Effects of polymorphic DNA genes involved in BER and caspase pathways on the clinical outcome of myeloproliferative neoplasms under treatment with hydroxyurea

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Abstract. Several single nucleotide polymorphisms (SNPs) influencing DNA repair capacity and apoptotic status may confer genetic predisposition to Philadelphia-chromosome negative myeloproliferative neoplasms (PN-MPNs), and influence therapeutic response and the clinical course. In the present study, whether SNPs in genes involved in apoptosis and the base excision repair (BER) pathway was evaluated. In addition, some known risk factors in PN-MPNs that may influence survival and therapeutic response to hydroxyurea (HU) were analyzed, taking into account three items: Disease progression, predisposition to new non-myeloid neoplasms and thrombotic events. The present study involved a total of 133 Caucasian Portuguese PN-MPNs patients treated with HU, whereby 17 cases showed progression to myelofibrosis/leukemia, 11 developed new non-myeloid neoplasms and 22 presented with thrombotic events. Progression to secondary myelofibrosis/leukemia is influenced by exposure to cytoreductive agents, and caspase and BER polymorphisms {globally, *CASP8* 3'untranslated region [odds ratio (OR)=0.24; 95% confidence interval (CI), 0.08-0.69], *XRCC*1 Arg194Trp [OR=3.58; 95% CI, 0.98-13.01]; for essential thrombocythemia patients *CASP9* Arg173His [OR=11.27; 95% CI, 1.13-112.28], *APEX1* Asp148Glu [OR=0.28; 95% CI, 0.74-1.03], and *XRCC*1 Arg194Trp [OR=6.60; 95% CI, 1.60-27.06]}. Moreover, globally caspase and BER polymorphisms influenced the development of new nonmyeloid malignancies [*CASP8* Asp270His (OR=5.90; 95% CI, 1.42-24.62) and *XRCC*1 Arg399Gln (OR=0.27; 95% CI, 0.07-1.03)]. On the other hand, only the BER pathway had a role in the presence of thrombotic events [*XRCC1* Gln399Arg (OR=0.35; 95% CI, 0.14-0.88)]. *JAK2* mutation had no influence on these complications. Larger studies are required to confirm these results, and to provide conclusive evidence of association between these and other variants with PN-MPNs therapeutic response and clinical evolution. However, this study may allow the development of drugs more directly targeted to the pathophysiology of the disease, with high efficacy, fewer adverse effects, contributing to compliance of patients with treatments. The clinical indication for classical drugs, including HU, may be guided by variant genes, which may provide additional beneficial effects.

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Introduction

Polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) are included in Philadelphia-chromosome negative myeloproliferative neoplasms (PN-MPNs).

There is an intrinsic tendency of PN-MPNs to progress to acute myeloid leukemia (AML), in occasions preceded by a phase of secondary myelofibrosis or myelodysplastic syndromes (MDS) (1), and to concurrently present thrombo-hemorrhagic complications, to an extent that is not fully known (2,3) and with a life expectancy reduced compared with the general population (4,5).

It is well known that high doses of alkylating agents and combined cytoreductive treatments are associated with enhancement of malignant transformation in PN-MPNs (2,3), reported in the literature to occur in 5-10% of patients 10 years following the initial diagnosis, correlated with a bad prognosis (6,7).

Moreover, there are other determinant factors not related to therapy that cannot be ruled out, affecting the clinical course of these disorders and the response to therapy, namely mutational burden, polymorphic variants of several genes, ambient/dietary exposure and immune system (8-12).

Another important aspect concerns the tendency of development of new nonhematological and nonmyeloid neoplasms in myeloproliferative neoplasms (MPNs), with an incidence ratio of 1.2-1.4 and 3.4, respectively, when compared to the general population (3). There is evidence that this risk is higher when *JAK2* V617F mutation is present and other patient factors may be also involved, although the association with cytoreductive therapy is not so well established (3,13).

Reported thromboembolic complications range from 7 to 57% at diagnosis and rise to 41-91% during follow-up, with arterial thrombotic events being much more common than venous ones in both PV and ET (14).

Thrombotic and hemorrhagic events are observed in up to 39 and 39.6% of PV patients, respectively (15). The cumulative rate of nonfatal thrombosis in PV is 3.8 events per 100 persons per year, and in ET the rate of fatal and nonfatal thrombotic events ranges from 2 to 4 events per 100 persons per year (14). PMF seems less susceptible for thrombotic events, with a cumulative percentage of 2.23 events per 100 persons per year. Age, previous thrombosis, leukocytosis and the presence of *JAK2* mutation are known risk factors for thrombosis occurrence in MPNs (14). Extreme thrombocytosis (count over 1,000 or $1,500x10^9$/l) is found to be related to hemorrhagic complications but not thrombosis, due to induced reduction of high-molecular-weight von Willebrand factor levels (14).

On the other hand, several single nucleotide polymorphisms (SNPs) at various loci, influencing DNA repair capacity and apoptotic status, and additional somatic genetic effects may confer genetic predisposition to PN-MPNs (16), influencing phenotype definition and determining therapeutic response (8,17-23). Moreover, despite the development of more efficient drugs in the last years, some patients with PN-MPNs still show disease progression to conditions more aggressive and difficult to treat (1,2). As a matter of fact, DNA damage induced to haematopoietic precursor cells would appear to be crucial for leukemic transformation, despite DNA repair systems act to repair the DNA lesions, thus maintaining genetic integrity (21,24,25). Several polymorphisms in DNA repair genes have been associated to protein dysfunction, compromising DNA damage repair (9,20,21,24,26).

Previous data described in the literature reported the identification and study of various SNPs in genes involved in the base excision repair (BER) pathway (*APEX1*, *MUTYH*, *OGG1*, *PARP1*, *PARP4* and *XRCC1*) for their association with progression to leukaemia and disease outcome (9,21,27) in patients with ET and PV. Likewise, there is also evidence that a nucleotide excision repair gene polymorphism is strongly associated with leukaemic transformation and development of non-myeloid malignancies in these disorders (3).

On the other hand, apoptosis is considered as the most important pathway of cell death, through regulatory proteins, the caspases, organized in both intrinsic and extrinsic pathways, acting as a defense mechanism against damaged, stressed, or stimulated cells by any agents, preventing accumulation of non-functional cells in the tissues.

The haematopoietic system is subject to a high cellular turnover rate, which makes it particularly sensitive to disturbance in the apoptosis process (28). Altered control of pro- and anti-apoptotic genes and in the relation with *JAK2* or *STAT5* signaling routes, seem to lead to myeloaccumulation and myeloproliferation, participating in the pathogenesis of MPNs (19,29-35).

