RESEARCH ARTICLE

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Molecular Profile of BCR-ABL1 Negative Myeloproliferative Neoplasm in a Moroccan Population

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Abstract

Introduction: Myeloproliferative neoplasms (MPN) are associated with clonal hematopoiesis, genomic instability, hemostasis dysregulation, and immune response. Classic BCR-ABL1 negative myeloproliferative neoplasms (BCR-ABL1 negative MPN), including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), are frequently associated with somatic abnormalities in *JAK2*, *CALR*, and *MPL*. Mutant clones induce an inflammatory immune response leading to immuno-thrombosis. **Objective:** The aim of this research was to investigate JAK2/STAT5 mutations in 49 Moroccan patients and to study their correlation with demographic and hematological parameters. **Method:** Genomic DNA was isolated from peripheral blood samples, and JAK2V617F, *JAK2* exon 12, *CALR* exon 9, and *MPL* exon 10 mutations were tested by qPCR, AS-PCR, and Sanger sequencing. Statistical analysis was performed using SPSS version 22. **Results:** Our results showed that 33 (67.35%) of our cohort was mutated for one of the investigated genes. JAK2V617F was detected in 25 cases, as well as two previously described CALR mutations: c.1092_1143del52 and c.1154_1155insTTGTC. Mutations in *JAK2* exon 12 (c.1622G>A/C and c.1641+6T>C) were observed and in PV and ET. MPL substitution c.1544G>T was detected in two cases. Mutations in *JAK2, CALR*, and *MPL* were mutually exclusive and benign single nucleotide variations/polymorphisms (SNV/SNP) have been identified in *CALR* and *JAK2*. The JAK2-PV subgroup was associated with higher red mass cell, hemoglobin, and hematocrit levels. Higher platelet counts correlated with the JAK2-ET subgroup, while higher leukocyte and neutrophil counts were associated with the JAK2-PMF subgroup. Complete blood count revealed hyperleukocytosis accompanied by hyperplateletosis and a lower level of hemoglobin in CALR-ET mutated patients, compared to JAK2-ET mutated or triple negative-ET cases. **Conclusion:** In conclusion, our study provides valuable insights into the prevalence and characteristics of *JAK2, CALR*, and *MPL* mutations in BCR-ABL1 negative MPNs in the Moroccan population, highlighting the importance of genetic characterization to optimize the clinical management of these diseases.

Keywords: Myeloproliferative neoplasm- *JAK2- CALR- MPL-* Morocco.

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Introduction

Myeloproliferative syndromes/neoplasms (MPN) are a heterogeneous group of chronic hematologic diseases defined by the overproduction of one or more myeloid cell lineages that can progress toward acute leukemic transformation. First described by William Dameshek in 1951, MPNs are characterized by enhanced proliferation of the erythrocytic, megakaryocytic, or granulocytic lineages, most often associated with thromboembolic events or myelofibrosis [1]. More than half of the patients are asymptomatic and incidentally diagnosed. MPNs are clonal disorders derived from hematopoietic stem cells (HSC) and are classified by the World Health Organization (WHO) based on hematological, anatomopathological, and cytological information [2–5]. Classic MPNs include chronic myeloid leukemia (CML), characterized by the presence of the BCR-ABL1 fusion gene (Philadelphia chromosome), resulting from translocation between chromosomes 9q34 and 22q11. BCR-ABL1 negative

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MPNs including polycythemia Vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), are considered rare conditions according to the widely accepted definition of rare cancers as subtypes of cancer with fewer than 6 new cases per 100,000 inhabitants per year [6, 7]. With a median age at diagnosis of 58 years, ET incidence is estimated at 1.2–3.0 per 100,000 inhabitants per year. PMF accounts for about 0.5–1.5 patients per 100,000 inhabitants per year while PV incidence ranges between 0.01 and 2.8 new cases per 100,000 inhabitants per year [8]. However, BCR-ABL1 negative MPN etiology remains poorly understood.

In 2005, the discovery by four independent teams [9–12] of a point mutation in the Janus kinase 2 (*JAK2*) gene (c.1849G>T) initiated the molecular discrimination of BCR-ABL1 negative MPNs. The JAK2V617F mutation is found in approximately 95% of PV cases and 50–60% of ET and PMF cases [13]. In addition to *JAK2*, calreticulin (CALR), and thrombopoietin (TPO) receptor (MPL) mutations, epigenetic alterations have been added to BCR-ABL1 negative MPNs catalog. Abnormalities in these genes would define specific phenotypes at the clinical-biological level and may affect the occurrence and development of the disease. Nevertheless, BCR-ABL1 negative MPNs remain associated in 95% of cases, with one of the three leading mutations occurring in *JAK2, CALR,* or *MPL*. In their absence, triple-negative BCR-ABL1 negative MPNs are associated with a low survival rate and greater susceptibility to acute transformation. For these reasons, the WHO expended the diagnostic and monitoring criteria for BCR-ABL1 negative MPNs since 2008 to include molecular data from *JAK2, CALR* and *MPL*.

The detection of these mutations is important for understanding and managing the disease. The lack of national databases for rare pathologies in low/middle-income countries contrasts with the evolution of diagnostic techniques. In Morocco [14–16], there is a paucity of literature on these diseases and patient genetic profiles have rarely been investigated. In this article, we present the mutational profile of forty-nine Moroccan patients for the driving genes *JAK2, CALR*, and *MPL* in BCR-ABL1 negative MPNs and investigate their significant correlation with Demographic and hematologic parameters. We investigated the mutation profiles of JAK2 (exons 14 and 12), exon 9 of *CALR*, and exon 10 of *MPL* in a Moroccan cohort with PV, ET, PMF, and unclassifiable MPNs. Our objective was to establish a standardized molecular diagnostic protocol for BCR-ABL1 negative MPN in order to enhance the predictive value of prognostic data and provide more in-depth estimates for regional and global comparisons.

Materials and Methods

Ethical considerations

Ethical approval for this prospective qualitative study was obtained from the University of Rabat Biomedical Research Ethics Committee (reference number CERB 62-21). Each participant was informed of the nature of the study by completing a survey during the interview, and informed consent was obtained from each participant. All the procedures performed in this study complied with the ethical standards of the 1964 Declaration of Helsinki and its subsequent amendments.

