Molecular landscape of the *JAK2* gene in chronic myeloproliferative neoplasm patients from the state of Amazonas, Brazil

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Abstract. *JAK2V617F* (dbSNP: *rs77375493*) is the most frequent and most-studied variant in *BCR::ABL1* negative myeloproliferative neoplasms and in the *JAK2* gene. The present study aimed to molecularly characterize variants in the complete coding region of the *JAK2* gene in patients with *BCR::ABL1* negative chronic myeloproliferative neoplasms. The study included 97 patients with *BCR::ABL1* negative myeloproliferative neoplasms, including polycythemia vera (n=38), essential thrombocythemia (n=55), and myelofibrosis (n=04). Molecular evaluation was performed using conventional PCR and Sanger sequencing to detect variants in the complete coding region of the *JAK2* gene. The presence of missense variants in the *JAK2* gene including *rs907414891, rs2230723, rs77375493 (JAK2V617F)*, and *rs41316003* were identified. The coexistence of variants was detected in polycythemia vera and essential thrombocythemia. Thus, individuals with high *JAK2V617F* variant allele frequency (≥50% VAF) presented more thrombo-hemorrhagic events and manifestations of splenomegaly compared with those with low *JAK2V617F* variant allele frequency (<50% VAF). In conclusion, individuals with *BCR::ABL1* negative neoplasms can display >1 variant in the *JAK2* gene, especially *rs2230722*, *rs2230724*, and *rs77375493* variants, and those with high *JAK2V617F* VAF show alterations in the clinical-laboratory profile compared with those with low *JAK2V617F* VAF.

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Introduction

The *BCR::ABL1* negative chronic myeloproliferative neoplasms (MPN) represent a heterogeneous group of clonal diseases of the hematopoietic progenitor cell, of which the most classic are polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) (1,2). In the 5th Classification of Hematolymphoid Tumors, published in 2022, the World Health Organization (WHO) revised certain aspects for the category of MPN (1), establishing as diagnostic criteria for the diagnosis of PV elevated hemoglobin concentration and/or hematocrit, accompanied by panmyelosis and detection of *JAK2V617F* or exon 12 variants in *JAK2*.

The primary diagnostic criterion of ET is marked thrombocytosis (platelet count >450x10^3/mm^3). PMF is characterized by a proliferation of abnormal megakaryocytes and granulocytes in the bone marrow, which is associated in fibrotic stages with a

polyclonal increase in fibroblasts that drive secondary reticulin and/or collagen marrow fibrosis, osteosclerosis, and extramedullary hematopoiesis. Thereby, these diseases are characterized by increased cell proliferation, development of chronic inflammation, and association with clonal hematopoiesis (1,3,4).

Missense mutations in the JAK/STAT pathway are the primary causes of the development of chronic MPN (5). Variants in the driver genes *JAK2, CALR*, and *MPL* are the most commonly associated with the development of MPN (6). According to National Center of Biotechnology Information (NCBI:https://www.ncbi.nlm.nih.gov/gene/3717), the *JAK2* gene is located on chromosome 9p24.1 and encompasses 145,559 nucleotides, distributed across 28 exons, and the *JAK2* coding sequence has a length of 3,399 nucleotides, distributed across 23 exons, from exon 3 to exon 25, which encodes a protein of 1,132 α. amino acids, a non-receptor tyrosine kinase named JAK2.

Most of the variants identified in *JAK2* result in a gain of function, and are characterized as somatic missense types that lead to unregulated production of hematopoietic cells in bone marrow and accumulation of mature cells in peripheral blood (7). *JAK2V617F* (dbSNP: *rs77375493*) is the most commonly identified variant in MPN and is found in up to 95% of cases of PV and between 50-60% of cases of ET and PMF (8). This variant is located in exon 14 of the *JAK2* gene and is characterized as a missense variant. It is a product of the substitution of a guanine by a thymine at position 1,849, that leads to a substitution of valine with phenylalanine at the amino acid position 617 (V617F) of the protein structure (9,10), a position that belongs to the pseudokinase domain, which is a region of the primary positive and negative regulation of the protein (10,11).

Variants in exon 12 of the *JAK2* gene are identified in ~3% of *JAK2V617F*-negative patients diagnosed with PV (12). Genetic alterations in this exon include missense and indel variations (13), which confer a marked erythrocytic picture in individuals with PV, and appear at younger ages when compared to the *JAK2V617F* variant (14).

The presence of coexisting non-driver variants can modulate the *JAK2V617F* variant allele frequency (VAF). In MPN, the determination of *JAK2V617F* VAF is pivotal when evaluating laboratory and clinical implications. It is worth mentioning that, in PV, a high VAF (≥50%) is associated with fibrotic progression and positively associated with total white blood cell count (WBC), neutrophil count, and thrombosis events, especially in the presence of coexisting non-driver variants (15), while in ET, a high VAF is correlated with increased thrombo-hemorrhagic events, hypercoagulable status, and low quantitation of hemostasis factors (16,17).

Sanger sequencing and next-generation sequencing have allowed the identification of variants in other *JAK2* exons (18,19). Several variants have been identified in the complete coding region of the *JAK2* gene, which affect other domains of the JAK2 protein (19,20) and lead to constitutive activation of the JAK/STAT pathway, with most of the described variants being somatic, with only a small fraction of them being germinal. This finding suggests that certain patients may develop a non-clonal myeloproliferative phenotype, with variable penetrance at the familial level (21).

Certain variants that are acquired in the coding region of *JAK2* are described as benign or of uncertain clinical significance, and the primarily affected exons are 6 (22), 9-10 (23), 11-15 (19), and 19 (24). According to certain studies, some variants in these regions have been found in coexistence, presenting cytokine-independent signaling (25), and are even associated with leukemic transformation and development of non-hematological solid tumors (23,24,26). Thus, the present study aimed to molecularly characterize variants in the complete coding region of the *JAK2* gene in individuals with *BCR::ABL1* negative chronic myeloproliferative neoplasms.

Materials and methods

Patients. In the present study, 97 patients from the state of Amazonas, Brazil, diagnosed with PV (n=38), ET (n=55) and MF (n=04), who were treated between July 2021 and March 2023 at Hospital Foundation for Hematology and Hemotherapy of Amazonas (which is the only reference institution in the state of Amazonas for the diagnosis and treatment of hematological diseases) were included. Participants showed an absence of *BCR::ABL1* transcripts. Additionally, all the patients with a MF diagnosis who agreed to participate in the investigation were included.

The prese study was performed in accordance with the Declaration of Helsinki and Resolution 466/12 of the Brazilian Ministry of Health. This study was approved by the National Ethics Committee, which is responsible for approving relevant human studies in Brazil (approval no. 4.450.813). Written informed consent was obtained from all subjects involved in the study.

Clinical and laboratory data. Clinical data were obtained from medical records, which included data regarding sex, age, splenomegaly, history of thrombotic or hemorrhagic events, and treatments administered. Laboratory data were obtained from blood samples and included red blood cell count (RBC), hematocrit (Ht), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), WBC, percentage of segmented neutrophils, monocytes and lymphocytes; Platelet count, prothrombin time-International Normalized Ratio (PT-INR), activated partial thromboplastin time (aPTT), fibrinogen (FIB), lactate dehydrogenase (LDH) and uric acid (UA). UA and LDH analyses were performed after diagnosis and during treatment, mentioning that several patients included in the study had received several years of hydroxyurea administration. The median optimal treatment regime in PV patients was 4 years (100-500 mg/per day of hydroxyurea or 2 mg/per day of Anagrelide), in ET patients it was 10.5 years (100-300 mg/per day of hydroxyurea or 2 mg/per day of Anagrelide), and in MF patients it was 2 years (2 mg/per day of Anagrelide). Of note, administration of hydroxyurea can significantly alter laboratory analysis.

Blood-sample processing and RNA extraction. Total RNA was extracted from peripheral blood samples with EDTA anticoagulant using TRIzol® (Ambion; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. cDNA was synthesized using SuperScript™ III Reverse Transcriptase (Promega Corporation). Reverse transcription was used to obtain cDNA, using the following thermocycling parameters: 5 min at 25˚C and 60 min at 42˚C. After the reaction, the cDNA was stored at -80˚C until used for PCR.

Table I. Sequences of the primers used for PCR and Sanger sequencing.

Primer name	Sequence (5'→3')	Annealing temperature
JAK2_Fow_1	GGCAACAGGAACAAGATGTGAA	69˚C
JAK2_Rev_691	AGCTGATAGAGTTATAGATGGC	64˚C
JAK2_Fow_691	AAACGATCAAACCCCACTGG	68˚C
JAK2_Fow_1325	CCCAATTTCGATGGATTTTGCCA	69˚C
JAK2_Rev_1340	TCCAGTCTGATTACCTGCTT	65˚C
JAK2_Fow_1981	ATTCTGGTTCAGGAGTTTG	62˚C
JAK2_Rev_1963	CAAACTCCTGAACCAGAAT	62˚C
JAK2_Fow_2715	GGTATGACCCTCTACAGGAC	66˚C
JAK2_Rev_2696	GTCCTGTAGAGGGTCATACC	65˚C
JAK_Rev1_3503	TTGGTCTCAGAATGAAGGTC	64˚C
Fow-JAK2-Confirmation	AGTGGTCCTTCAGGTGAGGAG	56˚C

Fow, forward; Rev, reverse.

PCR and Sanger sequencing analysis. Amplifications were performed using a total volume of 25 μl. Reaction products were visualized using electrophoresis on a 1.5% agarose gel stained with ethidium bromide. PCR products were purified with the DNA precipitate and purification protocol using polyethylene glycol 8000 (Promega Corporation) as described previously (27-29). A Sanger sequencing reaction (in both directions) was performed using BigDye® Terminator v3.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The sequences of the primers used are listed in Table I, and were designed using Primer-BLAST-NCBI and OligoAnalyzer Tool-IDTDNA to evaluate the percentage of GC, Tm, Hairpin capacity, and ΔG index, to flank the complete coding region of *JAK2*, spanning from exon 3 to exon 25 (Fig. 1). The products of the sequencing reaction were purified using the EDTA/ethanol protocol and were subsequently evaluated in an automatic sequencer (3500 XL Genetic Analyzer®, Applied Biosystems handbook; Thermo Fisher Scientific, Inc., pag. 12) using the POP-7 polymer.