Since the end of 60's, several groups as the American PV study Group (PVSG) and the Italian group for hematological diseases in adults (GIMEMA) devoted their efforts to the study of natural history of PV, ET, and PMF and the best treatment options (36).

Nowadays therapeutic goals in PV and ET are mostly directed to prevention of thrombo-hemorrhagic complications and relieve of symptoms, without curative potential nor capacity of prolonging life or preventing disease progression (15). Drug therapy towards PMF are intended especially to control symptoms and splenomegaly, with the possibility of remission in a limited number of patients undergoing allogeneic stem cell transplant (ASCT) (36,37).

According to present-day treatment algorithms, based on risk stratification (36), all the patients with PV require phlebotomies to a hematocrit target of <45%. In PV and ET patients it is very important to identify those whose risk of vascular and thrombotic complications is high enough to justify the use of risk-adapted treatment strategies, including aspirin, anticoagulants, hydroxyurea (HU), anagrelide, interferon alfa (IFN-alpha) or the recent JAK2 inhibitors (e.g., Ruxolitinib) (36). Patients are stratified and treatment is prescribed according to evaluation of the three major risk factors for thrombosis, namely previous history of arterial or venous thrombosis, possible presence of *JAK2* mutation and age >60 years (36,38). The risk of thrombosis in older *JAK2*-unmutated patients without thrombosis history is low enough to occasionally consider, on an individual basis, skipping cytoreductive therapy (36).

HU is widely used as first-line cytoreductive therapy in 'high risk' patients, as result of consensus of randomized clinical trials (37,38). This drug is an antimetabolite, capable of inhibition of ribonucleoside diphosphate reductase necessary for synthesis and repair of DNA, with a subsequent myelosuppressive activity (38). Moreover, HU affects polymorphonuclear leukocytes function and interferes in their interactions with platelets, having an antithrombotic effect (38). Several authors have shown that HU can also induce apoptosis in many types of cells (endothelial cells, human mesenchymal stem cells and mouse embryonic stem cells), promoting cell death by regulating the expression levels of Bcl-2 and the tumor suppressor p53 protein (38).

However, patients treated with HU can develop intolerance or become resistant to therapy, leading to an increased risk of death and progression to secondary myelofibrosis (15).

An alternative to first-line treatment is INF-alpha, which has antiproliferative effects on hematopoietic primordial cells, reduce *JAK2* V617F allele burden and induce cytogenetic remission, but it is also associated with unbearably adverse effects, leading to noncompliance and therapy discontinuation (15).

Second line cytoreductive therapy include busulfan or ^{32}P, but these agents have been associated with a very high propensity to leukemic transformation, being reserved to very specific situations (15).

The discovery of *JAK2* mutation led to the study and development of targeted agents with the propose of JAK2 inhibition (e.g., ruxolitinib), revealing better tolerability, and quality of life improvement and increased survival (15).

The present study was intended to characterize whether SNPs in caspase and BER genes might be relevant in PN-MPNs patients' survival and therapeutic response, concerning their role in disease progression and risk predisposition to new non-myeloid neoplasms and thrombotic events.

Materials and methods

Study subjects. The present study involved 133 Caucasian Portuguese patients diagnosed with PN-MPNs (80 with ET, 39 with PV and 14 with PMF), during the period of 1992-2016, for evaluation of the predisposition to fibrotic/leukemic progression, development of new primary non-myeloid neoplasms and thrombotic events. Among these patients, 104 were treated with isolated HU and 6 with HU in combination with other agents.

The study population included a total of 17 cases of ET/PV with fibrotic/leukemic (2^{ry} MF/AML) progression and 76 ET/PV patients who did not progress, 11 cases of ET/PV/PMF who developed new primary non-myeloid neoplasms and 30 patients who did not and 22 cases of ET/PV/PMF who presented thrombotic events and 56 patients who did not present this type of event.

For each case patient one to several control patients were selected from the group who did not present the event in question. Cases and controls were matched for the type of PN-MPN and duration of follow-up, ensuring that controls were followed at least for the same amount of time than the matched case, having the same chance to develop the event.

The patients were selected within the Portuguese population, with Portuguese ascendants, recruited in the Departments of Clinical Hematology and of Clinical Pathology, Hospital of São Francisco Xavier, West Lisbon Hospital Centre, a public general hospital that provides health care to the western population of Lisbon, where those patients were followed and treated. Diagnosis criteria for all patients were those updated by the World Health Organization (39,40) and all clinical, hematologic and treatment data were obtained from registries.

A written informed consent was obtained from all those involved, prior to blood withdrawal, in agreement with the Declaration of Helsinki. The blood samples were coded to guarantee anonymity. The present study was also conducted with approval by the institutional ethics' boards of the involved institutions (Hospital of São Francisco Xavier, West Lisbon Hospital Centre, reference no. 120/CE_2009 and NOVA Medical School/Faculty of Medical Sciences, Universidade Nova de Lisboa, reference number 34/2015/CEFCM). General characteristics for PN-MPNs patients at time of diagnosis and related to disease outcome are summarized in Table I.

DNA extraction, SNP selection and genotyping. SNP selection was previously reported for BER genes (16), for caspases genes (41) as well as for *JAK2* mutation (42). Peripheral blood samples (7-8 ml) of all patients and controls were collected and maintained thereafter at -80˚C until used. Genomic DNA was obtained from each blood sample (250 μl) using a commercially available kit (QIAamp® DNA mini kit (cat. no. 51306); Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. All DNA samples were stored at -20˚C until analysis.

Statistical analysis. The analysis of Hardy-Weinberg frequencies for all alleles in the control and patient populations was carried out using exact probability tests available in SNPStat website software (http://bioinfo.iconcologia.net/SNPstats). Differences in genotype frequency, gender, hematological values, therapeutic and pathology distributions, progression to secondary 2^{ry} MF/AML, development of a new primary nonmyeloid malignancy and thrombotic events occurrence distributions between PN-MPNs patient cases and controls were evaluated by the Chi-Square (χ^2) test. $P<0.05$ was considered to indicate a statistically significant difference.

Hazard ratios and 95% confidence intervals (95% CI) were estimated for each variable using the Cox univariate model. For the purpose of these calculations, the association between selected SNPs and their effect over 2^{ry} MF/AML progression, was evaluated using logistic regression conditional for the matched cases and controls. The covariates selected, including clinic-laboratorial data at the time of MPN diagnosis, *JAK2* mutational status and therapy related aspects (exposure to HU and/or other cytoreductive agents), were reported in the literature from previous studies as associated with disease progression (3,15).