Patients

This prospective study included 49 patients diagnosed between 2018 and 2022 and managed at the Cytogenetic department of the Pasteur Institute's in Casablanca. Patients exhibiting clinical features not suggestive of BCR-ABL1 negative MPNs with normal hemogram and those carrying the BCR-ABL1 transcript were excluded. Suspicion of BCR-ABL1 negative MPNs was retained based on the latest WHO 2022 classification diagnostic criteria [4, 5]. The parameters included in our study were platelet count, leukocytosis, red mass cells, hemoglobin, and hematocrit levels. We also included demographic criteria as sex and age, and molecular analysis results. However, other clinical parameters such as thrombosis, fibrosis, etc. were not considered.

Mutation screening

Mutations in *JAK2, CALR* and *MPL* were detected using real-time polymerase chain reaction (qPCR) for the JAK2V617F point mutation, allele-specific polymerase chain reaction (AS-PCR), and Sanger sequencing for *CALR* exon 9. Bidirectional Sanger sequencing was performed to screen for *JAK2* exon 12 and *MPL* exon 10.

Genomic extraction

Genomic DNA was extracted from whole blood using a silica membrane-based DNA purification kit (QIAamp DNA Blood Mini Kit, Germany), according to the manufacturer's instructions. Quantification and quality control of the extracted DNA was performed using a NanoVue Plus spectrophotometer. The measurements were performed at a wavelength of 260 nm. The acceptable DNA concentration was assessed to be ≥ 25 ng/μL. The absorbance ratio at 260 and 280 nm wavelengths (A260/A280) was measured with a threshold of 1.6 and 2. JAK2V617F was initially analyzed upon diagnosis, followed by the screening for *JAK2* exon 12, *CALR* exon 9, and *MPL* exon 10 in all samples.

Screening for JAK2 exon 14 and exon 12 mutations
JAK2V617F real-time PCR

The JAK2V617F mutation was detected using the "QClamp *JAK2* codon specific mutation test in codon 617" kit, following the manufacturer's instructions. This test is based on the principle of real-time PCR (qPCR) and was performed using a Cobas z 480 device.

JAK2 exon 12 sequencing

JAK2 exon 12 screening was conducted using direct bidirectional sequencing to amplify a 496 base pair (bp) region with primers forward (F): 5'-CTCCTCTTTGGAGCAATTCA-3' and reverse (R): 5'-GAGAACTTGGGAGTTGCGATA-3'. The analysis was performed according to previously published protocols by Xia et al. [17] and Afia Muhammad Akram et al. 2018 [18].

CALR exon 9 screening

CALR exon 9 mutations were detected by allelic discrimination (AS-PCR) coupled with agarose gel electrophoresis. Samples with suspicious electrophoretic profiles were analyzed by bidirectional Sanger sequencing.

Allele-specific PCR (AS-PCR)

Primers published by Zakaria et al. [19, 20] forward primer 1 (F1): 5′-GCA GCA GAG AAA CAA ATG AAG G-3′; forward primer 2 (F2): 5′- GCA GAG GAC AAT TGT CGG A-3’; and reverse primer (R): 5′-AGA GTG GAG GAG GGG AAC AA-3′ were used for allelic discrimination (AS-PCR). The cycling conditions were as follows: preheating at 95°C for 5 min followed by 40 cycles of denaturation at 94°C for 30 s, hybridization at 64°C for 30 s, and elongation at 72°C for 30 s. The cycle ended with an elongation at 72°C for 7 min. The PCR products were visualized on 3% agarose gel under a direct current of 135 volts for 30 min.

Sequencing

The sequencing reaction was carried out to identify the type of mutation presented by each patient (type 1 or type 2) using the CALR primers forward (F): 5′-CAT ACC GCT GAG GAG TTT GGC-3′ and reverse (R): 5 ‘-GAG TGG AGG GAG GGG AAC AAA-3’ designated on Primer 3 (https://primer3.ut.ee). PCR was performed according to previously described conditions [21]. Bidirectional sequencing was processed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on ABI 3500XL Genetic Analyzer (Applied Biosystems).

MPL exon 10 screening

Amplification of MPL exon 10 was performed according to the conditions described by Kang et al. [22]. The specific primers for MPL exon 10 forward (F): 5’- TGG GCC GAA GTC TGA CCC TTT-3’ and reverse (R): 5’- ACA GAG CGA ACC AAG AAT GCC TGT -3’, were used according to the following protocol: The PCR program began with a preliminary denaturation for 5 min at 98°C followed by 35 cycles including denaturation at 98°C for 20 s and annealing at 68°C for 1 min, concluded by 5 min of elongation at 68°C. Bidirectional sequencing was carried out using the forward primer (F): 5’-GGT GAC CGC TCT GCA TCT AGT GCT-3’ and reverse primer (R): 5’-CAC CTG GTC CAC CGC CAG TCT-3’.

Sequencing data analysis

The resulting data were compared with the reference sequences in GenBank for each of the screened genes. Sequencing results were compared to the wild-type sequences of *JAK2* (accession number: NM_004972.4), *CALR* (accession number: NM_004343.4), and *MPL* (accession number: NM_005373.3) from the EMBL-EBI database. Multiple nucleic acid sequence alignments and chromatogram analyses were performed using the UniPro UGENE v47.0 software.

Statistical analysis

Frequencies and means were calculated using Microsoft Excel 2016 and Statistical Package for Social Sciences software (SPSS) (IBM Corporation version 22, Chicago, IL, USA). To analyze parametric data, chi-square test (χ^2) was employed for categorical variables, and Student test (t) for numerical data. Additionally, to compare nonparametric and qualitative variables, the Mann-Whitney (U) and Fisher’s exact tests were performed. The threshold for statistical significance was set at $p \leq 5\%$.