Data analysis. The sequences obtained were initially analyzed using the Sequencing Analysis software (Applied Biosystems; Thermo Fisher Scientific, Inc.); only high-quality sequences were used for variant analysis (Q score ≥30). Geneious software 6.0.6 (Biomatters, Inc.) was used to obtain contigs and compare them to the *Homosapien JAK2* reference sequence, transcript 2, mRNA (NCBI: NM_001322194.2). Samples with the presence of rare variants were sequenced and confirmed at least twice. VAF was measured in *JAK2V617F*-positive individuals using Minor Variant Finder (Applied Biosystems, Thermo Fisher Scientific, Inc.) and Edit R software (moriaritylab.shinyapps.io/editr_v10). The clinical significance of the variants identified in the research was analyzed using the Polyphen2 tool and the ClinVar-NCBI site (https://www.ncbi.nlm.nih.gov/clinvar/).

Statistical analysis. Categorical variables are presented as the frequency (n, %). Continuous numeric variables are presented as the median and interquartile range (IQR). The distribution of continuous numerical variables was verified using a Shapiro-Wilk test. Statistical analysis of categorical variables was performed using a0020[2] test. Kruskal-Wallis and Mann-Whitney U tests were used to analyze numerical variables, when appropriate. Data from individuals with MF were excluded from the statistical analysis between groups due to the number of patients with MF. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analysis of the data was performed using GraphPad Prism version 8.2.1 (GraphPad Software, Inc.).

Results

Clinical and laboratory characteristics of patients. Samples from 97 patients diagnosed with MPN were evaluated, and these were distributed among PV (n=38), ET (n=55), and MF (n=04). During the length of the study, none of the patients showed transformation to acute leukemia, post-PV, or post-ET-MF. Clinically, ET showed a predominance in females (P=0.0276), compared with PV and MF. All individuals were between the fifth and sixth decade of life (P=0.565; comparing the age between the PV and TE groups). Splenomegaly was detected more frequently in MF, than in PV and ET (75, 23.6, and 16.3%, P=0.0212, respectively) patients.

Thrombotic and hemorrhagic events were more often observed in ET cases (16.3 and 21.8%, P=0.6406 and P=0.0205, respectively) when compared to PV cases. The thrombotic events included deep venous thrombosis, thrombosis of the splenic vein, esophageal varices, and miscarriage, and the following hemorrhagic events were evaluated in the study: Hypermenorrhagia, ocular and gingival hemorrhage, and hemorrhage of the gastrointestinal tract. All medical records of the patients included in this study were reviewed and none of these reported acquired von Willebrand syndrome.

In the blood count, an increase in the erythrocyte lineage was observed in individuals with PV compared to those with ET and MF, with an increased RBC (5.03 x mm^3, P<0.0001), a finding that is complemented by Ht values

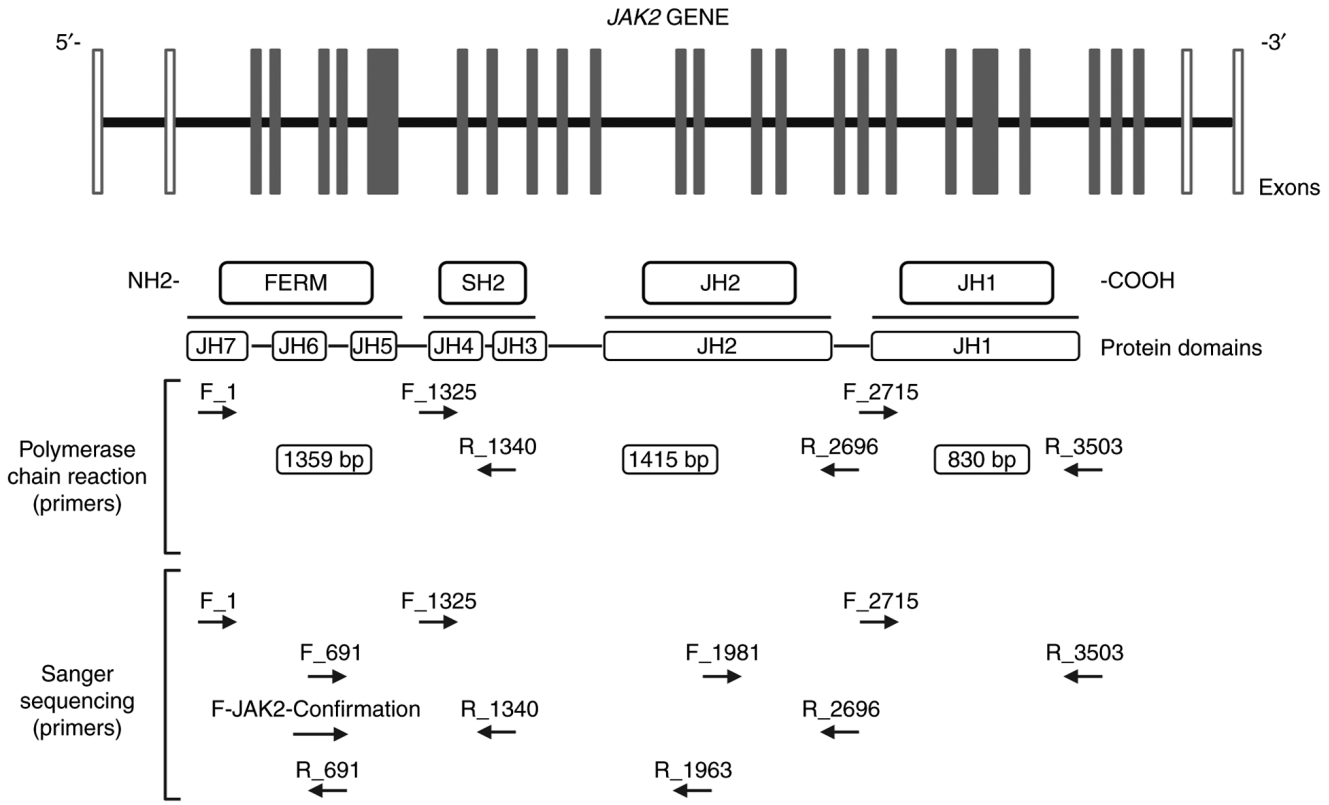

Figure 1. Structure of the *JAK2* gene. Gray boxes correspond to coding exons 3-25, which were analyzed using Sanger sequencing. Arrows indicate the primers used in the reactions. Text in bold type in the boxes indicates the size of the fragments.

(48%, P<0.0001) and Hb concentration (15.2 g/dl, P<0.0001). Hemometric values were found to be increased in ET cases [Mean Corpuscular Volume, MCV: 103.9 fl; P=0.0013; Mean Corpuscular Hemoglobin, MCH: 33.5 pg, P=0.006, and Mean Corpuscular Hemoglobin Concentration (MCHC): 32.5 g/dl, P=0.1160] when compared to PV and MF cases. The white blood cell count was within normal ranges in PV and TE cases, compared with those with MF (P=0.0134). However, the percentage of neutrophils was higher in MF patients (76.4%) when compared to ET and PV patients (P=0.0232), and the lymphocyte count was slightly higher in ET than in PV and MF patients (29.2%, P=0.0005). In ET patients, a high platelet count was observed when compared to PV and MF patients (470,500 x mm^3, P<0.0001). Erythropoietin measurements were not available in the present study.

Values in the hemostasis tests of individuals with PV, ET, and MF were closely related; however, a slight increase in fibrinogen concentrations was observed in individuals with MF (321 mg/dl, P=0.400). Biochemical analyses demonstrated higher concentrations of LDH and UA in subjects with MF (904.5 U/l, P=0.0295 and 6.8 mg/dl, P=0.006; respectively) compared with PV and ET patients. Clinical and laboratory values are described in Table II.

Variants detected in chronic MPN patients. In this study, missense variants were identified in the FERM domain (*rs907414891*); 1 variant in the FERM-SH2 linker region (*rs2230723*), 1 variant in the pseudokinase domain (*rs77375493*), and 1 variant in the kinase domain (*rs41316003*). This totals 4 missense variants identified in the complete coding region of the *JAK2* gene, as described in Table III. In addition, other synonyms and benign variants were detected in the complete coding region of the *JAK2* gene (*rs2230722*, *rs576746768*, *rs2230728*, *rs2230724*, and *rs55930140*). Conversely, the *rs10119726* variant is a synonymous variant and does not have a description of its clinical significance on ClinVar. These variants are shown in Figs. 2-11.

Frequency and distribution of missense variants in patients with variant alleles of the JAK2 gene. The frequency of variants was estimated in the population (PV=38, ET=55, and MF=04), and it was noted that most of them were in the first protein domains, especially in the FERM domain, followed by the pseudokinase domain. The variant *rs77375493* (*JAK2V617F*) showed a high frequency in individuals with PV when compared to those with ET (65.7 and 38.1%, respectively, P=0.0116). Variant *rs2230723* was found in sporadic cases of PV and ET. Interestingly, *rs907414891* and *rs41316003* were found only in cases of ET, but not in cases of PV or MF. The frequency of missense variants is presented in Table IV.

Mutational landscape of the JAK2 gene in individuals with chronic MPN. After estimating the frequency of the variants in the complete coding region of the *JAK2* gene, the mutational profile of the individuals was mapped. It was observed that patients with variant alleles in JAK2 simultaneously presented with 1-3 variants. Among the primary variants found simultaneously in the three types of MPN were *rs2230724*, *rs2230722*, and *rs77375493*, thus highlighting that most individuals with PV presented with the three variants when compared to those

Table II. Demographic, clinical, and laboratory characteristics of patients.