Because there were few patients who received other therapy than HU, exposure to cytoreductive therapy was considered as 'no exposure' (including IFN or anagrelide isolated, as these drugs are not leukemogenic), 'HU only' and 'other agents alone or in combination'.

All analyses were performed using the IBM SPSS v.22.0 (IBM Corp., Armonk, NY, USA). Since this is not a conclusive final study but an exploratory one on the role of apoptosis and BER pathway polymorphisms and some known risk factors in PN-MPNs clinical outcome, and the data to be obtained should be looked at as proof of concept, the Bonferroni adjustment was deemed as not necessary as it is too conservative.

Results

General characteristics of patients and survival. This study included 133 PN-MPNs patients, whose general characteristics are summarized in Table I. According to diagnosis criteria patients' distribution was as follows: 80 (60.2%) with ET, 39 (29.3%) with PV and 14 (10.5%) with PMF.

Globally, the study included 72 (54.1%) females and 61 (45.9%) male patients, with an overall mean age of 68 years, in agreement with the gender distribution usually observed in this type of pathology, as it was already published in our previous work (42).

Mean values for laboratorial data at time of diagnosis for each disorder are listed in Table I, reflecting the different clinic-hematological pattern characteristic of each one of them. Haemoglobin was significantly higher in PV than in other groups and significantly lower in PMF than in other groups. Platelet count was higher in ET than in other groups.

Concerning the presence of *JAK2* V617F mutation, 72.5% of ET, 87.2% of PV and 50.0% of PMF patients were positive (Table I).

HU was the first choice drug in the majority of ET, PV, and PMF cases, used alone in 104 patients and in combination with other agents in 6 patients. Anagrelide and interferon-alpha were used in a minority of patients. Acetylsalicylic acid was used in approximately 35% of both PV and ET cases (Table I).

Table I. Characteristics of patients according to the type of MPN.

Characteristics	ET	PV	PMF
No. of patients	80	39	14
Sex, n/%			
Male	32 (40.0)	20 (51.3)	9 (64.3)
Female	48 (60.0)	19 (48.7)	5 (35.7)
Age, years[a]	66 (33-100)	69 (46-96)	73 (55-84)
Hb, g/l[a]	131 (85-168)	165 (129-213)	114 (74-151)
HTC	0.4 (0.261-0.498)	0.5 (0.429-0.660)	0.4 (0.218-0.491)
WBC, $x10^9$/l[a]	10.0 (2.7-26.6)	12.7 (4.5-33.5)	10.8 (2.2-17.7)
Platelets, $x10^9$/l[a]	777 (241-2485)	411 (164-1316)	232 (34-461)
JAK2 V617F mutation, n (%)			
Val/Val	21 (25.6)	5 (12.8)	7 (50.0)
Val/Phe	56 (70.9)	31 (79.5)	5 (35.7)
Phe/Phe	2 (2.5)	3 (7.7)	2 (14.3)
Exposure to cytoreductive agents, n (%)			
No exposure	8 (10.1)	5 (12.8)	5 (35.7)
HU only	65 (82.3)	33 (84.6)	6 (42.9)
HU+other agents	6 (7.6)	0 (0.0)	0 (0.0)
Other agents	0 (0.0)	1 (2.6)	3 (21.4)
Follow-up, years[a]	8.6 (1-25)	7.4 (1-17)	6.0 (1-15)
Death[b], n (%)	11 (45.8)	8 (33.3)	5 (20.8)
≤5 years	6 (50.0)	2 (16.7)	4 (33.3)
6-10 years	1 (16.7)	4 (66.7)	1 (16.7)
10-20 years	4 (66.7)	2 (33.3)	0 (0.0)

[a]Median (range); [b]P=0.040. MPN, myeloproliferative neoplasms; ET, essential thrombocythemia; PV, polycythemia vera; PMF, primary myelofibrosis; Hb, hemoglobin; HTC, hematocrit; WBC, white blood cells; HU, hydroxyurea.

Patients were followed up for a mean of 7.6 years. Myelofibrosis patients presented the shorter survival, with the majority of them dying less than five years after diagnosis, followed by PV (20.5%) and ET (13.8%) patients (Table I).

Survival is influenced by progression to 2^{ry}MF/AML and the presence of *JAK2* V617F mutation (Table II). None of the other evaluated factors evidenced to influence PN-MPNs survival.

The characteristics of each SNP under study were previously described and published (16,41,42), while the genotype frequencies and therapeutic distribution determined according to survival are shown in Table II.

Analysis of patients who progressed to 2^{ry} MF/AML. The study included a total of 17 patients with PV (8 patients) or ET (9 patients) who progressed to 2^{ry}MF/AML, corresponding to 20.5 and 11.2% of each disorder population respectively, and 76 controls who did not (Tables I and III).

The characteristics of these cases/controls patients are summarized in Table III.

Median follow-up time for diagnosis of progression in cases patients was 7.6 years, ranging from 1 to 18 years.

There was no significant difference in age among cases and controls patients (median of 67.7 years), but the first ones had higher WBC counts at presentation and displayed more frequently *JAK2* mutation. *JAK2* V617F mutation was also tested, but no association was found with the propensity of leukemic transformation (Table III). Regarding the number of individuals with marked thrombocytosis (>1,000$x10^9$/l), there is no significant difference among cases and controls patients (Table III).

Globally, there is an association with progression to 2^{ry} MF/AML and the exposure to cytoreductive agents (Table III). Twelve of the total patients who progressed to 2^{ry}MF/AML were medicated with HU only (Table III). The result obtained in the group of patients medicated with 'other agents' than HU is not statistically relevant because it represents typical statistical error type I. Genotypes distribution were found to be in Hardy-Weinberg equilibrium.

The presence of at least one variant allele carriers for *CASP8* 3'UTR variant is associated with a lower effect in disease progression to 2^{ry} MF/AML (OR=0.24; 95% CI, 0.08-0.69) and the presence of *XRCC*1 Arg194Trp variant showed a border-line effect (OR=3.58; 95% CI, 0.98-13.01) (Table III) suggesting a higher risk of developing 2^{ry} MF/AML, representing a worse prognosis.

When stratified for ET patients, the presence of at least one variant allele carriers for *CASP9* Arg173His polymorphisms is associated with a worse effect in progression to 2^{ry} MF/AML (OR=11.27; 95% CI, 1.13-112.28). While for BER

Table II. Potential factors that might influence patient's survival.