Results

Among the entire cohort, 16 individuals exhibited a PV profile, 19 presented a ET profile, 10 had PMF, and 4 remained unclassified. The distribution according to sex was as follow: 27 males (55.1%) and 22 females (44.9%) with a sex ratio (M/F) of 1.22. The mean age at diagnosis was 60.85±15.879 years, with a higher mean age in females (M: 57.15±16.049; F: 65.65±14.673). Mutations in BCR-ABL1 negative MPNs were identified in 33 cases, which were distributed as 32.65% in PV,

Table 1. Distribution of BCR-ABL1 Negative *MPN* Subtypes According to Mutation Profile

Characteristics	PV (n=32.65%)	ET (n=38.78%)	PMF (n=20.41%)	Unclassified MPN (n=8.16%)	BCR-ABL1 negative MPNs (n= 100%)
Mean age ± SD	62.85 ± 19.685	60.79 ± 16.423	59.90 ± 9.231	55 ± 16.093	60.85 ± 15.879
JAK2V617F	12	9	4	0	25
	(n= 75%)	(n= 47.37%)	(n= 40%)	-	(n=51%)
JAK2 exon12+	1	1	0	0	2
	(n= 6.25%)	(n= 5.26%)	-	-	(n=4.08%)
CALR exon 9+	0	3	1	0	4
	-	(n= 15.79%)	(n= 10%)	-	-8.20%
MPL exon 10+	0	1	1	0	2
	-	(n= 5.26%)	(n= 10%)	-	(n=4.08%)
Triple-negative	3	5	4	4	16
	(n= 18.75%)	(n= 26.31%)	(n= 40%)	(n= 100%)	(n=32.65%)
Total	16	19	10	4	49
	(n=32.65%)	(n=38.78%)	(n=20.41%)	(n=8.16%)	(n=100%)

Table 2. Demographic and Hematological Characteristics of BCR-ABL1 Negative MPNs based on the Mutation Profile

	MPN * JAK2V617F (1)	MPN *JAK2 exon 12 (2)	MPN * CALR (3)	MPN * MPL (4)	MPN * Triple-negative (0)	P1vs2 value	P1vs3 value	P1vs4 value	P1vs0 value	P2vs3 value	P2vs0 value	P3vs4 value	P3vs0 value	P4vs0 value
Frequency (%)	51	4.08	8.2	4.02	32.65									
Number of cases (N)	25	2	4	2	16									
Sex ratio (M/F)	10:15	1 :1	4 :0	0 :2	12 :4									
Mean age (Years)	68.61	54.5	67.5	52.5	49.13	0.567	0.856	0.155	**<0,001**	0.380	0.643	0.148	**0.024**	0.131
	(30-100)	(37-72)	(53-77)	(47-58)	(30-72)									
Hematocrit (g/dL)	49.05	63.5	43.16	31.85	43.2	**0.021**	**0.047**	**0.007**	0.910	**0.020**	**0.015**	0.080	0.991	0.517
	(37,70-67)	(57,90-69,10)	(42-45,50)	(26,40-37,30)	(30,60-54,80)									
Red Blood cells (×10⁹/L)	5538.18	8420	4620	3815	4896.36	**0.014**	0.070	0.113	0.139	**0.022**	**0.001**	**<0,001**	0.361	0.150
	(3430-9360)	(7260-9580)	(4590-4680)	(2780-3850)	(3330-6250)									
Haemoglobin (g/dL)	15.63	19.6	13.96	9.9	14.5	**0.013**	**0.037**	**0.001**	0.233	**0.014**	**0.032**	0.077	0.580	0.053
	(1,60-19,70)	(18,40-20,80)	(13,50-14,90)	(8-11,80)	(9,40-18,20)									
Platelets (×10⁹/L)	511.68	562.5	1612	460	540.91	0.937	**<0,001**	0.922	0.819	0.415	0.947	0.837	**0.001**	0.360
	(36-1149)	(53-1072)	(1026-1905)	(41-879)	(174-1099)									
Leukocytes (×10⁹/L)	12.14	5.67	7.41	4.9	10.75	0.388	**0.042**	0.214	0.609	0.077	0.236	0.593	0.064	0.917
	(1,82-46)	(5,02-6,33)	(6,73-7,75)	(1,52-8,28)	(5,89-25,55)									
Neutrophils (×10⁹/L)	9.333	3.34	4.06	2.99	7.11	0.361	**0.016**	0.101	0.378	0.273	0.336	0.656	0.066	0.636
	(2,70-35)	-	(3,87-4,26)	(0,92-5,06)	(3,21-21,71)									

*All the results were within 95% confidence interval (CI); Statistically significant values ($P \leq 0.05$) are shown in bold.

Table 3. Demographic and Hematological Features of Patients with JAK2V617F Mutated BCR-ABL1 Negative MPNs

	JAK2V617F*PV (1)	JAK2V617F*ET (2)	JAK2V617F*PMF (3)	P1vs2 value	P2vs3 value	P1vs3 value
Frequency (%)	44	40	16			
Number of cases (N)	11	10	4			
Sex ratio (M/F)	6:7	4 :6	4:1			
Mean age (Years)	69.18	67.7	62.25	0.482	0.424	0.333
Hematocrit (Mean)	55.72	47.91	42.47	**0.002**	0.523	**<0,001**
Red Blood cells (×10⁹/L)	6717	5471	4200	**0.013**	0.180	**0.003**
Haemoglobin (g/dL)	17.39	15.13	14.5	**0.001**	0.761	**0.752**
Platelets (×10⁹/L)	295.3	839.8	257.75	**<0,001**	**<0,001**	0.140
Leukocytes (×10⁹/L)	11.41	14.45	4.99	0.556	**0.050**	0.044
Neutrophils (×10⁹/L)	9.75	10.36	2.9	0.761	**0.050**	0.082

*All the results were within 95% confidence interval (CI); Statistically significant values (P ≤ 0.05) are shown in bold.

38.78% in ET, and 20.41% in PMF (Table 1). JAK2V617F was observed in 25 samples, whereas in *JAK2* exon 12 c.1641+6T>C (rs182123615) and c.1622G>A/C (rs2130530074) were observed in two distinct cases. The CALR type 1 (c.1092_1143del52) mutation was found in two patients, while two other patients harbored the CALR type 2 (c.1154_1155insTTGTC) mutation. In our cohort, c.1544G>T (MPLW515L) was the only mutation detected in MPL exon 10. *JAK2, CALR*, and *MPL* mutations were mutually exclusive. The sex ratio (M/F) in ET-mutated favored males (10:9), while in PV-mutated and PMF-mutated, it favored females (6:7 and 1:2, respectively).

There was no statistically significant difference in patient mean age between the different pathologies. Nevertheless, BCR-ABL1 negative MPNs associated with JAK2V617F and *CALR* clonal abnormalities had a significantly higher mean age than triple-negative patients ($p < 0.05$) (Table 2). The student test revealed no significant difference in mean age between the different mutations. However, the mean age at diagnosis was higher in CALR type 2 and JAK2V617F mutated cases (70 and 68.61 years) than in CALR type 1 (65 years), *JAK2* exon 12 (54.5 years), and *MPL* exon10 (52.5 years). Statistical analysis yielded a p-value of less than 5% ($p \leq 0.05$), indicating a strong association between the variables (red blood cells, hemoglobin, hematocrit, platelets, white blood cells (WBC), and neutrophils) and pathology, as well as the type of mutation. Comparison between the *JAK2* exon 12-mutated and *MPL*-mutated subgroups was not conducted due to the insufficient sample size (less than 5 cases).

