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Bone marrow megakaryocytic activation predicts fibrotic evolution of Philadelphia-negative myeloproliferative neoplasms

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ABSTRACT

Philadelphia-negative chronic myeloproliferative neoplasms (MPN) have been traditionally considered as indistinctly slowly progressing conditions; recent evidence proves that a subset of cases have a rapid evolution, so that MPN prognosis needs to be personalized. We identified a new morphological parameter, defined as megakaryocytic activation (M-ACT) based on the coexistence of megakaryocytic emperipolesis, megakaryocytes (MK) cluster formation and evidence of arrangement of collagen fibers around the perimeter of MK. We retrospectively analyzed the bone marrow biopsy of two MPN cohorts of patients with polycythemia (PV) (n=64) and non-PV patients (including essential thrombocythemia, and early/prefibrotic primary myelofibrosis [PMF]) (n=222). M-ACT showed a significant correlation with splenomegaly, white blood cell count, and lactate dehydrogenase serum levels in both groups, with *JAK2* V617F allele burden in PV patients, and with *CALR* mutations, and platelet count in non-PV patients. Progression-free survival, defined as PV-to-secondary MF progression and non-PV-to-overt PMF, was worse in both PV and early/prefibrotic PMF patients with M-ACT in comparison to those without M-ACT ($P<0.0001$). Interestingly, M-ACT was not found in the subgroup of essential thrombocythemia patients. In conclusion, M-ACT can be helpful in the differential diagnosis of MPN and can represent a new morphologic parameter with a predictive value for progression of MPN.

Introduction

Philadelphia-negative chronic myeloproliferative neoplasms (MPN) represent a group of hematological disorders that originates from the neoplastic transformation of a pluripotent stem cell and are characterized by clonal proliferation of one or more hematopoietic progenitors in the bone marrow (BM) and in extramedullary sites.

According to the World Health Organization (WHO) 2017 classification, MPN can be divided into three main sets: polycythemia vera (PV), essential phrombocythemia (ET) and primary myelofibrosis (PMF), whose early stages' differential diagnosis is often challenging.[1]

While MPN have been traditionally considered as indistinct slow progressing conditions,[2,3] recent evidence, on the contrary, demonstrated that a subset of cases had a rapid evolution, leading different groups to develop several prognostic scores, mainly based on clinical and laboratory parameters with less emphasis on morphological, immunophenotypic and molecular data.[4] The first prognostic score was the International Prognostic Scoring System (IPSS), edited in 2009 by an international study group, which enabled survival estimation at the time of diagnosis primarily employing five clinical and hematologic parameters;[5] this model was further revised as the Dynamic International Prognostic Scoring System (DIPSS) and then as DIPSS-plus score.[6,7] The above, nonetheless, applied to already-established myelofibrosis (both PMF and post-PV/ET MF) only, determining survival from the time of disease progression/transformation to death without considering the heterogeneous disease history before the appearance of BM changes.[8]

On the basis of advances in MPN molecular profiling, in order to improve the prognostic prediction in PMF patients, novel models included *JAK2, CALR,* and *MPL* mutation status in addition to the IPSS parameters.[8] Moreover, novel insights were provided by in-depth analysis of genomic subsets with different clinical outcomes.[9] Recent publications have introduced new risk models for PMF, namely MIPSS70 (mutation-enhanced international prognostic scoring system for transplant-age patients),[10] MIPSS70+ version 2.0 (karyotype-enhanced MIPSS70) and GIPSS (genetically-inspired prognostic scoring system).[11,12] Similar risk models have been recently introduced for both ET and PV under the name of MIPSS-ET and MIPSS-PV, highlighting the prognostic contribution of spliceosome gene mutations.[13] However, all these predictive models do not consider morphological and phenotypical features, except BM fibrosis grade in the MIPPS70 model.