Factor	Alive (n=109)	Died: ≤5 years (n=12)	Died: 6-10 years (n=6)	Died: 10-20 years (n=6)	P-value[a]
Cases (n=17) with progression to 2ry MF/AML, n (%)	10 (58.8)	2 (11.8)	0 (0.0)	5 (29.4)	<0.001
Cases with non-myeloid neoplasms (n=11), n (%)	7 (63.6)	2 (18.2)	1 (9.1)	1 (9.1)	0.720
Cases with thrombotic events (n=22), n (%)	16 (72.7)	2 (9.1)	2 (9.1)	2 (9.1)	0.088
Exposure to cytoreductive agents, n (%)[b]					0.205
No exposure	13 (12.0)	2 (16.7)	3 (50.0)	0 (0.0)	
HU only	88 (81.5)	8 (66.7)	3 (50.0)	5 (83.3)	
HU+other agents	4 (3.7)	1 (8.3)	0 (0.0)	1 (16.7)	
Other agents	3 (2.8)	1 (8.3)	0 (0.0)	0 (0.0)	
JAK2 V617F mutation genotype, n (%)					0.009
Val/Val	26 (24.1)	5 (41.7)	2 (33.3)	0 (0.0)	
Val/Phe	79 (73.1)	6 (50.0)	2 (33.3)	5 (83.3)	
Phe/Phe	3 (2.8)	1 (8.3)	2 (33.3)	1 (16.7)	
Caspases SNPs					
CASP7 (Arg249Lys; rs2227309)					0.902
Arg/Arg	63 (57.8)	6 (50.0)	4 (66.7)	4 (66.7)	
Arg/Lys	40 (36.7)	6 (50.0)	2 (33.3)	2 (33.3)	
Lys/Lys	6 (5.5)	0 (0.0)	0 (0.0)	0 (0.0)	
CASP7 (Asp255Glu; rs2227310)					0.883
Asp/Asp	58 (53.7)	6 (50.0)	4 (66.7)	4 (66.7)	
Asp/Glu	43 (39.8)	6 (50.0)	2 (33.3)	2 (33.3)	
Glu/Glu	7 (6.5)	0 (0.0)	0 (0.0)	0 (0.0)	
CASP8 (3'UTR G>T; rs1035142)					0.668
G/G	41 (37.6)	5 (41.7)	3 (50.0)	1 (16.7)	
G/T	51 (46.8)	4 (33.3)	3 (50.0)	3 (50.0)	
T/T	17 (15.6)	3 (25.0)	0 (0.0)	2 (33.3)	
CASP8 (Asp270His; rs1045485)					0.570
Asp/Asp	82 (75.9)	9 (75.0)	5 (83.3)	5 (83.3)	
Asp/His	22 (20.4)	3 (25.0)	0 (0.0)	1 (16.7)	
His/His	4 (3.7)	0 (0.0)	1 (16.7)	0 (0.0)	
CASP9 (Arg173His; rs2308950)					0.989
Arg/Arg	105 (96.3)	12 (100.0)	6 (100.0)	6 (100.0)	
Arg/His	3 (2.8)	0 (0.0)	0 (0.0)	0 (0.0)	
His/His	1 (0.9)	0 (0.0)	0 (0.0)	0 (0.0)	
CASP9 (Phe136Phe; rs1132312)					0.902
TTC/TTC	25 (22.9)	3 (25.0)	2 (33.3)	1 (16.7)	
TTC/TTT	60 (55.0)	6 (50.0)	4 (66.7)	4 (66.7)	
TTT/TTT	24 (22.0)	3 (25.0)	0 (0.0)	1 (16.7)	
CASP9 (Ala28Val; rs1052571)					0.913
Ala/Ala	21 (19.3)	3 (25.0)	0 (0.0)	1 (16.7)	
Ala/Val	60 (55.0)	6 (50.0)	4 (66.7)	4 (66.7)	
Val/Val	28 (25.7)	3 (25.0)	2 (33.3)	1 (16.7)	
CASP10 (Ile522Leu; rs13006529)					0.611
Ile/Ile	29 (26.6)	6 (50.0)	1 (16.7)	2 (33.3)	
Ile/Leu	55 (50.5)	3 (25.0)	4 (66.7)	3 (50.0)	
Leu/Leu	25 (22.9)	3 (25.0)	1 (16.7)	1 (16.7)	
BER SNPs					
APEX1 (Asp148Glu; rs1130409)					0.141
Asp/Asp	28 (25.7)	2 (16.7)	3 (50.0)	4 (66.7)	
Asp/Glu	55 (50.5)	5 (41.7)	3 (50.0)	1 (16.7)	
Glu/Glu	26 (23.9)	5 (41.7)	0 (0.0)	1 (16.7)	

Table II. Continued.

Factor	Alive (n=109)	Died: ≤ 5 years (n=12)	Died: 6-10 years (n=6)	Died: 10-20 years (n=6)	P-value[a]
MUTYH (Gln335His; rs3219489)					0.381
His/His	59 (54.1)	6 (50.0)	1 (16.7)	2 (33.3)	
His/Gln	41 (37.6)	5 (41.7)	3 (50.0)	3 (50.0)	
Gln/Gln	9 (8.3)	1 (8.3)	2 (33.3)	1 (16.7)	
OGG1 (Ser326Cys; rs1052133)					0.837
Ser/Ser	67 (61.5)	8 (66.7)	5 (83.3)	3 (50.0)	
Ser/Cys	34 (31.2)	3 (25.0)	1 (16.7)	3 (50.0)	
Cys/Cys	8 (7.3)	1 (8.3)	0 (0.0)	0 (0.0)	
PARP1 (Val762Ala; rs1136410)					0.174
Val/Val	89 (81.7)	8 (66.7)	4 (66.7)	3 (50.0)	
Val/Ala	20 (18.3)	4 (33.3)	2 (33.3)	3 (50.0)	
Ala/Ala	-	-	-	-	
PARP4 (Gly1280Arg; rs13428)					0.506
Gly/Gly	45 (41.3)	4 (33.3)	4 (66.7)	1 (16.7)	
Gly/Arg	51 (46.8)	6 (50.0)	1 (16.7)	3 (50.0)	
Arg/Arg	13 (811.9)	2 (16.7)	1 (16.7)	2 (33.3)	
XRCC1 (Arg194Trp; rs1799782)					0.211
Arg/Arg	93 (85.3)	11 (91.7)	6 (100.0)	4 (66.7)	
Arg/Trp	7 (6.4)	1 (8.3)	0 (0.0)	2 (33.3)	
Trp/Trp	9 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)	
XRCC1 (Gln399Arg; rs25487)					0.292
Arg/Arg	38 (34.9)	6 (50.0)	5 (83.3)	3 (50.0)	
Arg/Gln	48 (44.0)	5 (41.7)	1 (16.7)	2 (33.3)	
Gln/Gln	23 (21.1)	1 (8.3)	0 (0.0)	1 (16.7)	