JAK2V617F was present in 51% of the samples: 75% in PV, 47.37% in ET, and 40% in PMF. Patients with JAK2V617F were compared based on their pathological subtypes (Table 3). The levels of red blood cells, hemoglobin, and hematocrit demonstrated a significant association with the JAK2V617F-PV subgroup. Conversely, platelet count was significantly higher in CALR-ET. Advanced age was significantly correlated with the presence of the JAK2V617F point mutation in ET (Table 4).

Screening of *CALR* exon 9 using AS-PCR/Sanger sequencing identified different types of mutations in four patients (two CALR type 1 and two CALR type 2). *CALR* was mutated in 8.2% of BCR-ABL1 negative MPNs (3

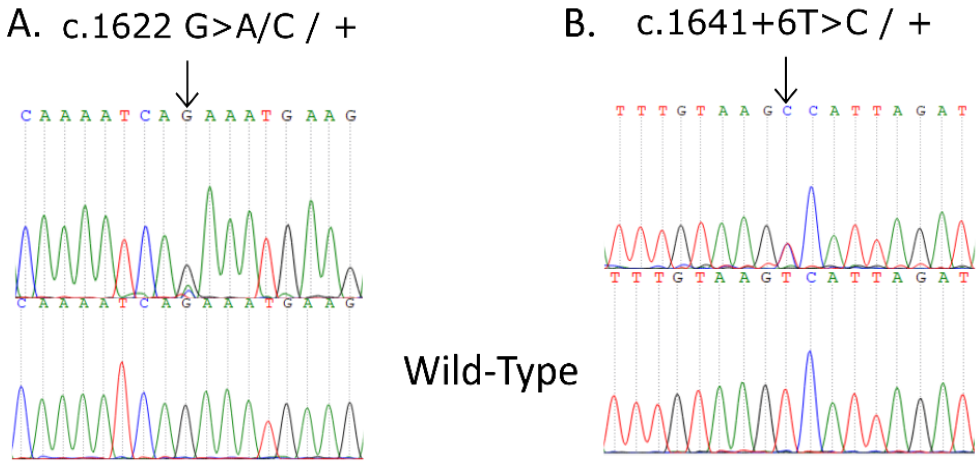

Figure 1. Sanger Sequencing Chromatograms of *JAK2* Exon 12 Ffeatures Arrows Indicating the Locations of the Mutation sites. A: c.1622G>A/C substitution resulting in amino acid substitution from Arginine to Lysine (R541K) along with the wild-type transcript. B: Nucleotide change c.1641+6T>C along with wild-type transcript.

Table 4. Demographic and Hematological Features of ET based on the Mutation Profile

	JAK2V617F*ET (1)	CALR*ET (2)	TRIPLE-NEGATIVE* ET (3)	P1vs3 value	P2vs3 value	P1vs2 value
Frequency (%)	47.40%	15.80%	26.30%			
Number of cases (N)	9	3	5			
Sex ratio (M/F)	4:05	3:00	3:02			
Mean age (Years)	67.22	66.33	46.4	**0.032**	0.631	0.879
Hematocrit (g/dL)	46.8	43.16	38.7	0.763	0.121	0.24
Red Blood cells (×10⁹/L)	5272.22	4620	4542	0.408	0.97	0.055
Haemoglobin (g/dL)	14.76	13.96	12.9	0.613	0.081	0.053
Platelets (×10⁹/L)	814	1612	947.8	0.558	**0.027**	**0.002**
Leukocytes (×10⁹/L)	15.35	7.41	13.96	0.598	0.127	0.094
Neutrophils (×10⁹/L)	11.09	4.06	9.98	0.53	0.206	0.07

*All the results were within 95% confidence interval (CI); Statistically significant values (P ≤ 0.05) are shown in bold.

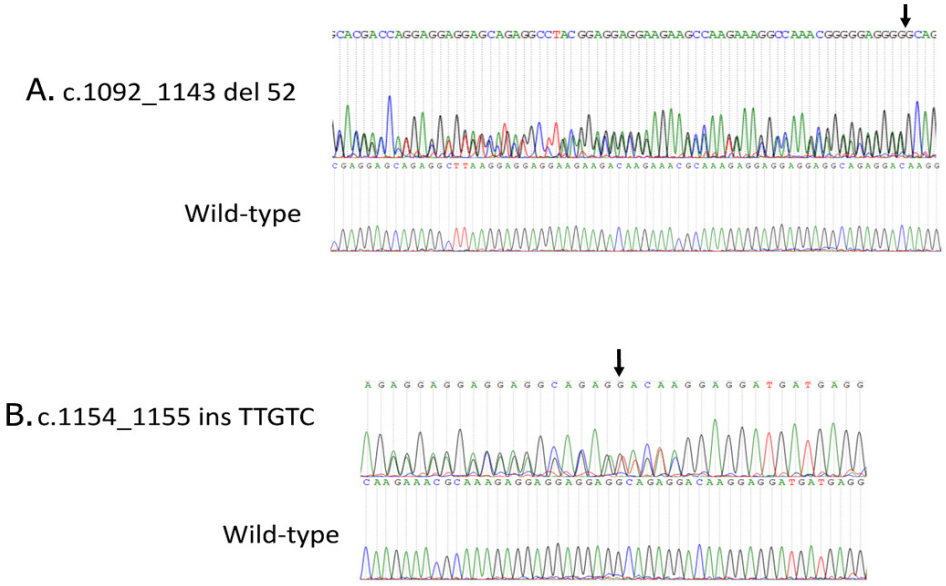

Figure 2. Sanger Sequencing Chromatograms of *CALR* Exon 9 Features Arrows Indicating the Locations of the Mutation sites. A: c.1092_1143 52 base pairs deletion resulting in a frameshift along with the wild-type transcript. B: c.1154_1155 TTGTC insertion along with wild-type transcript.