In this study we evaluated a new morphological parameter, defined by the coexistence of emperipolesis of megakaryocytes (MK) (i.e., the presence of an intact cell within the cytoplasm of another cell), MK clustering and peri-MK fibrosis in BM biopsy, which was named megakaryocytic activation (M-ACT). Larocca *et al*. in 2015 demonstrated that extensive BM emperipolesis associated to BM fibrosis was present in patients affected by gray platelet syndrome, with up to 65% MK containing two of four leukocytes engulfed within the cytoplasm;[14] a similar phenomenon has been described either in BM patients with PMF,[15] and in the BM of animal models of myelofibrosis.[16,17]

We demonstrated that M-ACT is a useful morphological parameter in forecasting both PV and early/prefibrotic PMF to myelofibrosis progression and could also help in the differential diagnosis between ET and early/prefibrotic PMF.[18]

Methods

Patients' features

Formalin-fixed, paraffin-embedded BM biopsy specimens, obtained from the posterior superior iliac spine,[19] were available in our Institute of Pathology for 460 patients clinically diagnosed with a MPN and followed at our Institute of Hematology (Fondazione Policlinico Universitario "A. Gemelli", IRCCS) from January 2005 to October 2019. The study was carried out in accordance with the Declaration of Helsinki and the consent for retrospective analysis of all clinical data, according to the Ethical Committee of the Università Cattolica del Sacro Cuore School of Medicine, and obtained by all the patients at the hospital admission. Patients were clinically followed-up over the observation time by one single team physician (VDS and ER as senior members).

All 286 cases were sorted until October 2019, according to three inclusion criteria: clinical diagnosis of either PV or non-PV MPN, first BM biopsy at diagnosis for non-PV cohort and within 0-24 months from the clinical diagnosis for PV cohort and no grade 2-3 BM fibrosis. Accordingly, patients with diagnosis of overt PMF or secondary myelofibrosis were excluded. Furthermore, BM biopsies were revised by two skilled pathologists (LMR and MM) and categorized according to the WHO 2017 criteria (PV, ET, early/prefibrotic PMF).

Clinical and hematological data (according to WHO 2017 criteria) were collected in order to trace lactose dehydrogenase (LDH) increase (i.e., LDH serum levels ≥250 UI/L), palpable splenomegaly, leukocytosis (i.e., white blood cell [WBC] count ≥ 11×10^9/L), high hemoglobin (Hg) level (i.e., Hgb >16 g/dL for women and Hgb > 16.5 g/dL for men) and thrombocytosis (i.e., PLT ≥ 450×10^9/L) for each patient at diagnosis. We also verified the occurrence of arterial/venous (A/V) thrombotic events and/or major bleeding events during the clinical course (until October 2019) for each case. Thrombotic and bleeding events were defined as previously described.[20] *JAK2* V617F mutation and allele burden analysis, *CALR* exon 9 mutations and *MPL* exon 10 mutations were performed as previously described.[20] Progression to secondary myelofibrosis was defined from the patient chart review and based on the International Working Group for Myelofibrosis Research and Treatment (IWG-MRT) consensus criteria.[21]

The main clinical, hematological and molecular characteristics of the 286 patients are shown in Table 1 for the PV cohort (64 patients), in Table 2 and the *Online Supplementary Table S1* for non-PV cohort (including 199 early/prefibrotic PMF patients [Table 2] and 23 ET patients [*Online Supplementary Table S1*]).

Bone marrow biopsy analysis and megakaryocytic activation histological parameters