[a]P-value determined by χ^2 test. [b]Patients who received IFN or anagrelide as the only cytoreductive drugs were included in the 'no exposure' group. 2^{ry} MF, secondary myelofibrosis; AML, acute myeloid leukemia; HU, hydroxyurea; CASP, caspase; SNPs, single nucleotide polymorphisms; BER, base excision repair; APEX1, apurinic/apyrimidinic endonuclease; MUTYH, MutY DNA glycosylase earlier mutY homolog (*E. coli*); OGG1, 8-oxoguanine DNA glycosylase 1; PARP, poly (ADP-ribose) polymerase; XRCC1, X-ray repair cross-complementing 1.

polymorphisms the *APEX1* Asp148Glu showed a border-line effect related to a probable better prognosis concerning the progression to 2^{ry} MF/AML for patients who present at least one variant allele (OR=0.28; 95% CI, 0.74-1.03), an increased effect in disease progression was found for *XRCC*1 Arg194Trp variant (OR=6.60; 95% CI, 1.60-27.06), which might be related to a worse prognosis (Table IV). No significant change in OR was observed for any of the other genotypes considered.

Analysis of patients who developed new primary non-myeloid malignancies. The study included 11 ET/PV/PMF patients who developed a new primary nonmyeloid malignancy during follow-up and 30 control patients who did not (Table I). One of these case patients developed a lymphoid malignancy and the others developed solid organ new malignancies, the most affected being lung, thyroid, adrenal gland and digestive tract. The characteristics of these cases/controls patients are summarized in Table V. There is a predominance of male patients among cases, but there is no significant difference in age among cases and controls patients (median of 70.1 years) (Table V).

PMF patients present the highest incidence of new primary nonmyeloid malignancies (21.4% against 10.2 and 5.0% of PV and ET patients, respectively) (Table I). No significant association with exposure to cytoreductive agents or the presence of *JAK2* mutation was found (Table V). Globally, the presence of *CASP8* Asp270His variant allele is associated with an increased incidence to develop new non-myeloid malignancies (OR=5.90; 95% CI, 1.42-24.62), while the presence of at least one variant allele carriers for *XRCC*1 Arg399Gln (OR=0.27; 95% CI, 0.07-1.03) showed a border-line effect related to a decreased incidence to develop new non-myeloid malignancies (Table V). Genotypes distribution was found to be in Hardy-Weinberg equilibrium. No significant change in OR was observed for any of the other genotypes considered.

Analysis of patients who presented with thrombotic event. The study included 22 ET/PV/PMF patients who developed a thrombotic event and 56 patients who did not (Tables I and VI). Thrombotic events are slightly more common in PV patients (17.9%), followed by ET and PMF patients (Table I). In our studied population contrariwise, more than a half are ET patients (Table VI).

In the studied population, the most frequent types of major thrombosis observed in our patients included stroke,

Table III. Factors investigated for their association with fibrotic/leukemic progression in ET and PV: Distribution in case patients who progressed to 2ry MF/AML and control patients who did not.

Characteristics	Progression to 2ry MF/AML cases (n=17)	Non progression controls (n=76)	P-value[a]	OR[b] (95% CI)
ET or PV, n (%)			0.149	-
ET	9 (52.9)	54 (71.1)		
PV	8 (47.1)	22 (28.9)		
Sex, n (%)			0.636	1.25 (0.46-3.42)
Male	8 (47.1)	31 (40.8)		
Female	9 (52.9)	45 (59.2)		
Age, years[g]	67.3 (46-91)	68.0 (37-100)	0.231	1.01 (0.97-1.05)
Years to progression[g]	7.6 (1-18)	-	-	-
WBC[g]				
>10x10^9/l	9 (52.9)	33 (43.4)	-	-
>1 x10^9/l	4 (23.5)	12 (15.8)	-	-
PLT >1000x10^9/l	4 (23.5)	16 (21.1)	-	-
JAK2 V617F mutation, n (%)			0.716	
Val/Val	2 (11.8)	15 (19.7		1 (Reference))
Val/Phe	14 (82.4)	58 (76.3)		1.59 (0.36-7.09)
Phe/Phe	1 (5.9)	3 (3.9)		0.69 (0.15-18.29)
Exposure to cytoreductive agentes[h]			0.011	
No exposure	1 (5.9)	9 (11.8)		1 (Reference)
HU only	12 (70.6)	65 (85.5)		1.12 (0.14-8.82)
HU+other agents	3 (17.6)	2 (2.6)		6.04 (0.61-60.15)
Other agents	1 (5.9)	0 (0.0)		29.41 (1.64-528.37)[c]
Caspases SNPs				
CASP8 (3'UTR; rs1035142)			0.084	
G/G	10 (58.8)	23 (30.3)		1 (Reference)
G/T	5 (29.4)	39 (51.3)		0.36 (0.08-1.64)
T/T	2 (11.8)	14 (18.4)		0.19 (0.05-0.70)[d]
G/T + T/T	7 (41.2)	53 (69.7)		0.24 (0.08-0.69)[e]
BER SNPs				
APEX1 (Asp148Glu; rs1130409)			0.128	
Asp/Asp	6 (35.3)	17 (22.4)		1 (Reference)
Asp/Glu	5 (29.4)	43 (56.6)		0.33 (0.09-1.16)
Glu/Glu	6 (35.3)	16 (21.0)		1.12 (0.35-3.59)
Asp/Glu+Glu/Glu	11 (64.7)	59 (77.6)		0.55 (0.20-1.55)
XRCC1 (Arg194Trp; rs1799782)			0.099	
Arg/Arg	14 (82.4)	64 (84.2)		1 (Reference)
Arg/Trp	3 (17.6)	4 (5.3)		3.58 (0.98-13.01)[f]
Trp/Trp	0 (0.0)	8 (10.5)		ND
Arg/Trp+Trp/Trp	3 (17.6)	12 (15.8)		1.68 (0.47-6.06)

[a]P-value determined by χ^2 test. [b]P-value determined by conditional logistic regression. [c]P=0.022; [d]P=0.013; [e]P=0.009; [f]P=0.053 (borderline effect). [g]Data are presented as the median (range). [h]Patients who received IFN or anagrelide as the only cytoreductive drugs were included in the 'no exposure' group. ET, essential thrombocythemia; PV, polycythemia vera; 2ry MF, secondary myelofibrosis; AML, acute myeloid leukemia; OR, odds ratio; WBC, white blood cells; PLT, platelets; HU, hydroxyurea; CASP, caspase; BER, base excision repair; SNPs, single nucleotide polymorphisms; APEX1, apurinic/apyrimidinic endonuclease; XRCC1, X-ray repair cross-complementing 1; ND, none determined.

myocardial infarction, peripheral arterial thrombosis and deep vein thrombosis. There is no significant difference in age (median of 67.9 years) and platelets counts (difference in median values of 100x10^9/l, higher in control patients) between cases and controls patients (Table VI). No significant association with the presence of *JAK2* V617F mutation, nor

Table IV. Risk effect in progression to 2ry MF/AML after pathology stratification.