Characteristic	PV, n=38	ET, n=55	MF, n=4	P-value	Reference values
Male/Female, n	18/20	12/43	2/2	0.0276[a]	
Age, median (IQR)	60.5 (48.75-70.25)	57 (42-72)	62 (54.2-75.7)	0.565	
RBC, x mm^3, median (IQR)	5.03 (4.3-6.2)	3.75 (3.2-4.5)	4.3 (3.4-6.1)	<0.0001[d]	3.9-5.3x10^3/mm^3
Ht, %, median (IQR)	48 (43.4-52.2)	37.9 (34.6-42.2)	37.05 (33.5-48.5)	<0.0001[d]	36-48%
Hb, g/dl, median (IQR)	15.2 (13.7-16.2)	12.7 (11.6-13.9)	11.7 (10.6-15.9)	<0.0001[d]	12-16 g/dl
MCV, fL, median (IQR)	92.3 (82.6-103.6)	103.9 (92.3-112.7)	85.9 (78.6-92.2)	0.0013[b]	80-100 fl
MCH, pg, median (IQR)	30.3 (27.1-33.2)	33.5 (30.1-36.7)	27.6 (24.6-30.3)	0.0006[c]	27-33 pg
MCHC, g/dl, median (IQR)	31.8 (30.3-33.3)	32.5 (32-33.6)	32.1 (30.6-33.4)	0.1160	32-36 g/dl
WBC, x mm^3, median (IQR)	6,540 (5,170-8,060)	5,370 (4,170-7,200)	12,930 (5,783-15,678)	0.0134[a]	3,600-11,000x10^3/mm^3
Neutrophils, %, median (IQR)	68 (56.7-77.1)	61.9 (56.1-69.2)	76.4 (65.7-79.0)	0.0232[a]	
Lymphocytes, %, median (IQR)	21.5 (15.9-29.8)	29.2 (22.5-35.4)	11.0 (11.0-17.6)	0.0005[c]	
Monocytes, %, median (IQR)	5 (3.5-7.0)	4.8 (3.9-6)	2.4 (1.2-5.2)	0.169	
Platelets, x mm^3, median (IQR) x10^3/mm^3	301,000 (180,000-403,000)	470,500 (369,000-577,000)	439,000 (253,250-839,250)	<0.0001[d]	150,000-400,000
LDH, U/l, median (IQR)	439.8 (324.7-552.9)	423.1 (348.5-494.2)	904.5 (568.1-1210)	0.0295[a]	214-450 U/l (male) 195-453 U/l (female)
Uric acid, mg/dl, median (IQR)	4.4 (3.4-5.6)	4.1 (2.9-4.8)	6.8 (5.8-7.6)	0.006[b]	3.5-7.2 mg/dl (male) 2.6-6.0 mg/dl (female)
PT, sec, median (IQR)	11.5 (10.9-12.6)	11.4 (11.0-12.3)	13.8 (13.0-14.1)	0.0360[a]	12-14 sec
INR, median (IQR)	0.99 (0.93-1.08)	0.98 (0.95-1.06)	1.18 (1.11-1.21)	0.0342[a]	
aPTT, sec, median (IQR)	31.7 (27.9-36.3)	30.7 (28.1-33.6)	37.9 (35.1-42.7)	0.0336[a]	35-40 sec
Fibrinogen, mg/dl, median (IQR)	278 (228-320)	291 (220-362)	321 (218.8-493.8)	0.400	180-350 mg/dl
Splenomegaly, n (%)	9 (23.6)	9 (16.3)	3 (75)	0.0212[a]	
Thrombotic events, n (%)	5 (13.1)	9 (16.3)	0	0.6406	
Bleeding events, n (%)	1 (2.6)	12 (21.8)	0	0.0205[a]	
Treatment with HU, n (%)	27 (71)	49 (89.09)	0	<0.0001[d]	
Treatment with Anagrelide, n (%)	0	5 (9.09)	1 (25)	0.0565	
Therapy with phlebotomy, n (%)	7 (18.4)	0	0	0.0029[b]	

[a]P<0.05, [b]P<0.01, [c]P<0.001, [d]P<0.0001. PV, polycythemia vera; ET, essential thrombocythemia; MF, myelofibrosis; RBC, red blood cell count; Ht, hematocrit; Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cell count; LDH, lactate dehydrogenase; PT-INR, prothrombin time-international normalized ratio; aPTT, activated partial thromboplastin time; HU, Hydroxyurea; IQR, Interquartile range; sec, seconds.

with ET (P=0.0023). In contrast, individuals with ET showed a predominance of two variants (*rs2230722* and *rs2230724*) compared to those with PV (P=0.0253). The mutational landscape of the patients is presented in Table V. Individuals with four variants were not found.

JAK2V617F VAF in patients with PV and ET. Of the 97 patients included in this study, the allele burden of *JAK2V617F* was measured in 46 individuals who were *JAK2V617F*-positive (PV, n=25 and ET, n=21). The allele burden of *JAK2V617F* was compared in individuals with PV and ET. In each disease, two groups were considered to describe the VAF of *JAK2V617F*: High VAF (≥50%) and low VAF (<50%). Individuals with ET showed a low VAF *JAK2V617F* (<0.0001) when compared to those with PV who showed VAF ≥50% (0.0477). Individuals with MF were excluded from this comparison. The comparison of the VAF of *JAK2V617F* among the groups is presented in Table VI.

Comparison of the clinical and laboratory profile according to the VAF of JAK2V617F in patients with PV. The clinical and laboratory profile of individuals with PV and ET with *JAK2* variants were compared considering the VAF of *JAK2V617F* in both groups [high VAF (≥50%) and low VAF (<50%)].

Table III. Missenses variants detected by Sanger sequencing in the entire coding region of the *JAK2* gene in patients with myeloproliferative neoplasms.

Variant	Allele	Variation in cDNA	Exon localization	Variation in the protein	Affected domain	Functional consequence	Clinical significance[a]	Type of variant[b]
rs907414891	A>G	c.496	6	(p.Ile166Val)	FERM	Missense, no functional evidence registered	No description	Somatic
rs2230723	C>G	c.1177	9	(p.Leu393Val)	FERM-SH2	Missense, no functional evidence registered	Uncertain clinical significance	Germline
rs77375493	G>T	c.1849	14	(p.Val617Fen)	Pseudokinase	Missense, no functional evidence registered	Pathogenic	Somatic
rs41316003	G>A	c.3188	24	(p.Arg1063His)	Kinase	Missense, no functional evidence registered	Benign	Germline

[a]Clinical significance was described according to ClinVar reports. [b]Variant type was described according to dbSNP reports.

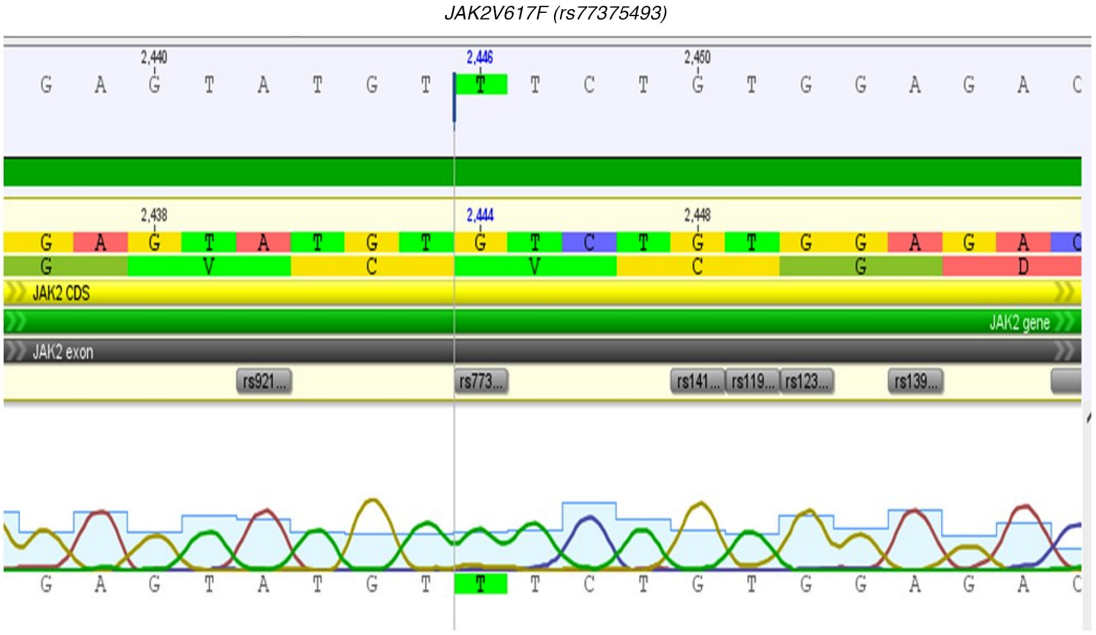

Figure 2. Chromatogram of the *JAK2V617F* variant by DNA sequence analysis.

Regarding the clinical profile in individuals with PV, thrombotic and hemorrhagic events were evenly distributed among both groups. However, in the PV patients, splenomegaly was more frequent in individuals with a high VAF. The clinical data of the individuals with PV according to VAF of *JAK2V617F* are presented in Table VII.

The comparison of laboratory profiles in individuals with PV, according to their VAF of *JAK2V617F*, showed an increase in hematimetric values (RBC, 4.7 x mm^3; Ht, 46.9%; Hb, 14.9) in individuals who presented a VAF of *JAK2V617F* ≥50%, compared with those with a VAF of <50%. WBC and platelet count were slightly augmented in individuals with a VAF of ≥50%. Likewise, LDH was elevated in individuals with a VAF of *JAK2V617F* of ≥50% (486.5 U/l). Hemostasis tests were relatively equivalent between both groups in PV patients. The laboratory profiles of the individuals with PV, according to the VAF of *JAK2V617F*, are presented in Table VII.