All biopsy specimens had a suitable length (at least 1.5-2 cm) in order to obtain at least ten partially preserved intertrabecular areas, since subcortical medullary lacunae are less cellular than deep ones (especially in the elderly) and since focal pathologies can have a deep localization.[22] After collection, each biopsy specimen was kept in a properly-labeled clean container filled with 10% natural buffered formalin at pH 7.6 for 12 hours for fixation, was then decalcified with a Decalcifier II solution (Leica Biosystems, Milan, Italy) for 1 hour at room temperature, then fixed with 10% natural buffered formalin at pH 7.6 for 2 hours and finally embedded in paraffin. Sections (3-5 μm thick) were cut from each block for staining with hematoxylin and eosin (H&E) and Gordon&Sweet's silver staining to evaluate morphological features and fibrosis.[23,24] The specimens were concurrently examined and reviewed by two pathologists experienced in BM biopsy interpretation (LML and MM), who were blinded toward the patients' characteristics and survival. Cases with disagreement were discussed using a multiheaded microscope until agreement was achieved. The agreement indices (Cohen's K) between the two pathologists were very good: k=0.83 and k=0.85 for PV group and for non-PV group, respectively.

In the definition of M-ACT the following parameters were examined in detail (as shown in Figure 1): (i) MK emperipolesis, (ii) MK clustering and (iii) peri-MK fibrosis: i) MK emperipolesis was defined as the presence of one or more leukocyte or a precursor of hematopoiesis within the cytoplasm of at least 30% MK in the specimen; ii) MK clustering was defined as an aggregation of three or more megakaryocytes in close contact with each other and at least 25% of MK distributed in clusters in the specimen; iii) peri-MK fibrosis was defined as the arrangement of collagen fibers around the perimeter of the vast majority of MK, underlining their primary role in the genesis of fibrosis.

M-ACT positive patients showed the contemporary presence of all three parameters and M-ACT was evaluated only on the first BM biopsy at diagnosis and before any treatment.

Table 1. Correlation between megakaryocytic activation and the main polycythemia vera patient's clinical and molecular features.

PV	Overall (n=64)	M-ACT - (n=26)	M-ACT + (n=38)	HR [CI 95%]	P
Age[1]	59.1 (40-74)	59.3 (42-74)	58.8 (40-73)	1.00 [0.821 - 1.036]	1.00
Male	38 (59.4%)	15 (23.4%)	23 (35.9%)	1.01 [0.791 – 1.059]	1.00
Female	26 (40.6%)	11 (17.2%)	15 (23.4%)		
JAK2-RT > 50%	43 (67.2%)	16 (25.0%)	27 (42.2%)	1.24 [0.058 - 2.589]	0.059
Secondary MF-progression	41 (64.1%)	6 (9.4%)	32 (50%)	2.87 [0.332-1.591]	0.0001
Time-progression[2]	78.3 (26-116)	95.5 (72-116)	44.7 (26-48)	2.78 [0.036-2.589]	0.0001
Hgb[3]	18.7 (15.2-19.9)	16.2 (15.2-18.4)	17.1 (15.4-19.9)	1.31 [0.228-2.746]	0.06
LDH[4]	351.7 (210-522)	241.3 (210-380)	410.5 (238-522)	1.54 [0.025-3.532]	0.002
Palpable splenomegaly	36 (56.2%)	16 (25.0%)	20 (31.3%)	1.83 [0.179-3.795]	0.001
WBC[5]	11.3 (7.2-20.5)	9.8 (7.2-13.4)	15.6 (10.3-20.5)	1.81 [0.054-3.273]	0.001
PLT[6]	590.8 (380-851)	507.2 (380-623)	720.4 (430-851)	2.39 [0.082-2.629]	0.0001
A/V thrombosis	24 (37.5%)	8 (12.5%)	16 (25.0%)	1.02 [0.882 – 1.069]	0.43
Major bleeding	12 (18.7%)	1 (1.6%)	11 (17.2%)	1.54 [0.332-1.591]	0.06

JAK2-RT: *JAK2* V617F allele burden; Hgb: hemoglobin, serum levels; LDH: lactate dehydrogenase, serum levels; WBC: white blood cell count; PLT: platelet count; A/V: arterial/venous; HR: hazard ratio; CI: Confidence Interval. [1]In years; [2]In months; [3](g/dL); [4](UI/L); [5]($x10^9$/L); [6]($x10^9$/L).

Table 2. Correlation between megakaryocytic activation and the main early/prefibrotic primary myelofibrosis patient's clinical and molecular features.