Pathology stratification	n	SNP progression to 2ry MF/AML (n=17)	OR (95% CI)	P-value
ET	9	*CASP9* (Arg173His; rs2308950)		
		Arg/Arg[a]	1 (Reference)	
		Arg/His	12.73 (1.30-124.41)	0.029
		Arg/His+His/His	11.27 (1.13-112.28)	0.039
		APEX1 (Asp148Glu; rs1130409)		
		Asp/Asp[a]	1 (Reference)	
		Asp/Glu	0.19 (0.04-1.0)	0.049
		Asp/Glu+Glu/Glu	0.28 (0.74-1.03)	0.055
		XRCC1_194 (Arg194Trp; rs1799782)		
		Arg/Arg[a]	1 (Reference)	
		Arg/Trp	6.60 (1.60-27.06)	0.009

[a]The genotype considered as reference class. 2ry MF, secondary myelofibrosis; AML, acute myeloid leukemia; SNPs, single nucleotide polymorphisms; OR, odds ratio; CI, confidence interval; ET, essential thrombocythemia; CASP, caspase; APEX1, apurinic/apyrimidinic endonuclease; XRCC1, X-ray repair cross-complementing 1.

the exposure to cytoreductive agents were found (Table VI). Globally, the thrombotic events where less frequent, revealing a decreased incidence, in patients with at least one variant allele carriers for *XRCC1* Gln399Arg (OR=0.35; 95% CI, 0.14-0.88) (Table VI). Genotypes distribution was found to be in Hardy-Weinberg equilibrium. No significant change in OR was observed for any of the other genotypes considered.

Discussion

This study was aimed to evaluate the general characteristics and clinical outcome of PN-MPNs in a Portuguese population, the majority of patients under therapeutic scheme with HU.

Median age of 68 years old at time of diagnosis and gender distribution are similar to what is usually described in the literature (14,43). Moreover, published data shows that ET is more common in women and PV more common in men (44,45), sustained in our study especially in the case of ET patients.

General laboratorial characteristics of the patients included in this study were in agreement with diagnostic WHO classification criteria (6,40). Hemoglobin, hematocrit and leukocytes' levels were higher in PV and platelet levels were higher in ET, and they were lower in PMF, as expected.

We found *JAK2* mutation in 87.2% of the PV group, 72.5% of the ET group, and 50.0% of PMF group. Although, the frequency of *JAK2* mutation in the PV and ET groups is similar to that reported by another author, from a work developed in Turkey (14), these results differ from what is described in the general literature (42). Regarding PMF group, the current results are in accordance with previous ones we had obtained (42).

Survival was highest in ET and lowest in PMF cases. However, the majority of deaths occurred through five years after diagnosis and involved ET and PMF patients, followed by PV patients six to ten years after diagnosis. According to literature, PV has a life expectancy of 10 to 20 years (4), ET patients ranges from 13 to 22.3 years (45,46), and PMF patients have a mean overall survival of 5.5 years (47).

Progression to 2ry MF/AML and the presence of *JAK2* V617F mutation shortened the survival significantly, consistent with the literature (14). However, according to our results development of new primary nonmyeloid malignancies and occurrence of thrombotic events did not influenced survival, a datum not in accordance with what is described in the literature (14), probably because of the dimension of the studied populations. Likewise, none of the caspase and BER pathway studied polymorphisms influenced survival.

It is known that prognosis of MPNs is determined by progression to secondary MF and AML, development on new primary nonmyeloid malignancies and thromboembolic and hemorrhagic complications, reflecting the impact that therapeutics and other inherited genetic factors may play in disease outcome (3,14).

Therapy should be directed towards preventing leukemic transformation in PN-MPNs. Progression to AML is a relatively rare complication, usually appearing late in the clinical course of ET and PV, requiring studies involving large cohorts of patients and extended follow-up periods (3). We here describe the development of a case control-study, comparing a population composed of ET and PV patients demonstrating fibrotic/leukemic progression with a control population including patients who did not progress, monitored for at least the same period of time, to investigate the role of clinical, genetic and therapy related factors potentially involved in fibrotic/leukemic transformation in PN-MPNs.

According to our results, 12.8% of all PN-MPNs patients progressed to 2ry MF/AML, with a mean time of 7.6 years. ET and PV revealed the same incidence of progression, but none of the PMF cases transformed to leukemia, similarly with other authors (14). Patients who developed leukemia were not significantly different from others by means of sex and age.

Leukemic transformation was influenced by the exposure to cytoreductive agents (3), but the mutagenic and carcinogenic potential of HU, through reduction of DNA repair, in PN-MPNs is controversial, with significant discrepancies among the several studies reported in the literature (3).

Table V. Factors investigated for their association with new nonmyeloid malignancy in ET, PV and PMF: Distribution in case patients who developed a new nonmyeloid cancer and control patients who did not.