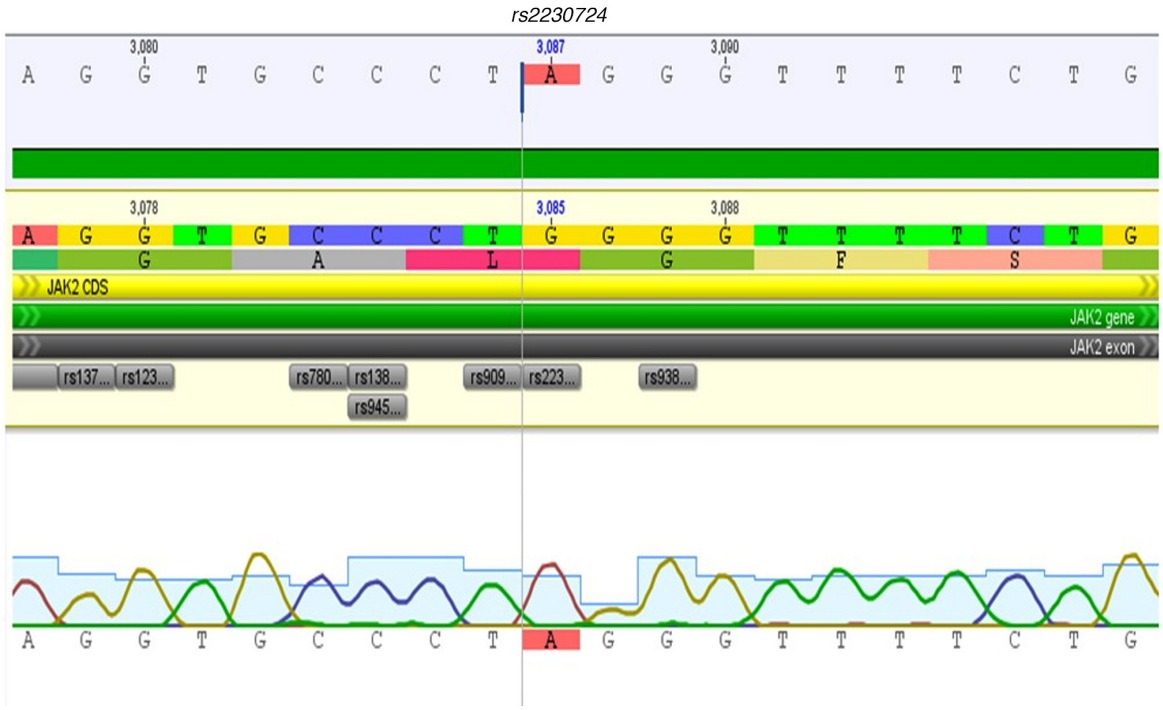

Figure 3. Chromatogram of the *rs2230724* variant by DNA sequence analysis.

Figure 4. Chromatogram of the *rs2230722* variant by DNA sequence analysis.

Comparison of the clinical and laboratory profiles according to the VAF of JAK2V617F in patients with ET. In the individuals with ET, the clinical and laboratory profiles were also described based on the VAF of *JAK2V617F*. Regarding the clinical characteristics in individuals with ET, thrombo-hemorrhagic episodes were the most commonly recorded clinical events in the patients, especially in those with VAF of *JAK2V617F* of ≥50%; however, despite this fact, it was not statically significant. Just as in the PV individuals, splenomegaly was more frequent in individuals with a high VAF. The clinical data of the individuals with ET according to the VAF of *JAK2V617F* are presented in Table VII.

The laboratory profiles of individuals with ET, according to the VAF of *JAK2V617F*, showed an increase in hematimetric values (RBC, 5.1 x mm^3; Ht, 47.0%; and Hb, 15.5 g/dl) in individuals who presented a VAF of *JAK2V617F* of ≥50% when compared to those with a VAF of <50%. The WBC showed equivalence in both groups. Interestingly, the platelet count was increased in individuals with a VAF of <50%. Likewise, for individuals with ET, LDH was elevated in

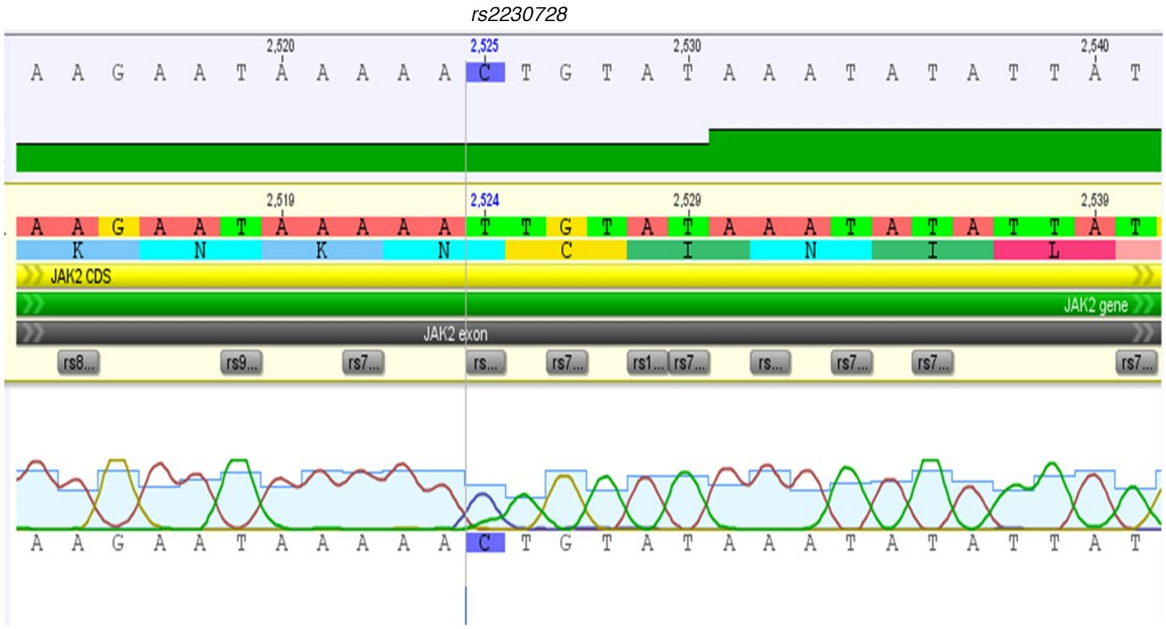

Figure 5. Chromatogram of the *rs2230728* variant by DNA sequence analysis.

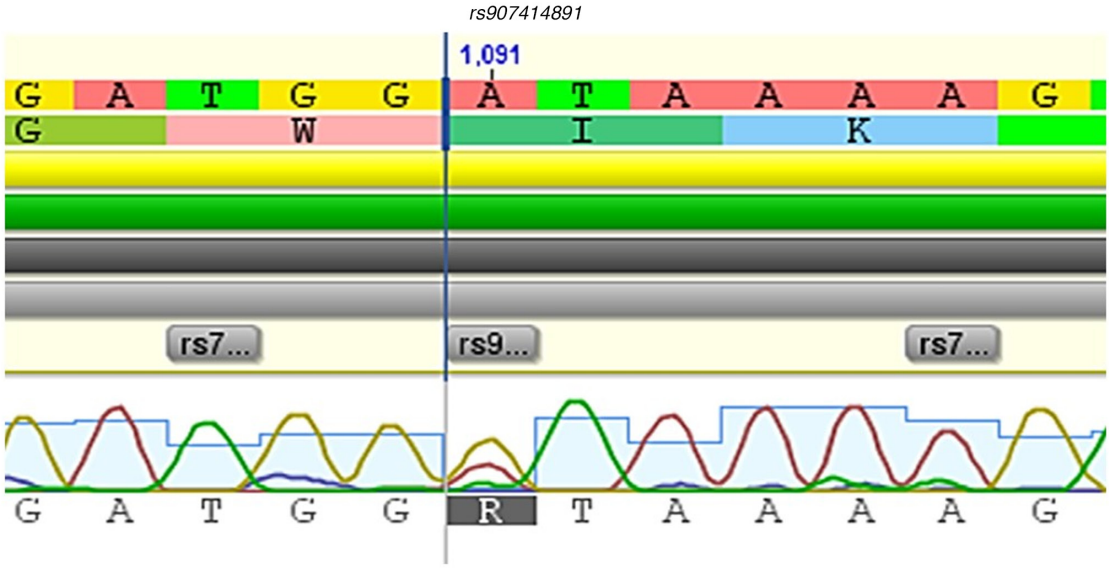

Figure 6. Chromatogram of the *rs907414891* variant by DNA sequence analysis.

individuals with a VAF of *JAK2V617F* of ≥50% (412.1 U/l). Hemostasis was slightly prolonged in individuals with a VAF of *JAK2V617F* of ≥50%. The laboratory profiles of individuals with ET, according to VAF *JAK2V617F*, are presented in Table VII.

Discussion

MPNs are generally characterized by an increase in cell counts in the blood, which can lead to clonal evolution and disease progression. Despite investigations in other Brazilian states (30-32), this study is the first to address *JAK2V617F* mutation detection and the hematologic profile according to *JAK2V617F* VAF in patients from the state of Amazonas diagnosed with MPN.

Regarding the proportion of MF patients, which is a multifactorial issue, previous studies in Brazil have shown a lower proportion of MF patients compared with PV and ET (30-32), and it is noteworthy that MF is the most aggressive MPN, and shows a high ratio of leukemic transformation. Silva *et al* (32) determined the prevalence of *JAK2V617F* in MPN in Pernambuco, Brazil, and found that few patients had MF diagnosis compared with those with PV and ET. Similarly, Macedo *et al* (30) investigated the association between the *JAK2* 46/1 haplotype and acquisition of *JAK2V617F*. They observed the lowest number of cases of MF. Furthermore, they concluded that the JAK2 46/1 haplotype was present in *JAK2V617F* positive individuals and associated with MPN phenotype in Brazilian patients. Likewise, in another study, Macedo *et al* (31) assessed the association of TNF

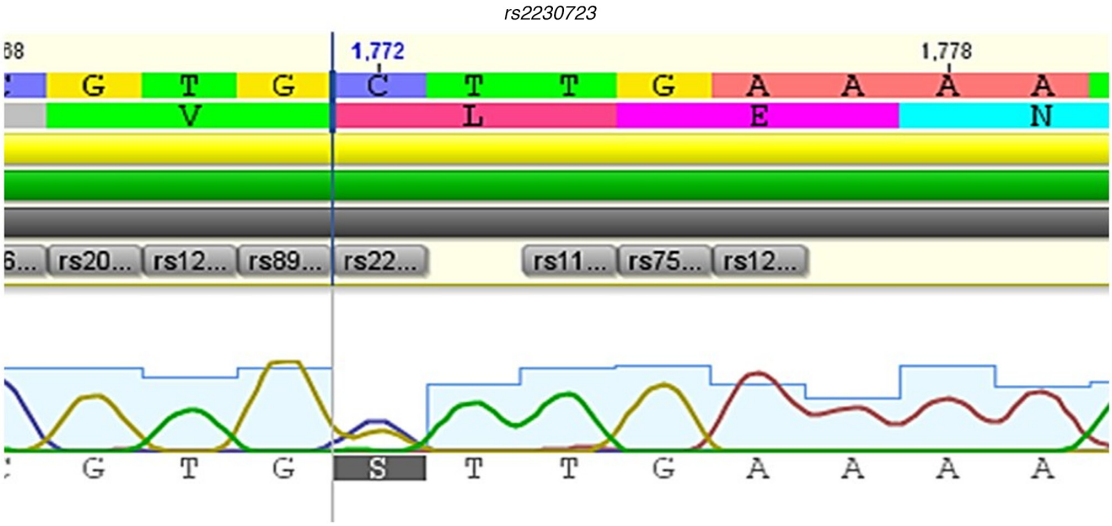

Figure 7. Chromatogram of the *rs2230723* variant by DNA sequence analysis.