ET and 1 PMF cases). *CALR* mutations are associated with higher platelet counts (Table 2). Univariate analysis showed no significant difference in age between the CALR-ET and JAK2V617F-ET subgroups. Two cases with mutations in *JAK2* exon 12 were identified in PV and ET. *JAK2* exon 12 mutations are associated with high levels of hemoglobin, hematocrit, and red blood cell levels. Two samples, one from ET and the other from PMF,

Table 5. Demographic and Hematological Features of PMF Depending on the Presence or Absence of Mutations

	MUTATED*PMF	TRIPLE-NEGATIVE*PMF	P-value
Frequency (%)	60	40	
Number of cases (N)	6	4	
Sex (M/F)	2 :4	3 :1	
Mean age (Years)	63	55.25	0.371
Hematocrit (g/dL)	39.26	40.33	0.683
Red Blood cells (×10⁹/L)	4116	4563.33	0.361
Haemoglobin (g/dL)	13.2	14.3	0.947
Platelets (×10⁹/L)	214.4	284.5	0.594
Leukocytes (×10⁹/L)	4.29	8.55	**0.022**
Neutrophils (×10⁹/L)	2.51	5.11	**0.049**

*All the results were within 95% confidence interval (CI); Statistically significant values (P ≤ 0.05) are shown in bold.

Table 6. Comparative Occurrence of BCR-ABL1 Negative MPN in Different Populations

Populations	Sex ratio (M/F)	Mean age (Years)	PV	ET	PMF	References
Present study	27/22	60.85	16/49 (n= 32,65%)	19/49 (n= 38,78%)	10/49 (n= 20,41%)	-
Morocco	2/1	-	-	22/33 (n=66,66%)	11/33 (n=33,33%)	[15]
Morocco	38/30	60.78	19/70 (n=27,14%)	08/70 (n=11,42%)	12/70 (n=17,14%)	[14]
Algeria	185/159	52.83	98/344 (n=28,48%)	75/344 (n=21,80%)	13/344 (n=3,78 %)	[39]
Malaysia	517/500	54.2	385/1010 (n=38,1%)	408/1010 (n=40,4%)	93/1010 (n=9,2%)	[40]
South-Africa	-	-	87/373 (n=23,32%)	39/373 (n=10,45%)	76/373 (n=20,37%)	[54]
China	413/516	-	234/929 (n=25,18%)	428/929 (n=46,07%)	187/929 (n=20,13%)	[55]
Egypt	20/20	56.4	92/200 (n=46%)	68/200 (n=34%)	40/200 (n=20%)	[38]

displayed mutations in *MPL*, accounting for 4.08% of our cohort. Patients with *MPL* mutations did not demonstrate any significant association with hematological parameters or age. The presence of mutations in PMF was associated with significantly higher leukocyte and neutrophil counts than in triple-negative PMF (Table 5).

None of the PV cases was positive for *MPL* or *CALR* mutations, and no coexistence of two or more gene mutations was observed. No mutation was identified in sixteen cases, bringing the percentage of triple-negative cases to 32.65%. Triple-negative patients had a mean age of 49.13±14.131 years. None of the unclassified MPN cases harbored mutations in *JAK2, CALR,* or *MPL*.

Discussion

MPN is associated with cytopenia, splenomegaly, and myelofibrosis. Several studies [23–25] have revealed BCR-ABL1 negative MPNs to be characterized by clonal myeloproliferation originating from stem cells and mutually exclusive driver mutations in *JAK2, CALR*, or *MPL*. Criteria for distinguishing BCR-ABL1 negative MPNs are based on clinical, hematological, and genetic characteristics and are organized into major and minor criteria according to the WHO classification [5, 6]. Mutations in *CALR, JAK2*, and *MPL* lead to constitutive activation of JAK2/STAT, PI3K/AKT, RAS/MAPK and MAPK/ERK signaling. These pathways promote the transport of several transcription factors such as signal transducer and activator of transcription (STAT) and forkhead box class O (FOXO) to the nucleus.

PV is predominantly associated with mutations in *JAK2,* whereas ET and PMF exhibit a broader range of mutations, including those in *JAK2, CALR,* and *MPL*. The

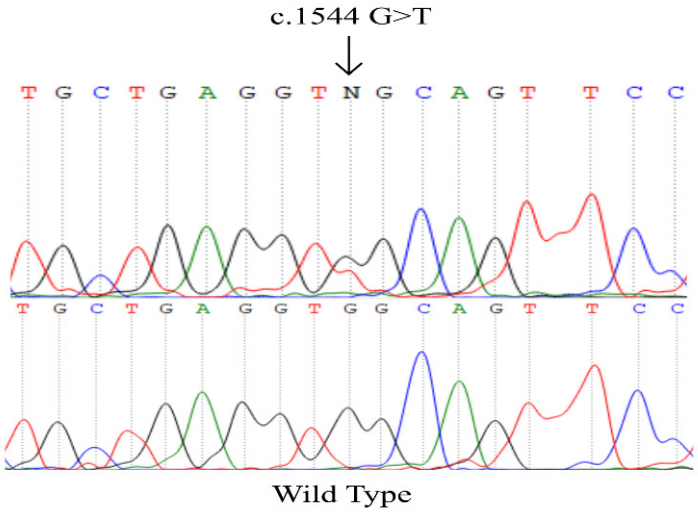

Figure 3. Sanger Sequencing Chromatogram of *MPL* Exon 10 Features Arrows Indicating the Locations of the Mutation Sites. The c.1544G>T substitution resulting in amino acid substitution from Tryptophane to Leucine (W515L) along with the wild-type transcript.

Table 7. Comparison of BCR-ABL1 Negative MPN Frequencies in Different Populations According to Mutation Profile