Characteristics	Development of new nonmyeloid malignancy cases (n=11)	No development controls (n=30)	P-value[a]	OR[b] (95% CI)
ET/PV/PMF, n (%)			0.509	-
ET	4 (36.4)	17 (56.7)		
PV	4 (36.4)	7 (23.3)		
PMF	3 (27.3)	6 (20.0)		
Sex, n (%)			0.350	-
Male	8 (72.7)	17 (56.7)		
Female	3 (27.3)	13 (43.3)		
Age, years[e]	70.3 (60-84)	69.9 (45-100)	0.552	-
JAK2 V617F mutation, n (%)			0.027	
Val/Val	5 (45.5)	3 (10.0)		1 (Reference)
Val/Phe	6 (54.5)	23 (76.7)		0.62 (0.18-2.07)
Phe/Phe	0 (0.0)	4 (13.3)		ND
Exposure to cytoreductive agents[f]			0.649	
No exposure	1 (9.1)	5 (16.7)		
HU only	9 (81.8)	24 (80.0)		
Other agents alone or in combination	1 (9.1)	1 (3.3)		
CASP8 (Asp270His; rs1045485)			0.011	
Asp/Asp	6 (54.5)	25 (83.3)		1 (Reference)
Asp/His	2 (18.2)	5 (16.7)		0.90 (0.17-4.69)
His/His	3 (27.3)	0 (0.0)		5.90 (1.42-24.62)[c]
Asp/His+His/His	5 (45.5)	5 (16.7)		1.96 (0.59-6.54)
XRCC1 (Gln399Arg; rs25487)			0.047	
Arg/Arg	8 (72.7)	10 (33.3)		1 (Reference)
Arg/Gln	3 (27.3)	12 (40.0)		0.51 (0.13-1.95)
Gln/Gln	0 (0.0)	8 (26.7)		ND
Arg/Gln+Gln/Gln	3 (27.3)	20 (66.7)		0.27 (0.07-1.03)[d]

[a]P-value determined by χ^2 test. [b]P determined by conditional logistic regression. [c]P=0.015; [d]P=0.056. [e]Median (range). [f]Patients who received IFN or anagrelide as the only cytoreductive drugs were included in the 'no exposure' group. ET, essential thrombocythemia; PV, polycythemia vera; PMF, primary myelofibrosis; OR, odds ratio; CI, confidence interval; ND, none determined; HU, hydroxyurea; CASP, caspase; XRCC1, X-ray repair cross-complementing 1.

Our results revealed that previous treatment with HU was not found to be an influencing factor for leukemic transformation. Although not statistically significant in our results, it appears that leukocytosis and marked thrombocytosis could be involved in leukemic transformation, in agreement with the literature (3,4,15,45).

There is published evidence of the involvement of genetic polymorphisms and the susceptibility to leukemic progression (3), consistent with our results, in which an association with caspase and BER gene polymorphisms was found. Altogether, the presence of at least one variant allele in carriers for *CASP8* 3'UTR variant is associated with a lower effect in disease progression, and the presence of *XRCC*1 Arg194Trp variant showed a border-line effect, suggesting a higher effect associated with a worse prognosis for 2[ry] MF/AML development.

When stratified for ET patients, the presence of at least one variant allele carriers for *CASP9* Arg173His polymorphisms is associated with an increased effect and a worse prognosis. Regarding BER polymorphisms, *APEX1* Asp148Glu showed a border-line effect related to an ensuing better prognosis for the presence of at least one variant allele carriers. A worse prognosis in disease progression was found for *XRCC*1 Arg194Trp variant.

Development of new nonmyeloid malignancies was observed in 8.3% of all PN-MPNs patients, approximately equally distributed among ET, PV and PMF, with a male predominance. Regarding age, it appears that there is no significant difference among case and control patients.

The majority of cases corresponded to solid organ new malignancies, the most affected being lung, thyroid, adrenal gland and digestive tract, and one case developed a lymphoid malignancy.

The association of long-term HU use with development of nonmyeloid malignancies is controversial, with some authors

Table VI. Factors investigated for their association with thrombotic events in ET, PV and PMF: Distribution in case patients who developed a thrombotic event and control patients who did not.

Characteristics	Development of thrombotic event cases (n=22)	No development controls (n=56)	P-value[a]	OR[b] (95% CI)
ET/PV/PMF, n (%)			0.830	
ET	13 (59.1)	34 (60.7)		
PV	7 (31.8)	19 (33.9)		
PMF	2 (9.1)	3 (5.4)		
Sex, n (%)			0.196	
Male	13 (59.1)	24 (42.9)		
Female	9 (40.9)	32 (57.1)		
Age, years[e]	68.5 (45-88)	67.3 (54-96)	0.810	
Platelets, $x10^9/l$[e]	576.6 (134-1518)	676.9 (63-1473)	-	-
JAK2 V617 mutation, n (%)			0.945	
Val/Val	5 (22.7)	12 (21.4)		1 (Reference)
Val/Phe	15 (68.2)	40 (71.4)		1.03 (0.37-2.90)
Phe/Phe	2 (9.1)	4 (7.1)		0.70 (0.13-3.69)
Exposure to cytoreductive agents[f]			0.624	
No exposure	2 (9.1)	8 (14.3)		
HU only	17 (77.3)	45 (80.4)		
HU+other agents	2 (9.1)	2 (3.6)		
Other agents	1 (4.5)	1 (1.8)		
Caspases SNPs		No significant results		
BER SNPs				
XRCC1 (Gln399Arg; rs25487)			0.386	
Arg/Arg	10 (45.5)	19 (33.9)		1 (Reference)
Arg/Gln	10 (45.5)	25 (44.6)		0.40 (0.15-1.07)
Gln/Gln	2 (9.1)	12 (21.4)		0.22 (0.05-1.05)[c]
Arg/Gln+Gln/Gln				0.35 (0.14-0.88)[d]

[a]P-value determined by χ^2 test. [b]P determined by conditional logistic regression. [c]P=0.058; [d]P=0.025. [e]Median (range). [f]Patients who received IFN or anagrelide as the only cytoreductive drugs were included in the 'no exposure' group. ET, essential thrombocythemia; PV, polycythemia vera; PMF, primary myelofibrosis; OR, odds ratio; CI, confidence interval; SNPs, single nucleotide polymorphisms; CASP, caspase; XRCC1, X-ray repair cross-complementing 1.

reporting no association and others a weak relation (3,13). In fact, in the present study, no association was observed. According to previous studies (3), *JAK2* V617F mutation and caspase and BER polymorphisms constitute significant influencing factors for the occurrence of new primary nonmyeloid malignancies in PN-MPNs, conditioning these disorders clinical evolution. Our results revealed no association with the presence of *JAK2* mutation. However, globally, an increased incidence to develop new non-myeloid malignancies was found for the presence of *CASP8* Asp270His variant, while a border-line effect related to a decreased incidence to develop new non-myeloid malignancies was observed for the presence of at least one variant allele carriers for *XRCC*1 Arg399Gln.