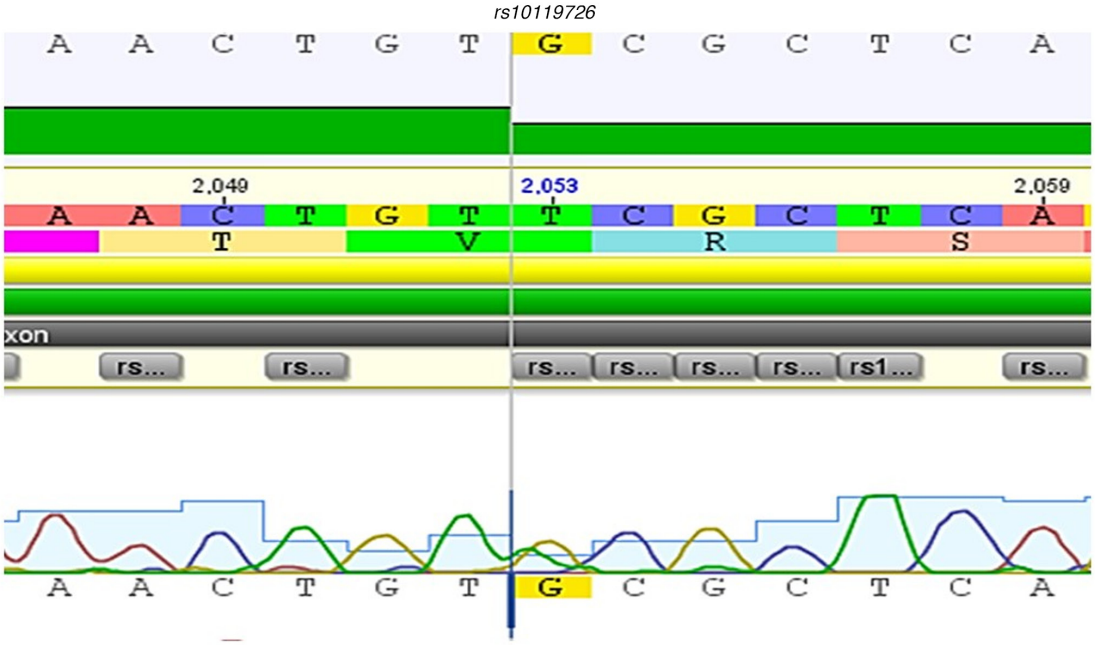

Figure 8. Chromatogram of the *rs10119726* variant by DNA sequence analysis.

polymorphisms with *JAK2V617F* MPN in Brazilian patients finding a low number cases of MF.

The present study showed that the increase in the erythrocyte lineage was in fact a characteristic of individuals with PV and that the increase in the platelet count was an indicator that is suggestive of ET, according to the indicators established by the WHO (1). RBC counts are directly related to Hb and Ht concentrations; it is hypothesized that these two hematological parameters are reliable indices for the diagnosis of PV (33).

Currently, erythropoietin measurement is considered a major diagnostic criterion for PV diagnosis (1,34). In the present study, these measurements were not available; however, MCV is considered a marker that can be used to differentiate between PV and ET (33). In the present study, MCV was found to be lower in patients with PV than in those with ET. This finding may explain the iron deficiency and the accelerated time for renewal of red blood cells in these patients (33,35).

The role of the lymphocyte count in MPN is not well described. Stefaniuk *et al* (36) found that there was little evidence for the prognostic significance of the neutrophil-lymphocyte ratio and lymphocyte-monocyte ratio in MPN, but they both may be higher in patients with PMF compared to healthy individuals, and may be associated with chronic inflammation and tumorigenesis. Likewise, Mulas *et al* (37) described that high a neutrophil-lymphocyte ratio had been reported in JAK2-positive patients and this parameter could be used as an indicator of chronic inflammation in MPN.

In addition, Vannucchi *et al* (38) reported that individuals with MPN have an increased risk of developing lymphoproliferative neoplasms, particularly in those that were

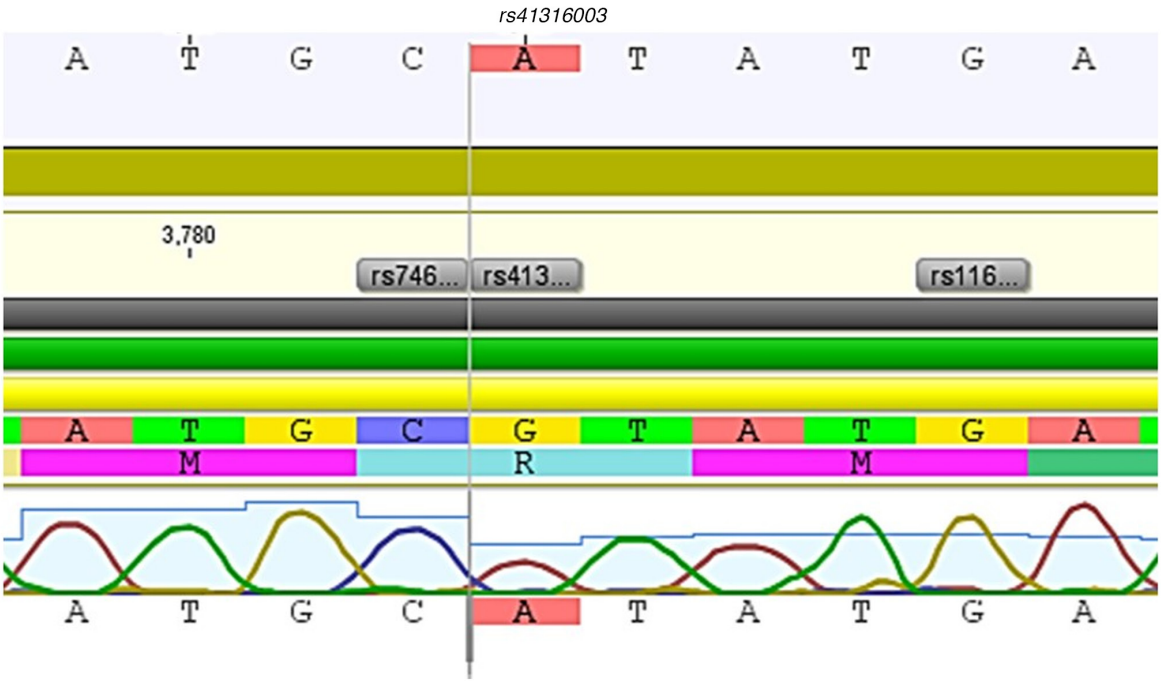

Figure 9. Chromatogram of the r*s41316003* variant by DNA sequence analysis.

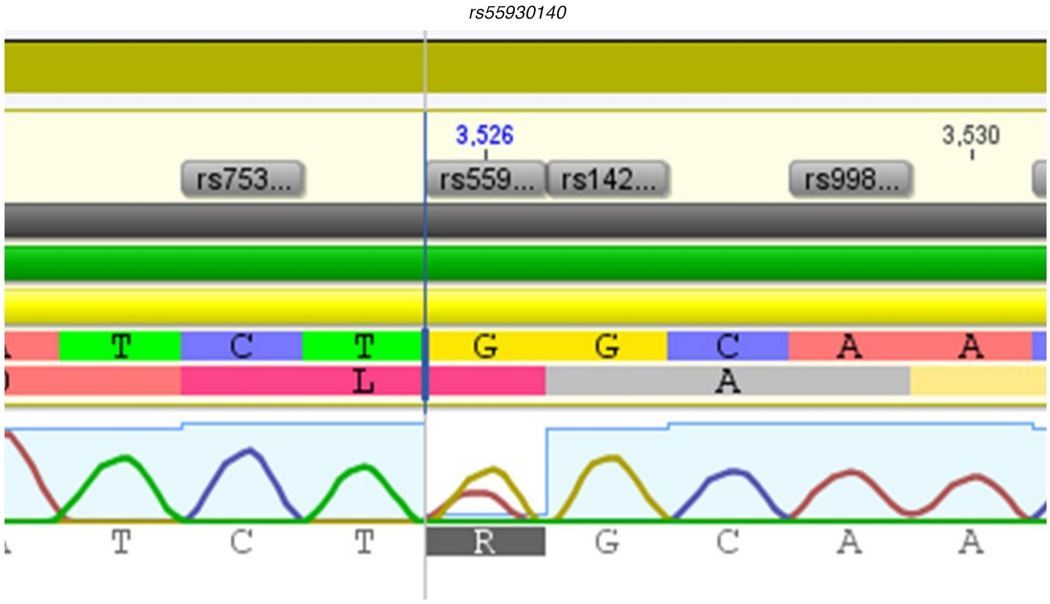

Figure 10. Chromatogram of the *rs55930140* variant by DNA sequence analysis.

JAK2V617F-positive. Similarly, Garcia-Gisbert *et al* (39) found that certain patients with a diagnosis of MPN showed CD3+ *JAK2V617F*-positive lymphocytes, These findings may support the hypothesis that *JAK2V617F*-positive lymphocytes may be related to leukemic transformation.

Furthermore, it has been highlighted that MPN is associated with a high risk of thrombotic and thromboembolic events when compared with the general population, and is also associated with increased hematopoietic counts (40), which was also observed in the present study. This fact may be explained by the presence of a high VAF of *JAK2V617F* (≥50%), which likely stimulates deregulation signaling in hematopoietic progenitor cells and may be potentialized by the presence of other variants in genes such as *CALR* and *MPL*; these are directly implicated in platelet activation and increased platelet account (40).

