count compared to CALR mutated patients without statistically significant. Our results were supported by previous reports and other studies.[33–35]

Tefferi et al have also shown that patients with CALR mutations were younger and had higher platelet count and lower hemoglobin and leucocytes counts compared with JAK2V617F patients.[32]

Several studies have shown that JAK2V617F may increase the likelihood of thrombotic events in ET patients. Therefore, it has been included in prognostic models and its presence may guide treatment decisions.[36,37] Subsequent meta analyses and systematic reviews of the literature showed that among patients with ET, the risk of thrombosis is about twice as high in those with the JAK2V617F mutation compared to those without. Based on this data, patients with CALR mutation have better prognosis with lower incidence of thrombotic events relative to patients bearing JAK2V617F mutation associated with an increased risk of thrombosis.[6]

In this study, JAK2 V617F mutation was associated with a higher increased incidence of thrombosis compared to CALR mutations in patients with ET which was consistent with previous studies.[36,38]

These results indicate that patients with CALR mutated ET display a phenotype favoring megacaryopoiesis as opposed to the erythropoiesis favored in patients with JAK2V617F mutated ET. Despite the fact that CALR mutation involve high platelet count, the risk of thrombosis of these patients is relatively low and the overall survival is long compared with those of JAK2V617F mutant.[24,33,32,39]

In conclusion, this study is the first comprehensive investigation of the prevalence of JAK2V617F and CALR mutations in Tunisian patients with ET. Molecular status might improve the diagnosis and contribute to stratify patients according to the risk of thrombosis.

A limitation of our study is that this was a retrospective study with a limited sample size without clinical data and long terms follow up, thus more detailed studies with assessment of thrombotic risk will be necessary in order to improve our knowledge.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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