Thrombotic complications were seen in 16.5% of all PN-MPNs patients in our study, the majority of them occurring during follow-up, with a similar incidence between PV, ET and PMF patients, slightly lower in the last group. In the literature, it is reported that about 41% of all deaths in PN-MPNs (1.5 deaths per 100 persons per year) were due to cardiovascular complications (48), with an increase of thromboembolic events during follow-up (ranging from 41 to 91%), in comparison with a variation from 7 to 57% at PN-MPN diagnosis, with fewer PMF patients being affected compared to other PN-MPNs (14). In our patients, arterial events were more frequent than venous, in agreement to what is described by other authors (14), including stroke, myocardial infarction, peripheral arterial thrombosis and deep vein thrombosis. Apparently, according to our results, the occurrence of this type of events is not related to age, sex, platelet counts, the presence of *JAK2* mutation, nor the exposure to cytoreductive agents, differing from the results previously published by some authors (14).

Leukocytosis and *JAK2* V617F allele burden have been identified as thrombotic risk factors and in the case of PV patients, leukocytosis at diagnosis has been considered to be associated with an increased risk to develop arterial

thrombosis and progress to acute leukemia, resulting in shorter survival (15).

In the present study, only BER pathway showed a role in the presence of thrombotic events, revealing a decreased incidence when at least one variant allele carriers for *XRCC1* Gln399Arg is present, making thrombotic episodes less frequent in these patients.

The caspases, a specific group of cysteine aspartate proteases, are a family of intracellular proteins involved in the initiation and execution of apoptosis processes, responsible for the dismantling and destruction of the cell (49,50). There are 14 different caspases and they can be classified as initiator, effector and cytokine activators (51). The initiators caspases (caspase-2, 8, 9, 10) activate the effectors caspases (caspase 3, 6, 7 and 14), which are capable of degrading direct multiple substrates leading to deregulation of vital cellular processes and cellular death (49,51-54), and also the cytokine activators caspases (caspase 1, 4, 5, 11, 12 and 13). Several proteins have been described that promote pro- or anti-apoptotic activity in the cell. The ratio of these pro-and anti-apoptotic proteins plays an important role in the regulation of cell death, and disruption in the balance between these proteins has been established to contribute to carcinogenesis by reducing apoptosis in malignant cells (35,55,56).

Several studies described modifications in the expression of molecules that participate in the regulation of intrinsic and extrinsic routes of apoptosis, as well as functional studies that showed resistance to apoptosis, indicating that the deregulation of apoptosis in MPNs is a mechanism involved in the pathophysiology and clinic-hematological outcome of these diseases (19,29,30).

Uphold of genetic integrity, through DNA repair mechanisms, is essential for preventing cellular damage and the development of leukaemia (16). Protein function and thus DNA damage repair may be affected by several polymorphisms in DNA repair genes, leading to susceptibility to malignancy (16). Previous reports have identified BER pathway polymorphisms associated solid tumors development (16), and a nucleotide excision repair gene polymorphism displayed strong association with leukaemic transformation and development of non-myeloid malignancies in ET and PV patients (3).

The BER pathway typically repairs a small region (1-13 nucleotides) around the damaged base, and several polymorphisms have been identified and studied for their association with progression to leukemia and disease outcome (16).

Previous studies performed by our group in which we tested the contribution of apoptosis and BER related genes to individual susceptibility to MPNs, also revealed involvement of the same family of polymorphisms (16) that were found to be associated with disease outcome as described in this present work.

HU, an antimetabolite, and anagrelide are the most commonly used drugs in the treatment of all PN-MPNs groups. The former was shown to reduce the incidence of thrombotic events in several studies, but there is some evidence that it may increase the risk of leukemic transformation (3,14). Anagrelide is effective in reducing platelet counts in ET and PV patients who are resistant or intolerant to HU. Increment of leukemia progression has not been shown for this drug (14).

HU is the initial choice of treatment because of its proven efficiency, especially in reducing thrombotic complications. However, HU is recommended to be used with caution in young patients regarding the data showing an increment of leukemia incidence in long-term usage of HU (14). In the ANAHYDRET study, it was shown that anagrelide is as effective as HU. Secondary leukemia has not been reported with anagrelide treatment yet. Interferon alpha was the least commonly used agent in all MPN groups, probably because of its parenteral usage and poor tolerability (14).

In the present study, we did not find any relation between the complications of MPN and the treatment options.

In summary, in the present study there is evidence of influence of fibrotic/leukemic transformation and the presence of *JAK2* mutation in PN-MPNs survival. Besides driver mutations, many other factors, such as apoptotic mechanisms and a set of proteins for DNA repair, are involved in therapeutic response and clinical outcome of PN-MPNs. Caspase (*CASP8* and *CASP9*) and BER pathway (*XRCC1* and *APEX1*) polymorphisms are associated with leukemic transformation and occurrence of new primary nonmyeloid malignancies, whereas only BER pathway (*XRCC1*) polymorphisms are associated with the presence of thrombotic events.

Apparently, therapeutics only influences the tendency to 2^{ry}MF/AML transformation.

Despite all the advances in the last few years, leading to the development of more targeted treatments, long-term effective and well-tolerated therapies are still lacking for both low and high risk patients (15).

Larger studies are required to confirm these results and to provide conclusive evidence of association between these and other variants and PN-MPNs and therapeutic response.

Identification of the main molecules that are altered in MPNs may have potential value as predictive biomarkers, contributing to pathophysiology clarification, identification of patients' subgroups and elaboration of tighter therapeutic strategies, which may allow to predict in advance what will be the potential response to a given therapeutic protocol, with high efficacy, fewer adverse effects, contributing to compliance of the patients with treatments and with foreseeable implications in survival increase.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

APA, SNS and JR contributed to the conception and design of the work, acquisition, analysis and interpretation of data; drafted and wrote the manuscript, revising it critically for important intellectual content; were accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work were appropriately investigated and resolved. AR and FL made substantial contributions to the acquisition of data, revised the manuscript and gave final approval of the manuscript version to be published. EJ contributed to acquisition of data, revised the manuscript and gave final approval of the manuscript version to be published. All authors approved the final manuscript.

Ethics approval and consent to participate

A written informed consent was obtained from all those involved, prior to blood withdrawal, in agreement with the Declaration of Helsinki. The blood samples were coded to guarantee anonymity. This study was also conducted with approval by the institutional ethics' boards of the involved institutions (Hospital of São Francisco Xavier, West Lisbon Hospital Centre, reference number 120/CE_2009 and NOVA Medical School/Faculdade de Ciências Médicas, Universidade Nova de Lisboa, reference number 34/2015/CEFCM).

Patient consent for publication

Patients and controls anonymity and consent were guaranteed, in agreement with the Declaration of Helsinki.

Competing interests

The authors declare that they have no competing interests.

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