Administration of hydroxyurea is frequently used in cases of PV and ET for the normalization of hematological counts (41,42). The results of the present study showed that the high platelet count observed in individuals with ET was directly related to the increase in the frequency of thrombo-hemorrhagic events, which indicates that platelets could in fact be the primary mediators of thrombotic activation in these patients. As such, the study by

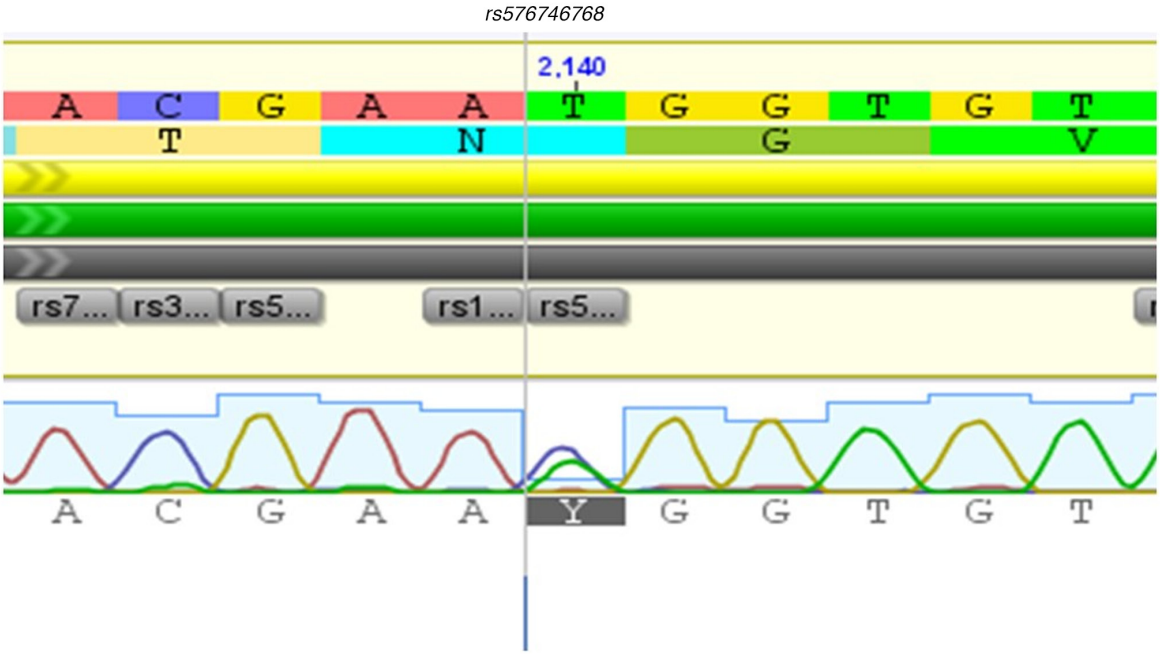

Figure 11. Chromatogram of the *rs576746768* variant by DNA sequence analysis.

Buxhofer-Ausch *et al* (43) demonstrated that platelet count normalization is an important factor in reducing thrombotic risk, regardless of the leukocyte count. However, further studies are needed to confirm what the cut-off point in the platelet count is to trigger these risks.

Esophageal and gastric complications are often described in patients with myeloproliferative neoplasms diagnosis (44), and this is typically due to portal system hypertension or von Willebrand syndrome, which is the result of excessive thrombocytosis. However, in the present study, bleeding complications were relatively high, especially in patients with ET. This fact may be due to an increased platelet count with functional platelet disorders, such as impaired platelet aggregation response to collagen and reduced number of dense granules in platelets (45). In addition, current literature notes that ET is more common in females, and bleeding and thrombotic risks are the major complications in MPN patients (40,46). Nevertheless, female biology may play a role in the development of bleeding and thrombotic events, likely due to pregnancy and the use of contraceptives interfering with the interactions of platelets and other molecules in the endothelium.

Other variants in the *JAK2* gene have been reported, and most of these variants are of the somatic type (21,22). The existence of germline variants in MPN has also been described, and this includes showing patterns of erythropoietin (EPO) hypersensitivity and weak constitutive signaling of the JAK2/STAT5 pathway compared to *JAK2V617F* (47).

Therefore, by applying Sanger sequencing in the complete coding region of the *JAK2* gene, the results of the present study demonstrated the existence of somatic and germline variants in individuals with MPN other than *JAK2V617F*, with somatic variants being the most frequent. This is also corroborated by previous studies (19,48,49). Moreover, germline variants in individuals with MPN at an early age in individuals with a familial predisposition, compared with those with somatic variants, have been described (50). Age differences between patients with somatic and germline mutations were not investigated in the present study, and this will form a future research direction.

JAK2V617F is the most common variant in *BCR::ABL1* negative MPN (51), with constitutive activity of the JAK2/STAT5/STAT3 pathway, and it is highly associated with the development of cardiovascular and thrombotic complications (15). In the present study, *JAK2V617F* was identified in 65.7% of the patients with a diagnosis of PV. This may be related to the median optimal treatment regimes, as these individuals have been treated with cytoreductive therapy for several years.

The effects of *JAK2* VAF are well established; however, the specific populations affected are poorly understood. Through the comparison of *JAK2V617F* VAF, it was shown that patients from the state of Amazonas with PV had a *JAK2V617F* VAF that was higher than those diagnosed with ET, and individuals with a VAF of ≥50% had more thrombo-hemorrhagic events and a slight prolongation in coagulation tests, especially in PT-INR and aPTT when compared with those with a VAF of <50%, which is that not dissimilar to previous studies (40,46). This fact directly suggests that individuals with a high *JAK2V617F* VAF exhibit increased intracellular signaling, cellular activation, and possible alterations in coagulation factors, thus contributing to the deregulation of hemostasis.

Furthermore, the results of the present study are in agreement with the results of Hu *et al* (16) who demonstrated that individuals with PV had a high *JAK2V617F* VAF (≥50%) compared with those with ET. In addition, the results of the present study demonstrated that patients from the state of Amazonas with a diagnosis of PV had a mutational landscape that was more complex than that of individuals with ET from

Table IV. Frequency and distribution of missenses variants in patients.

Variant	PV, n=38	ET, n=55	MF, n=4	P-value
rs2230723, n (%)	1 (2.6)	2 (3.6)	0	>0.9999
rs77375493, n (%)	25 (65.7)	21 (38.1)	2 (50)	0.0116[a]
rs907414891, n (%)	0	1 (1.8)	0	NA
rs41316003, n (%)	0	1 (1.8)	0	NA

[a]P<0.05. PV, polycythemia vera; ET, essential thrombocythemia; MF, myelofibrosis.

Table V. Frequency and distribution of variants in patients.

Number of variants	PV, n=38	ET, n=55	MF, n=4	P-value
1[c], n (%)	6 (15.7)	11 (19.6)	0	0.786
2[d], n (%)	7 (18.4)	22 (40.7)	0	0.0253[a]
3[e], n (%)	21 (55.2)	13 (23.6)	3	0.0023[b]

[a]P<0.05, [b]P<0.01. PV, polycythemia vera; ET, essential thrombocythemia; MF, myelofibrosis. [c]*rs2230724*; [d]*rs2230722/rs2230724;* [e]*rs2230722/rs77375493/rs2230724*.

Table VI. *JAK2V617F* variant allele frequency in patients with PV and ET.

Myeloproliferative neoplasm	VAF <50%, n (%)	VAF ≥50%, n (%)	P-value
PV, n=25	9 (36%)	16 (64%)	0.0477[a]
ET, n=21	17 (80.9)	4 (19)	<0.0001[b]

[a]P<0.05, [b]P<0.0001. PV, polycythemia vera; ET, essential thrombocythemia; VAF, variant allele frequency.

the same state. This landscape showed at least three mutations in concomitance in the *JAK2* gene, suggesting genomic instability and, subsequently, the instability of regulatory mechanisms at the protein level and possibly in the myeloproliferative phenotype of individuals with MPN.

According to data available on the ClinVar-NCBI website, a number of the acquired variants located in the extension of the *JAK2* coding region are either benign or of uncertain clinical significance. This indicates that most of the variants reported to date are in the FERM domains, kinase, and binding regions (19,22-24), and this finding relates to the present study, since the detected variants are located in the aforementioned regions.

Thus, it is highlighted that the presence of variants in the FERM domain may result in increased basal activity of JAK2 (52,53), which is a phenomenon that may explain the myeloproliferative phenotype in *JAK2V617F*-negative individuals who present with other variants in the *JAK2* gene, and could possibly be related to the clinical phenotype in the different subtypes of neoplasms, a phenomenon that is still not well understood. The present study identified the *rs907414891* variant, located in the FERM domain, which results in the exchange of isoleucine for valine at position 166 of the JAK2 protein (p. Ile166Val). Currently, this variant has no description in the literature regarding its clinical impact. However, the exclusive presence of *rs907414891*, *rs576746768*, *rs413160003*, and *rs55930140* in ET individuals may represent novel clonal biomarkers in ET. Nevertheless, it is necessary to perform additional molecular and functional tests to verify their possible association with MPN.

The SNV *rs2230722*, located in exon 6 of *JAK2*, was frequently observed in the present study and had a higher predominance in females, in agreement with Sokol *et al* (22). This variant was more frequent in women with platelet aggregation syndrome compared to men; and was significantly associated with deep vein thrombosis. As such, the variant could be correlated with the clinical picture of MPN, especially in individuals with thrombotic complications. The SNV *rs2230724*, a variant that is present in exon 19 of *JAK2*, was detected in the present study in the JH2-JH1 linker region. Although variants in this region are not frequently described in MPN, alterations in the JH1-JH2 interaction may generate dysregulation in the inhibition of catalytic activity and, therefore, alter its function. This SNV, together with *rs2230728,* are reported in hematologic cancers and associated with the progression to acute leukemia, especially in individuals older than 45 years old (23); and may thus serve as genetic markers of leukemic progression in MPN.

Table VII. Clinical data in individuals with PV according to the VAF of *JAK2V617F*.