	Authors	PV	ET	PMF
JAK2V617F	This study	12	9	4
		(n= 75%)	(n= 47,37%)	(n= 40%)
	Lin Yani et al. [54]	199/234	250/428	123 /187
		(n=85%)	(n=58,4%)	(n=65,8%)
	Soliman et al. [38]	45	30	13
		(n=48,9%)	(n=44,1%)	(n=32,5%)
	Benguella-Benmansour et al. [39]	80	44	6
		(n=81,6%)	(n=58,7%)	(n=46,1%)
	Shires et al. [37]	84	28	59
		(n=97%)	(n=72%)	(n=78%)
JAK2 exon 12	This study	1	1	
		(n= 6,25%)	(n= 5,26%)	-
	Lin Yani et al. [54]	10	-	-
		(n= 4,3%)		
CALR exon 9	This study		3	1
		-	(n= 15,79%)	(n= 10%)
	Lin Yani et al. [54]	-	97/428	33/187
			(n=22,7%)	(n=17,6%)
	Soliman et al. [38]	-	13	7
			(n=19,1%)	(n=17,5%)
	Smaili et al. [15]	-	4	11
			(n=36,36%)	(n=50%)
	Shires et al. [37]	-	Sep-39	Dec-72
			(n=23%)	(n=10%)
MPL exon 10	This study		1	1
		-	(n= 5,26%)	(n= 10%)
	Lin Yani et al. [54]	-	5	5
			(n=1,2%)	(n=17,6%)

heterogeneous phenotypes observed among patients have highlighted the importance of a comprehensive knowledge of the molecular mechanisms underlying these diseases. The Janus kinase 2 signaling pathway promotes the expression of oncostatin M (OSM) in neoplastic myeloid cells. Using recombinant purified JAK2 JH1-JH2 proteins, Varghese et al. [26] showed that when activated, both wild-type and MPN-associated *JAK2* mutants display comparable enzymatic activity and inhibition by SOCS3 in *in-vitro* kinase assays. This validates a cell-intrinsic mechanism whereby, differential protein phosphorylation causes splicing-dependent alterations in JAK2-ERK signaling and the maintenance of JAK2 malignant clones [27].

Calreticulin is a chaperone protein located in the endoplasmic reticulum that is involved in the quality control of N-glycosylated proteins and calcium ion storage. The wild-type and mutated CALR (Type 1 and Type 2) exhibit significant structural differences. *CALR* mutations lead to the loss of the KDEL motif and generation of a novel positively charged C-terminus. Three-dimensional structural analysis revealed that the C-terminal region of the wild-type CALR protein (including the KDEL motif) had an open conformation compared to the mutated CALR forms [28]. In summary, wild-type CALR provides structural stability in contrast to mutated CALR. Although homozygous mutations may occur, *CALR* mutations are typically heterozygous. They all have the common effect of creating a +1 base pair (bp) frameshift in *CALR* exon 9, which results in a gain of function. CALR type 2 mutations eliminate approximately half of the negatively charged amino acids, whereas CALR type 1 mutations delete all the negatively charged amino acids in the C-terminus [29]. Since 2005, other mutations have been described in *CALR* exon 9, which can be categorized as type 1-like or type 2-like depending on the extent of amino acid deletion. Nonetheless, *CALR* type 1 and type 2 mutations account for 80% of all mutations in *CALR* exon 9 [29]. Type 1-like mutations are significantly more common in PMF, whereas type 2-like mutations are more common in ET [30]. The differences in phenotypes observed between BCR-ABL1 negative MPN patients with type 1-like and type 2-like mutations may be attributed to varying levels of MPL signaling activation and/or the greater loss of calcium-

binding sites associated with type 1-like mutations.

Increasing evidence suggests that BCR-ABL1 negative MPNs are disorders characterized by multiple signaling pathways that are significantly disrupted. Mutant *CALR* also activates *JAK2*-dependent signaling pathways, which are the primary mechanisms driving *CALR*-mediated transformation. Several studies have defined *CALR* mutant as a context-dependent oncogene that requires MPL. *CALR* mutations constitutively activate the thrombopoietin (TPO) receptor (MPL), even in the absence of TPO. The underlying mechanism of MPL activation is thought to involve the multimerization of *CALR* mutants [31–33]. Araki et al. 2016 [34, 35] demonstrated that *CALR* mutant promotes MPN development by activating c-MPL and its downstream pathways. Mutated CALR forms have higher binding affinities for TPO receptors [33]. Mediated by the N-glycan binding affinity of wild-type *CALR*, this interaction leads to structural modifications that facilitate interactions between the mutant *CALR* and MPL [35]. The CALR-MPL connection activates platelet-related transcription factors as calcium (Ca2+) plays a crucial role in platelet production [28]. Whether in *JAK2, CALR*, and *MPL*, the main pathogenic mechanism appears to be the constitutive activation of JAK2/STAT5 and JAK2-related signaling pathways (MAPK/ERK, PI3K/AKT…) [27]. The pathogenic mechanisms of *JAK2, MPL*, and *CALR* mutants appear to be simple, although they can cause complex disturbances. The characterization of new somatic and epigenetic alterations affecting the genes involved in these signaling pathways may contribute to phenotypic heterogeneity.

In this single-center study, 49 participants were enrolled to analyze BCR-ABL1 negative MPN driver mutations to either confirm or establish a diagnosis. Sixteen unmuted cases (32,65%) were observed, despite a complete blood count (CBC) suggestive of BCR-ABL1 negative MPNs. The BCR-ABL1 negative MPN patients negative for the three gene mutations, classified as triple-negative, represented a third of our cohort. Our results are in accordance with previously published data on South African (37.26%) and Malaysian (20%) populations [36, 37]. Triple-negative patients were distributed as 18.75% of PV, 26.31% of ET, and 40% of PMF, which is lower than the frequencies (51.1% PV – 36.8% ET – 50% PMF) reported in the Egyptian population [38] (Tables 6 and 7).

Our report presents the rates of BCR-ABL1 negative MPNs at the Pasteur Institute of Morocco – Casablanca, as well as the associations between mutations and hemogram data in 49 patients. In our cohort, approximately 67.35% of patients carried mutations in one of the investigated genes. The proportion of patients with hematological confirmation was 91.8%, with a preponderance of PV, followed by ET, PMF, and unclassified MPNs. BCR-ABL1 negative MPNs mainly appeared in subjects over 50 years of age. Consistent with previous studies [14], the JAK2V617F frequencies in PV, ET, and PMF were 75%, 47.37%, and 40%, respectively. The percentage of *CALR* mutant was 15.79% in ET and 10% in PMF, while the percentage of MPL mutant was 5.26% in ET and 10% in PMF. Mutations in *JAK2, CALR*, and *MPL* can be heterozygous or homozygous. In our study, quantification was not addressed, therefore the impact of allelic burden could not be discussed.