Early/prefibrotic PMF	Overall (n=199)	M-ACT - (n=109)	M-ACT + (n=90)	HR [CI 95%]	P
Age[1]	66.2 (46-78)	64.2 (48-76)	66.3 (46-78)	1.01 [0.67078-1.036]	1.00
Male	91 (45.7%)	57 (28.6%)	34 (17.1%)	1.04 [0.872-1.055]	0.05
Female	108 (54.3%)	52 (26.7%)	56 (28.1%)		
JAK2-RT > 50%	37 (18.6%)	26 (13.1%)	11 (5.5%)	1.27 [0.073-2.879]	0.04
CALR mut.	60 (30.1%)	19 (9.5%)	41 (20.6%)	2.14 [0.301-1.902]	0.001
CALR type 1	46 (23.1%)	11 (5.5%)	35 (17.6%)	3.01 [1.527-3.812]	0.0001
CALR type 2	14 (7.0%)	8 (4.0%)	6 (3.0%)	0.97 [0.833-1.056]	1.00
MPL mut.	3 (1.5%)	2 (1.0%)	1 (0.5%)	1.00 [0.663-1.044]	1.00
Time-progression[2]	67.3 (15-109)	70.2 (53-109)	33.7 (15-56)	2.83 [0.328-1.913]	0.0001
Hgb[3]	15.3 (12.8-16.4)	14.8 (12.8-15.5)	15.2 (13.6-16.4)	1.04 [0.923-1.088]	0.43
LDH serum levels[4]	402.7 (205-612)	230.3 (205-390)	407.9 (288-612)	1.54 [0.045-3.235]	0.003
Palpable splenomegaly	111 (55.8%)	49 (24.6%)	62 (31.1%)	1.82 [0.1797-3.759]	0.001
WBC[5]	11.8 (7.5-18.7)	10.3 (7.5-14.8)	14.4 (9.9-18.7)	1.80 [0.045-3.733]	0.002
PLT[6]	620.1 (366-861)	520.6 (366-650)	710.3 (490-861)	2.46 [0.087-2.279]	0.0001
A/V thrombosis	74 (37.2%)	30 (15.1%)	44 (22.1%)	1.03 [0.243-1.912]	0.001
Major bleeding	30 (15.1%)	14 (7.0%)	16 (8.0%)	1.00 [0.928-1.079]	0.42

JAK2-RT: *JAK2* V617F allele burden; *CALR* mut: *CALR* exon 9 mutations (type 1 + type 2); MPL mut: *MPL* exon 10 mutations; Hgb: hemoglobin: serum levels; LDH: lactate dehydrogenase, serum levels; WBC: white blood cell count; PLT: platelet count; A/V: arterial/venous; HR: hazard ratio; CI: Confidence Interval. [1]In years; [2]In months; [3](g/dL); [4](UI/L); [5]($x10^9$/L); [6]($x10^9$/L).

Statistical analysis

Statistical analysis was performed using GraphPad-Prism 5 software (Graph Pad Software, San Diego, CA) and MedCalc version 10.2.0.0 (MedCalc Software, Mariakerke, Belgium).[25] Statistical comparison of continuous variables was performed by the Mann-Whitney U test (*t*-test), as appropriate. Comparison of categorical variables was performed by χ^2 statistic, using the Fisher's exact test. In order to evaluate the agreement between the two pathologists about the presence or absence of M-ACT in BM biopsies, the inter-rater agreement (Kappa) using MedCalc software was calulated.

The endpoint was progression-free survival (PFS), defined as the time between the first diagnosis and PV-to-secondary MF progression and early/prefibrotic PMF-to-overt PMF progression, respectively.

We followed the WHO 2017 criteria to establish the progression for PV-to-secondary MF and for early/prefibrotic PMF to overt myelofibrosis progression.[1]

Kaplan-Meier survival curves were plotted and differences in survival between groups of patients were compared using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards regression analysis including only those clinical and biological variables with a *P*-value of 0.10 or lower at the univariate analysis. *P*-values less than 0.05 were considered as statistically significant.