	PV, n=25			ET, n=21		
Parameter	VAF <50%	VAF ≥50%	P-value	VAF <50%	VAF ≥50%	P-value
Thrombotic events, n (%)	1 (4.0)	2 (8.0)	0.5515	2 (9.5)	5 (23.8)	0.214
Hemorrhagic events, n (%)	1 (4.0)	0	0.3124	6 (28.5)	7 (33.3)	0.738
Splenomegaly, n (%)	0	7 (28.0)	0.0043[c]	1 (4.7)	3 (14.2)	0.293
RBC, x mm^3, median (IQR)	4.5 (4.05-5.6)	4.7 (3.9-5.7)	0.834	3.7 (3.2-4.4)	5.1 (4.7-6.5)	0.006[b]
Ht, %, median (IQR)	42.6 (40.1-49.1)	46.9 (44.4-51.0)	0.2325	40 (36.6-43.0)	47.0 (44.6-54.2)	0.0022[b]
Hb, g'dl, median (IQR)	14.5 (13.1-15.6)	14.9 (13.6-16.1)	0.7989	13.4 (12.0-13.8)	15.5 (14.6-16.7)	0.0023[b]
WBC, x mm^3, median (IQR)	6,615 (4,748-8,065)	6,860 (5,673-10,430)	0.343	5,760 (4,645-7,335)	5,320 (3,968-7,115)	0.6977
PLT, x mm^3, median (IQR)	310,500 (190,500-448,000)	373,500 (255,250-562,750)	0.3576	429,000 (365,500-491,500)	333,500 (167,250-458,500)	0.1718
LDH, U/l, median (IQR)	394.5 (331.1-623.6)	486.5 (421.4-564.4)	0.4523	387.1 (316.9-462.6)	412.1 (386.5-493.7)	0.517
Uric acid, mg/dl, median (IQR)	4.1 (3.4-5.4)	3.7 (2.7-5.1)	0.4077	3.8 (2.7-4.3)	4.0 (3.5-4.2)	0.682
PT (sec), median (IQR)	11.5 (10.8-11.7)	12.0 (11.2-12.8)	0.2576	11.1 (10.6-11.6)	13.0 (12.1-14.5)	0.0132[a]
INR, median (IQR)	0.98 (0.93-1.0)	1.03 (0.96-1.10)	0.2184	0.95 (0.91-1.00)	1.11 (1.04-1.25)	0.013[a]
aPTT (sec), median (IQR)	31.5 (28.2-35.6)	34.4 (31.5-37.5)	0.2076	28.4 (27.0-33.05)	38.8 (33.8-40.3)	0.0057[b]
Fibrinogen, mg/dl, median (IQR)	293.0 (209.8-365.0)	257.5 (225.5-283.0)	0.4438	315.0 (268.5-414.5)	224.5 (124.5-296.0)	0.0847

[a]P<0.05, [b]P<0.01 and [c]P<0.001. ET, essential thrombocythemia; VAF, variant allele frequency; RBC, red blood cell count; Ht, hematocrit; Hb, hemoglobin; WBC, white blood cell count; LDH, lactate dehydrogenase; PT, prothrombin time; INR, international normalized ratio; aPTT, activated partial thromboplastin time; IQR, interquartile range.

The coexistence of *JAK2* variants is not often described in MPN; however, this could have greater repercussions in the individual's clinical picture (50). In the present study, concomitance was observed in up to three variants, in the presence of *JAK2V617F*, and presented laboratory profiles with slight increases in cell counts, including red blood cells and platelet counts, which indicates that these variants may confer genomic instability and increase intracellular signaling of the JAK/STAT, PI3K, MAPK, NF-κB, and HIF1-α pathways, to induce tumorigenesis and facilitate the acquisition of other variants within the same gene (50,54).

Using Sanger sequencing, Lanikova *et al* (55) demonstrated the presence of SNV *rs2230723* in coexistence with *JAK2V617F* and, in this case, described normalized hematological counts after administration of hydroxyurea. In other experiments, both variants showed increased STAT1, STAT3, and STAT5 signaling, which suggested the potential of both variants in the predisposition to malignancies. Likewise, other variants in *JAK2* may confer weak constitutive signaling of the JAK/STAT pathway, resulting in a 'more attenuated' myeloproliferative phenotype, with slightly altered cell counts. However, further studies are needed to assess the functional behaviors of these variants, both individually and when combined.

Although the present study highlights the importance of detecting other variants in the entire coding region and the coexistence of variants in the same gene with possible repercussions on the clinical and laboratory status of individuals with MPN, it has several limitations. Among the primary limitations of this study is the small sample size due to the lack of patients from various centers. Future studies will aim to recruit a larger cohort from several centers to confirm the results. Here, only patients from the Hospital Foundation of Hematology and Hemotherapy of Amazon were included (a unique reference institution in the state of Amazonas for the diagnosis and treatment of hematological diseases). Another limitation is the lack of functional studies that confirm the myeloproliferative activity of these variants, the lack of allelic association of variants with outcomes, which may explain the possible predispositions for the development of MPN, and *JAK2* analysis was performed once along of the study. Likewise, the individuals included in the present study were treated with hydroxyurea and anagrelide, decreasing the probability of the detection of *JAK2V617F* mutations. The results also may be affected by the low sensitivity of Sanger sequencing.

In conclusion, individuals with negative *BCR::ABL1* MPN may present with more than one variant in the *JAK2* gene, in particular *rs2230722*, *rs2230724*, and *rs77375493* variants, both separately and together, and those with a high *JAK2V617F* VAF show alterations in the clinical-laboratory profiles compared with those with a low *JAK2V617F* VAF.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request. The GenBank accession nos. for the nucleotide sequences are ON706985 and ON706994.

Authors' contributions

AMT designed the study. DGT, GAVS, LPDSM and AMT prepared the manuscript and performed the literature search. LPDSM, AM, EVBA, MADS, WHL, JP, EA, DC, NAF, RA and LN acquired all the data. AGC, GAVS, AM, EVBA, MADS, WHL, JP, EA, DC, and AMT interpreted the data. AMT, AGC, GAVS, and DGT analyzed the data. AMT, AGC, NAF, RA, LN, and GAVS edited the manuscript. AMT, DGT, GAVS, LPDSM confirm the authenticity of all the data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was performed in accordance with the Declaration of Helsinki and Resolution 466/12 of the Brazilian Ministry of Health. The present study was approved by the National Ethics Committee, which is responsible for approving relevant human studies in Brazil (approval no. 4.450.813). Written informed consent was obtained from all subjects involved in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Barbui T, Thiele J, Gisslinger H, Kvasnicka HM, Vannucchi AM, Guglielmelli P, Orazi A and Tefferi A: The 2016 WHO classification and diagnostic criteria for myeloproliferative neoplasms: Document summary and in-depth discussion. Blood Cancer J 8: 15, 2018.
2. Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM, Bloomfield CD, Cazzola M and Vardiman JW: The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood 127: 2391-2405, 2016.
3. Lussana F and Rambaldi A: Inflammation and myeloproliferative neoplasms. J Autoimmun 85: 58-63, 2017.
4. Challen GA and Goodell MA: Clonal hematopoiesis: Mechanisms driving dominance of stem cell clones. Blood 136: 1590-1598, 2020.
5. Bousoik E and Montazeri Aliabadi H: 'Do we know jack' about JAK? A closer look at JAK/STAT signaling pathway. Front Oncol 8: 287, 2018.
6. Palumbo GA, Stella S, Pennisi MS, Pirosa C, Fermo E, Fabris S, Cattaneo D and Iurlo A: The role of new technologies in myeloproliferative neoplasms. Front Oncol 9: 321, 2019.
7. Campbell PJ and Green AR: The myeloproliferative disorders. N Engl J Med 355: 2452-2466, 2006.
8. Tefferi A: Myeloproliferative neoplasms: A decade of discoveries and treatment advances. Am J Hematol 91: 50-58, 2016.
9. Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S, Vassiliou GS, Bench AJ, Boyd EM, Curtin N, *et al*: Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet 365: 1054-1061, 2005.
10. Chen E and Mullally A: How does JAK2V617F contribute to the pathogenesis of myeloproliferative neoplasms? Hematology Am Soc Hematol Educ Program 2014: 268-276, 2014.
11. Hubbard SR: Mechanistic insights into regulation of JAK2 tyrosine kinase. Front Endocrinol (Lausanne) 8: 361, 2018.
12. Geay A, Aral B, Bourgeois V, Martin P, Airaud F, Garrec C, Bézieau S, Gardie B and Girodon F: Diagnosis of exon 12-positive polycythemia vera rescued by NGS. Clin Case Rep 8: 790-792, 2020.
13. Bader MS and Meyer SC: JAK2 in myeloproliferative neoplasms: Still a protagonist. Pharmaceuticals (Basel) 15: 160: 1-13, 2022.
14. Scott LM, Tong W, Levine RL, Scott MA, Beer PA, Stratton MR, Futreal PA, Erber WN, McMullin MF, Harrison CN, *et al*: JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. N Engl J Med 356: 459-468, 2007.
15. Kanduła Z, Janowski M, Więckowska B, Paczkowska E and Lewandowski K: JAK2V617F variant allele frequency, non-driver mutations, single-nucleotide variants and polycythemia vera outcome. J Cancer Res Clin Oncol 149: 4789-4803, 2023.
16. Hu L, Pu L, Ding Y, Li M, Cabanero M, Xie J, Zhou D, Yang D, Zhang C, Wang H, *et al*: Relationship between JAK2V617F mutation, allele burden and coagulation function in Ph-negative myeloproliferative neoplasms. Hematology 22: 354-360, 2017.
17. Arellano-Rodrigo E, Alvarez-Larrán A, Reverter JC, Colomer D, Villamor N, Bellosillo B and Cervantes F: Platelet turnover, coagulation factors, and soluble markers of platelet and endothelial activation in essential thrombocythemia: Relationship with thrombosis occurrence and JAK 2 V617F allele burden. Am J Hematol 84: 102-108, 2009.
18. Villanueva A, Poon KS, Gallardo CA, Chai CN, Chiu L, Yan B, Ding CSL, Yong KJ, Zhou J, Lee J, *et al*: A novel JAK2 R564* variant in a patient with thrombocytosis. Int J Lab Hematol 42: e38-e41, 2020.
19. Alghasham N, Alnouri Y, Abalkhail H and Khalil S: Detection of mutations in JAK2 exons 12-15 by Sanger sequencing. Int J Lab Hematol 38: 34-41, 2016.
20. Skov V: Next generation sequencing in MPNs. Lessons from the past and prospects for use as predictors of prognosis and treatment responses. Cancers (Basel) 12: 2194, 2020.
21. Loscocco GG, Guglielmelli P and Vannucchi AM: Impact of mutational profile on the management of myeloproliferative neoplasms: A short review of the emerging data. Onco Targets Ther 13: 12367-12382, 2020.
22. Sokol J, Skerenova M, Ivankova J, Simurda T and Stasko J: Association of genetic variability in selected genes in patients with deep vein thrombosis and platelet hyperaggregability. Clin Appl Thromb Hemost 24: 1027-1032, 2018.