Phenotypically, functional differences linked to the patient's mutational status have been reported (*JAK2* mutated, *CALR* mutated, *MPL* mutated, or triple-negative). Our results showed significant differences in age, hemoglobin, hematocrit, platelet, and red blood cell counts depending on the patient's mutational profiles. Recent studies have correlated JAK2V617F with older age regardless of sex [39]. It has been reported that patients carrying only the wild-type allele of JAK2V617F were younger than those carrying the mutation [20, 22]. Our results are following the literature with a mean age at diagnosis of 68.61 years. Mutations in *JAK2*, specifically the c.1849G>T substitution, are in BCR-ABL1 negative MPNs associated with higher hemoglobin and leukocyte levels and lower platelet count in comparison to wild-type *JAK2* [40]. These patients also exhibit increased rates of thrombosis and hemorrhage as well as a high risk of fibrosis.

In our study, JAK2V617F was present in 51% of the cases, while *JAK2* exon 12 mutations were identified in two cases (4.08%). *JAK2* exon 12 mutations are rare (0-5%) in most cohorts, although occasional studies have noted higher levels (13-16%), indicating a possible testing bias [37, 41]. Over 37 distinctive mutations, comprising both point and insertion-deletion abnormalities, have been described in the *JAK2* exon 12 region, which encodes aa534-547. Two substitutions in *JAK2* exon 12 c.1641+6T>C and c.1622G>A/C were identified in the present study (Figure 1). The c.1641+6T>C (rs182123615) substitution was observed in ET. This mutation has been described in congenital erythrocytosis and familial forms of BCR-ABL1 negative MPN in the Tunisian population. The c.1641+6T>C *JAK2* variant was found in 1.5% of healthy probands and controls, suggesting it may be a rare polymorphism rather than pathogenic [42, 43]. Therefore, *in-vitro*, predictions do not always align with *in-vivo* outcomes. Alamut identified rs182123615 as a deleterious variant because of the degenerative nature of splice sites. Intronic variants outside conserved GT/AG positions at the 5′ and 3′ intron boundaries of the acceptor and donor splice sites are usually classified as variants of unknown significance, unless there is some functional evidence of their pathogenicity [43]. The second JAK2 exon 12 mutant, c.1622G>A/C (rs2130530074), has been described in solid tumors [44] as a gain-of-function mutation that leads to ligand-independent signaling through *JAK2*. While the effects of this mutation remain uncertain according to the ACMG classification, both COSMIC and Likelihood ratio test (LRT) have classified it as a pathogenic variant. In their 2013 study Gong et al. identified a hotspot in *JAK2* exon 12, including the region spanning residues R541-E543, which accounts for approximately 10% of all mutations in this exon. This hotspot is also associated with the rs2130530074 variant [45, 46]. Lastly, characteristic absolute erythrocytosis was observed in the two *JAK2* exon 12 mutants, despite CBC being suggestive of JAK2V617F mutated PV/ET, without associated erythroid hyperplasia.

Single nucleotide variations (SNV) and single

nucleotide polymorphisms (SNP) in *JAK2* exon/intron 12 were identified: c.1576T>C, c.1616A>G, c.1641+10T>A, and c.1641+179_183delTCTTA. *JAK2* exon 12 c.1616A>G, and c.1641+179_183 del TCTTA were found in 3 and 11 patients, respectively. These anomalies do not appear to correlate with disease, although alterations in regulatory signals can have pathogenic effects. In silico analysis [47] showed that c.1616A>G had a likely benign effect on the structure and function of the JAK2 protein. In contrast, c.1641+179_183delTCTTA, and c.1641+10T>A had benign effects on the protein. However, their location in a splice site region may result in continuous activation of the protein [42, 48] and may be part of disease initiation. The c.1576T>C substitution was found to be synonymous variation. No clinical information has been associated with these abnormalities in the COSMIC, HGMD, LOVD, HGVS, OMIM, and ClinVar databases. Despite the absence of a pathogenic effect on tyrosine kinase function according to in silico prediction, the impact of abnormalities (SNP or SNV) in *JAK2* exon 12 is still unclear and needs further investigation.

According to the literature, more than 50 variants of *CALR* exon 9 mutant have been identified with a common consequence: loss of the terminal KDEL binding domain [49] following a shift in the reading frame. In our series, *CALR* exon 9 sequencing enabled the identification of two somatic mutation variants, in four distinct samples (12.12%) (Table 1). Two patients carried CALR type 1 (c.1092_1143del52), and the other two carried *CALR* type 2 mutants (c.1154_1155insTTGTC) (Figure 2). This was lower than that reported in a previous Moroccan cohort [15] but is following the observations of Zulkeflee et al. [36] where 7.31% of ET and 14.3% of PMF patients carried mutations in *CALR* [15, 36]. Exclusively observed in males, *CALR* mutant mean age at diagnosis was 67.5 years. Mutations in *CALR* exon 9 are associated with younger age, higher platelet count, lower risk of thrombosis, lower DIPSS-plus score, and less leukocytosis [50]. No significant association was observed in CALR-ET despite a lower mean age (CALR-ET 61.22 years vs JAK2-ET 68.2 years), which is consistent with previous reports [21, 30]. Blood tests revealed hyperleukocytosis, which was accompanied by hyperplateletosis in CALR-ET mutated patients, than in JAK2-ET mutated. Previous studies [39, 40] suggested that patients with JAK2-ET and CALR-ET exhibit similar long-term survival rates, although those with *JAK2* mutations are more susceptible to thrombotic events. *CALR* mutations in ET have been associated with a decreased risk of thrombosis, which has been attributed to the younger age distribution of affected cases [51]. Therefore, recent research indicates that *JAK2*'s prothrombotic impact, in contrast to *CALR,* might also contribute to the decreased risk of arterial thrombosis in CALR-ET mutated. The impact of *CALR* mutations on long-term survival in ET remains unclear, and further research is needed to determine whether these mutations have a favorable or unfavorable prognosis. In PMF, a comparison of the clinical and hematological characteristics of *CALR*-mutated and JAK2-mutated subgroups showed that CALR-PMF mutated cases present higher WBC and leukocyte counts. In respectively six cases with an heterozygous form and two cases with an homozygous form, a SNV was detected in *CALR*: c.*54G>T.. This SNV has not been linked to BCR-ABL1 negative MPNs; however, it may play a role in the pre-disease development stage.