Results

Megakaryocytic activation in the polycythemia vera cohort

Twenty-six of the 64 PV did not meet histological criteria for M-ACT (40%), *versus* 38 who did (60%). In the PV cohort, M-ACT showed a significant correlation with one clinical parameter, i.e., palpable splenomegaly (*P*=0.001),

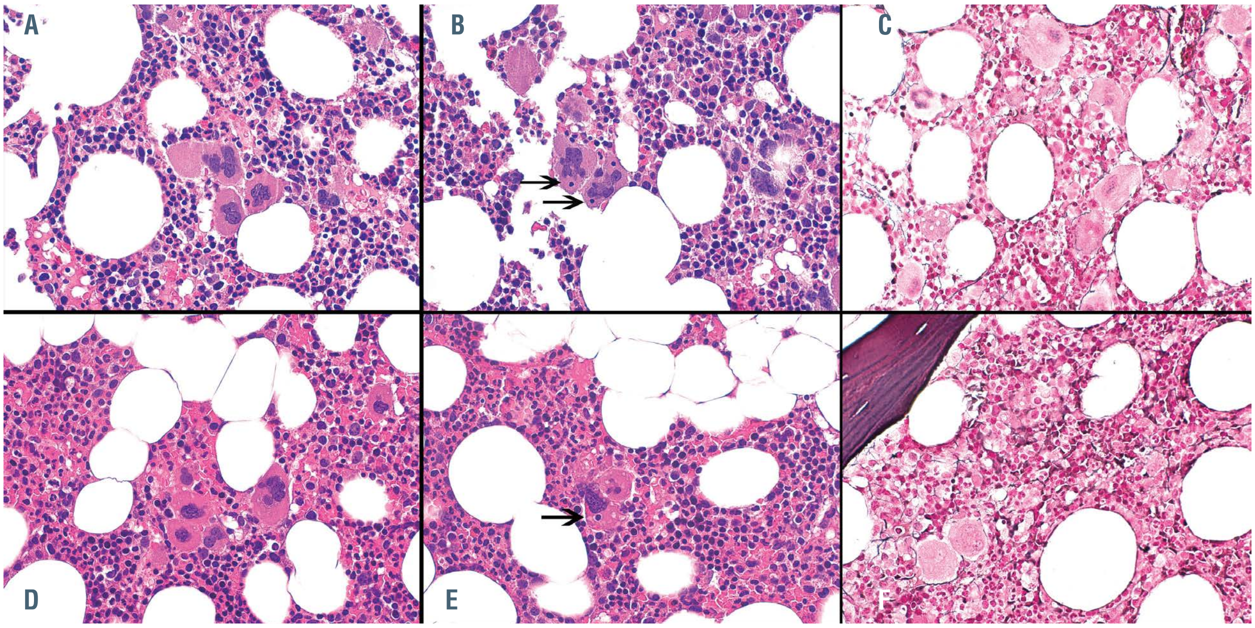

Figure 1. Representative images of megakaryocytic activation in polycythemia vera (A to C) and in early/prefibrotic primary myelofibrosis (D to F). (A and D) Emperipolesis, defined as the presence of at least one leukocyte (indicated by black arrows) or a precursor of hematopoiesis within the cytoplasm of a megakaryocyte (MK) (400X magnification); (B and E) MK clustering, defined as an aggregation of three or more MK in close contact with each other (200X and 400X magnification for panels B and E, respectively); (C and F) peri-MK fibrosis, defined as the arrangement of collagen fibers around the perimeter of activated MK (400X and 200X magnification for panel C and F, respectively).

Table 3. Polycythemia vera cohort: progression-free survival multivariate analysis.