23. Zhong Y, Wu J, Ma R, Cao H, Wang Z, Ding J, Cheng L, Feng J and Chen B: Association of Janus kinase 2 (JAK2) polymorphisms with acute leukemia susceptibility. Int J Lab Hematol 34: 248-253, 2012.
24. Yang L, Liu D, Liang S, Guo R, Zhang Z, Xu H, Yang C and Zhu Y: Janus kinase 2 polymorphisms are associated with risk in patients with gastric cancer in a Chinese population. PLoS One 8: e64628, 2013.
25. Maslah N, Verger E, Schlageter MH, Miclea JM, Kiladjian JJ, Giraudier S, Chomienne C and Cassinat B: Next-generation sequencing for JAK2 mutation testing: Advantages and pitfalls. Ann Hematol 98: 111-118, 2019.
26. De Carvalho TG, De Carvalho AC, Maia DC, Ogawa JK, Carvalho AL and Vettore AL: Search for mutations in signaling pathways in head and neck squamous cell carcinoma. Oncol Rep 30: 334-340, 2013.
27. Silva GA, Ramasawmy R, Boechat AL, Morais AC, Carvalho BK, Sousa KB, Souza VC, Cunha MG, Barletta-Naveca RH, Santos MP and Naveca FG: Association of TNF-1031 C/C as a potential protection marker for leprosy development in Amazonas state patients, Brazil. Hum. Immunol 76: 137-141, 2015.
28. Lis JT: Fractionation of DNA fragments by polyethylene glycol induced precipitation. Methods Enzymol 65: 347-353, 1980.
29. Paithankar KR and Prasad KS: Precipitation of DNA by polyethylene glycol and ethanol. Nucleic Acids Res 19: 1346, 1991.
30. Macedo LC, Santos BC, Pagliarini-e-Silva S, Pagnano KB, Rodrigues C, Quintero FC, Ferreira ME, Baraldi EC, Ambrosio-Albuquerque EP, Sell AM and Visentainer JE: JAK2 46/1 haplotype is associated with JAK2 V617F-positive myeloproliferative neoplasms in Brazilian patients. Int J Lab Hematol 37: 654-660, 2015.
31. Macedo LC, de Cesare Quintero F, Pagliari-E-Silva S, Pagnano KB, Rodrigues C, de Alencar JB, Sell AM and Visentainer JE: Association of TNF polymorphisms with JAK2 (V617F) myeloproliferative neoplasms in Brazilian patients. Blood Cells Mol Dis 57: 54-57, 2016.
32. da Silva RR, Domingues Hatzlhofer BL, Machado CG, Lima AS, de Albuquerque DM, dos Santos MN, Fertrin KY, Costa FF, Araújo Ada S and Bezerra MA: JAK2 V617F mutation prevalence in myeloproliferative neoplasms in Pernambuco, Brazil. Genet Test Mol Biomarkers 16: 802-805, 2012.
33. Hasselbalch HC: Time for revival of the red blood cell count and red cell mass in the differential diagnosis between essential thrombocythemia and polycythemia vera? Haematologica 104: 2119-2125, 2019.
34. Langabeer SE: The role of a low erythropoietin level in the diagnosis of JAK2 exon 12-mutated polycythemia vera. Blood Cells Mol Dis 80: 102377, 2020.
35. Maslah N, Soret J, Dosquet C, Vercellino L, Belkhodja C, Schlageter MH, Cassinat B, Kiladjian JJ, Chomienne C, Giraudier S. Masked polycythemia vera: analysis of a single center cohort of 2480 red cell masses. Haematologica 105: e95-e97, 2020
36. Stefaniuk P, Szymczyk A and Podhorecka M: The neutrophil to lymphocyte and lymphocyte to monocyte ratios as new prognostic factors in hematological malignancies-a narrative review. Cancer Manag Res 12: 2961-2977, 2020.
37. Mulas O, Mola B, Madeddu C, Caocci G, Macciò A and Nasa GL: Prognostic role of cell blood count in chronic myeloid neoplasm and acute myeloid leukemia and its possible implications in hematopoietic stem cell transplantation. Diagnostics (Basel) 12: 2493, 2022.
38. Vannucchi AM, Masala G, Antonioli E, Chiara Susini M, Guglielmelli P, Pieri L, Maggi L, Caini S, Palli D, Bogani C, *et al*: Increased risk of lymphoid neoplasms in patients with Philadelphia chromosome-negative myeloproliferative neoplasms. Cancer Epidemiol. Biomarkers Prev 18: 2068-2073, 2009.
39. Garcia-Gisbert N, Camacho L, Fernández-Ibarrondo L, Fernández-Rodriguez C, Longarón R, Gibert J, Angona A, Andrade-Campos M, Salar A, Besses C and Bellosillo B: Analysis of saliva samples and cluster of differentiation 3 (CD3)+ lymphocytes as a source of germline DNA in myeloproliferative neoplasms. Br J Haematol 189: e204-e207, 2020.
40. Sunu C, Gunes AK, Akat GK, Kalpakci Y, Ceran F, Dagdas S and Ozet G: The evaluation of patients with essential thrombocythemia in terms of risk of thrombosis. Rev Assoc Med Bras (1992) 67: 385-389, 2021.
41. Yahouédéhou SCMA, da Guarda CC, Figueiredo CVB, Santiago RP, Carvalho SP, Fiuza LM, Ndidi US, Oliveira RM, Carvalho MOS, Nascimento VML, *et al*: Hydroxyurea alters hematological, biochemical and inflammatory biomarkers in Brazilian children with SCA: Investigating associations with βS haplotype and α-thalassemia. PLoS One 14: e0218040, 2019.
42. Davidson TM: The good, the bad, and the ugly. Arch Otolaryngol Head Neck Surg 123: 115, 1997.
43. Buxhofer-Ausch V, Steurer M, Sormann S, Schloegl E, Schimetta W, Gisslinger B, Ruckser R, Gastl G and Gisslinger H: Influence of platelet and white blood cell counts on major thrombosis-analysis from a patient registry in essential thrombocythemia. Eur J Haematol 97: 511-516, 2016.
44. Kaifie A, Kirschner M, Wolf D, Maintz C, Hänel M, Gattermann N, Gökkurt E, Platzbecker U, Hollburg W, Göthert JR, *et al*: Bleeding, thrombosis, and anticoagulation in myeloproliferative neoplasms (MPN): Analysis from the German SAL-MPN-registry. J Hematol Oncol 9: 18, 2016.
45. Matsuura S, Thompson CR, Belghasem ME, Bekendam RH, Piasecki A, Leiva O, Ray A, Italiano J, Yang M, Merill-Skoloff G, *et al*: Platelet dysfunction and thrombosis in $JAK2^{V617F}$-mutated primary myelofibrotic mice. Arterioscler Thromb Vasc Biol 40: e262-e272, 2020.
46. Accurso V, Santoro M, Mancuso S, Napolitano M, Carlisi M, Mattana M, Russo C, Di Stefano A, Sirocchi D and Siragusa S: The essential thrombocythemia in 2020: What we know and where we still have to dig deep. Clin Med Insights Blood Disord 13: 2634853520978210, 2020.
47. Chang YC, Lin HC, Chiang YH, Chen CG, Huang L, Wang WT, Cheng CC, Lin J, Chang YF, Chang MC, *et al*: Targeted next-generation sequencing identified novel mutations in triple-negative myeloproliferative neoplasms. Med Oncol 34: 83, 2017.
48. Milosevic Feenstra JD, Nivarthi H, Gisslinger H, Leroy E, Rumi E, Chachoua I, Bagienski K, Kubesova B, Pietra D, Gisslinger B, *et al*: Whole-exome sequencing identifies novel MPL and JAK2 mutations in triple-negative myeloproliferative neoplasms. Blood 127: 325-332, 2016.
49. Schulze S, Stengel R, Jaekel N, Wang SY, Franke GN, Roskos M, Schneider M, Niederwieser D and Al-Ali HK: Concomitant and noncanonical JAK2 and MPL mutations in JAK2V617F-and MPLW515 L-positive myelofibrosis. Genes Chromosom Cancer 58: 747-755, 2019.
50. Marty C, Saint-Martin C, Pecquet C, Grosjean S, Saliba J, Mouton C, Leroy E, Harutyunyan AS, Abgrall JF, Favier R, *et al*: Germ-line JAK2 mutations in the kinase domain are responsible for hereditary thrombocytosis and are resistant to JAK2 and HSP90 inhibitors. Blood 123: 1372-1383, 2014.
51. Etheridge SL, Cosgrove ME, Sangkhae V, Corbo LM, Roh ME, Seeliger MA, Chan EL and Hitchcock IS: A novel activating, germline JAK2 mutation, JAK2R564Q, causes familial essential thrombocytosis. Blood 123: 1059-1068, 2014.
52. Gou P, Zhang W and Giraudier S: Insights into the potential mechanisms of JAK2V617F somatic mutation contributing distinct phenotypes in myeloproliferative neoplasms. Int J Mol Sci 23: 1013, 2022.
53. Zhao L, Ma Y, Seemann J and Huang LJ: A regulating role of the JAK2 FERM domain in hyperactivation of JAK2(V617F). Biochem J 426: 91-98, 2010.
54. Kapralova K, Horvathova M, Pecquet C, Fialova Kucerova J, Pospisilova D, Leroy E, Kralova B, Milosevic Feenstra JD, Schischlik F, Kralovics R, *et al*: Cooperation of germ line JAK2 mutations E846D and R1063H in hereditary erythrocytosis with megakaryocytic atypia. Blood 128: 1418-1423, 2016.
55. Lanikova L, Babosova O, Swierczek S, Wang L, Wheeler DA, Divoky V, Korinek V, Prchal JT. Coexistence of gain-of-function *JAK2* germ line mutations with $JAK2^{V617F}$ in polycythemia vera. Blood 128: 2266-2270, 2016.