Identified in two cases, *MPL* mutation c.1544G>T was present in 5.26% and 10% of ET and PMF cases, respectively. This is in agreement with previously published data that estimated MPL-ET and MPL-PMF to have a frequency between 2 to 10% [15, 50, 52]. *MPL* substitution MPLW515L (Figure 3) was exclusively observed in females. Our results demonstrated a frequency of 4.08% in *MPL*, which aligns with Shams et al. [53] findings in an Iranian population (4.6%). Mutations in *MPL* seem to be more frequent in populations from the Middle East than in those from the West. In MPL-*PMF* mutated cases, we observed a higher age, as well as significantly low platelet counts, hematocrit, and neutrophil levels. According to the latest research, *MPL* mutations increase the likelihood of thrombotic issues in contrast to the reduced risk associated with *JAK2* mutations. In ET, the risk of arterial thrombosis is significantly higher when associated with *JAK2* or *MPL* mutations than in *CALR*-mutated or triple-negative patients; the same might be true in terms of vascular risk in PMF [52]. As a result, patients with *MPL* mutations are more likely to be diagnosed at a younger age owing to the increased frequency of thrombotic events. *MPL*-mutated patients might also be at risk for accelerated fibrotic progression.

We conducted a comparative analysis of the sex ratio, mean age, and frequencies of JAK2V617F, *JAK2* exon 12, *CALR* exon 9, and *MPL* exon 10 in our cohort with those reported in other studies [14, 15, 38–40, 37, 54] (Tables 6 and 7). The mean age was comparable across the studies. The sex ratio favored males, except in Lin et al. [54] and Soliman et al. [38] reports. The frequencies of JAK2V617F, *JAK2* exon 12, and *CALR* exon 9 were similar to our findings, except for those reported by Smaili et al. [15] and Shires et al. [37]. The frequency of the *MPL* exon 10 was found to be dissimilar in our cohort as compared to Lin et al. [37]. The differences observed may likely be attributed to variations in population characteristics across different ethnic backgrounds or to the sensitivity of detection methods.

Strengthens and Limitations

The strength of this study lies in the fact that it is the first step in understanding the genetic profile of BCR-ABL1 negative MPNs in patients received at the Pasteur Institute of Morocco in the Greater Casablanca region. However, the limitations of the current study include the fact that it was a single-center study, with a relatively small sample size, without patient follow-up. Therefore, information bias is unavoidable. Diagnosis of BCR-ABL1 negative MPN in our institution was mainly based on CBC and molecular aspect of the disease.

In conclusions, in BCR-ABL1 negative MPNs, the determination of the mutation profile is part of the major criterion for diagnosis validation. Until now, there has been a lack of data on the correlation between JAK2V617F, *JAK2* exon 12, *CALR*, and *MPL* mutations

and demographic as well as hematologic parameters in the Moroccan region. The present research constitutes the initial investigation to unveil the existence of the three key genes associated with BCR-ABL1 negative MPNs in Morocco. Our study has certain limitations that prevent us from exploring certain areas, such as ethnicity, race, origin, thrombosis, fibrosis, or survey. Based on our findings, two cases displayed *JAK2* exon 12 mutations, one with PV and the other with an ET diagnosis. However, it is challenging to ascertain whether the ET case's JAK2 exon 12 mutation is accurate or if it represents a misdiagnosis of PV without additional data.

Despite the relatively small sample size, we demonstrated the presence of the three driver mutations in the BCR-ABL1 negative MPNs. Therefore, it would be advantageous to implement a larger sample size in future analyses to make more definitive conclusions. Our analysis showed that the mean age at diagnosis was similar to that reported in other populations. JAK2V617F-ET has been linked to an older mean age compared to triple-negative-ET. Patients with CALR-ET mutated presented with a higher platelets count than those with JAK2-ET or triple negative-ET, whereas patients with JAK2-ET had a higher platelet count than those with JAK2-PV or JAK2-PMF. Red blood cells, hematocrit and hemoglobin levels were observed to be significantly elevated in JAK2-PV cases, while leukocyte and neutrophils counts displayed a significant increase in *PMF* mutated cases. In PMF, *CALR* and *MPL* mutations are associated with higher leukocyte and neutrophil counts. In sum, our observations are comparable to those reported in the literature despite the absence of mutations in the unclassified MPNs group. The analysis also revealed potential pathogenic SNPs and SNVs in the *JAK2* and *CALR* exons and splicing regions. Recently, mutations in epigenetic regulation genes have been identified. Tumor suppression, transcriptional regulation splicing, and other signaling pathway alterations leading to the modification of some disease features add a layer of complexity to BCR-ABL1 negative MPNs molecular pathogenesis. Therefore, the investigation of these genes could result in a better understanding of triple-negative BCR-ABL1 negative MPNs.

What is Known about This Topic

• Genetic abnormalities in somatic, tumor suppression, transcriptional regulation, splicing, and other signaling pathways can lead to specific clinical-biological phenotypes that may influence the emergence and progression of MPNs;

• Classic BCR-ABL1 negative MPN include polycythemia Vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). They are cancers that affect mature blood cells of the myeloid lineage;

• The development of BCR-ABL1 negative MPN is associated with molecular alterations in *JAK2, CALR,* or *MPL;*

• BCR-ABL1 negative MPNs are accompanied by fibrosis or thrombotic events which can evolve into acute forms.

What This Study Adds

• This is the first study to investigate the three genes, *JAK2, CALR*, and *MPL*, in a Moroccan population;

• Our study reports the first cases of *MPL* exon 10 and *JAK2* exon 12 mutations associated with MPNs in Morocco.

Author Contribution Statement

S.G.C.S. was the principal investigator. S.G.C.S. designed the research and took primary responsibility for this paper. S.G.C.S., B.B.A and A.G. performed experiments and reported the results. S.G.C.S., G.A., and S.B. analyzed the data and wrote the manuscript. H.C. and M.O. supervised statistical analyses and provided conceptual advice. A.E.H reviewed the manuscript and gave conceptual advice. H.L. and S.N. conceived and supervised the project. R.S., H.L., and S.N. gave final approval for the submitted version. The manuscript was reviewed and approved by all authors.

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Declarations

This study was conducted as part of academic research and is part of the doctoral thesis of Dr. SORO Somda Georgina Charlène. It is scientifically approved by Hassan II University, Casablanca.

Ethical Approval

The present study has been approved by the Ethics Committee for Biomedical Research Mohammed V University - Faculty of Medicine Pharmacy and Dentistry of Rabat by the approval code CERB 62-21.

Consent For Publication

We confirm that we have obtained the necessary approvals from all authors and participants before submitting the manuscript for publication.

Data Availability

The data presented in this study are available from the corresponding author on reasonable request.

Conflict of Interest

The authors declare that they have no financial or non-financial competing interests.

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