Covariate	*b*	SE	Wald	*P*	Exp(b)	95% CI of Exp(b)
Major bleeding	-0.1562	0.3890	0.1614	0.6879	0.8554	0.3991 to 1.8333
JAK2 status	1.0726	0.4191	6.5500	0.0105	2.9229	1.2855 to 6.6460
M-ACT	2.3435	0.4761	24.2335	<0.0001	10.4180	4.0978 to 26.4858
WBC	-0.1520	0.3604	0.1780	0.6731	0.8590	0.4239 to 1.7407

b: coefficient estimates; SE: standard error for coefficient estimates b; Exp(b): hazard ratio value; 95% CI of Exp(b)= 95% Confidence Interval of hazard ratio. Major bleeding *vs.* no major bleeding; *JAK2* burden≥50% *vs. JAK2* burden<50% (*JAK2* status); megakaryocyte activation vs. no megakaryocyte activation (M-ACT); WBC≥11.0x10^9/L *vs.* WBC<11.0x10^9/L; WBC: whitel blood cell count.

Table 4. Early/prefibrotic primary myelofibrosis subset: progression-free survival multivariate analysis.

Covariate	*b*	SE	Wald	*P*	Exp(b)	95% CI of Exp(b)
WBC	0.4334	0.1879	5.3207	0.0211	1.5425	1.0673 to 2.2294
M-ACT	0.7659	0.1640	21.8235	<0.0001	2.1510	1.5598 to 2.9661
CALR status	0.3690	0.1685	4.7956	0.0285	1.4463	1.0395 to 2.0124
Sex	0.4071	0.1520	7.1702	0.0074	1.5024	1.1153 to 2.0240
LDH	0.1831	0.1749	1.0958	0.2952	1.2009	0.8524 to 1.6919
Palpable splenomegaly	0.3084	0.1746	3.1217	0.0773	1.3613	0.9668 to 1.9167
PLT	0.2415	0.1638	2.1729	0.1405	1.2731	0.9235 to 1.7551

b: coefficient estimates; SE: standard error for coefficient estimates b; Exp(b): hazard ratio value; 95% CI of Exp(b): 95% Confidence Interval of hazard ratio; WBC: white blood cell count; PLT: platelet count; LDH: lactose dehydrogenase; M-ACT: megakaryocyte activation. *CALR* mutations *vs.* no *CARL* wt (*CARL*); sex male *vs.* female (sex); megakaryocyte activation *vs.* no megakaryocyte activation (M-ACT); WBC count ≥11.0x10^9/L *vs.*: WBC<11.0x10^9/L; LDH≥250 UI/L *vs.* LDH<250 UI/L; palpable splenomegaly *vs.* no palpable splenomegaly; PLT≥450x10^9/L vs. PLT<450x10^9/L.

and with hematologic parameters, like platelet count (*P*=0.0001), LDH serum levels (*P*=0.002) and WBC count (*P*=0.001). On the other hand, no significant correlation was found between M-ACT and age (*P*=1.00), sex (*P*=1.00), A/V thrombosis (*P*=0.43), while major bleeding (*P*=0.06), Hgb level (*P*=0.06) and *JAK2* V617F burden>50% (*P*=0.059) showed a certain associative trend (Table 1).

We found that patients with M-ACT had a significant lower PFS than those without M-ACT (Table 1; Figure 2 panel A, for PFS: median PFS for M-ACT positive patients 58 months *vs.* median PFS for M-ACT negative patients 108 months, *P*<0.0001, hazard ratio [HR] 6.81, 95% Confidence Interval [CI]: 3.48-13.32). Moreover, *JAK2* V617F allele burden ≥50% and history of major bleeding had a significant correlation with a worse PFS (*P*=0.0225 and *P*=0.0174, respectively, *Online Supplementary Figure S1*), while WBC count>11x10^9/L showed a certain trend toward significance (*P*=0.0823, *Online Supplementary Figure S1*). Conversely, age (*P*=0.3718), sex (*P*=0.3645), LDH serum level (*P*=0.1305), PLT count (*P*=0.5643), Hgb level

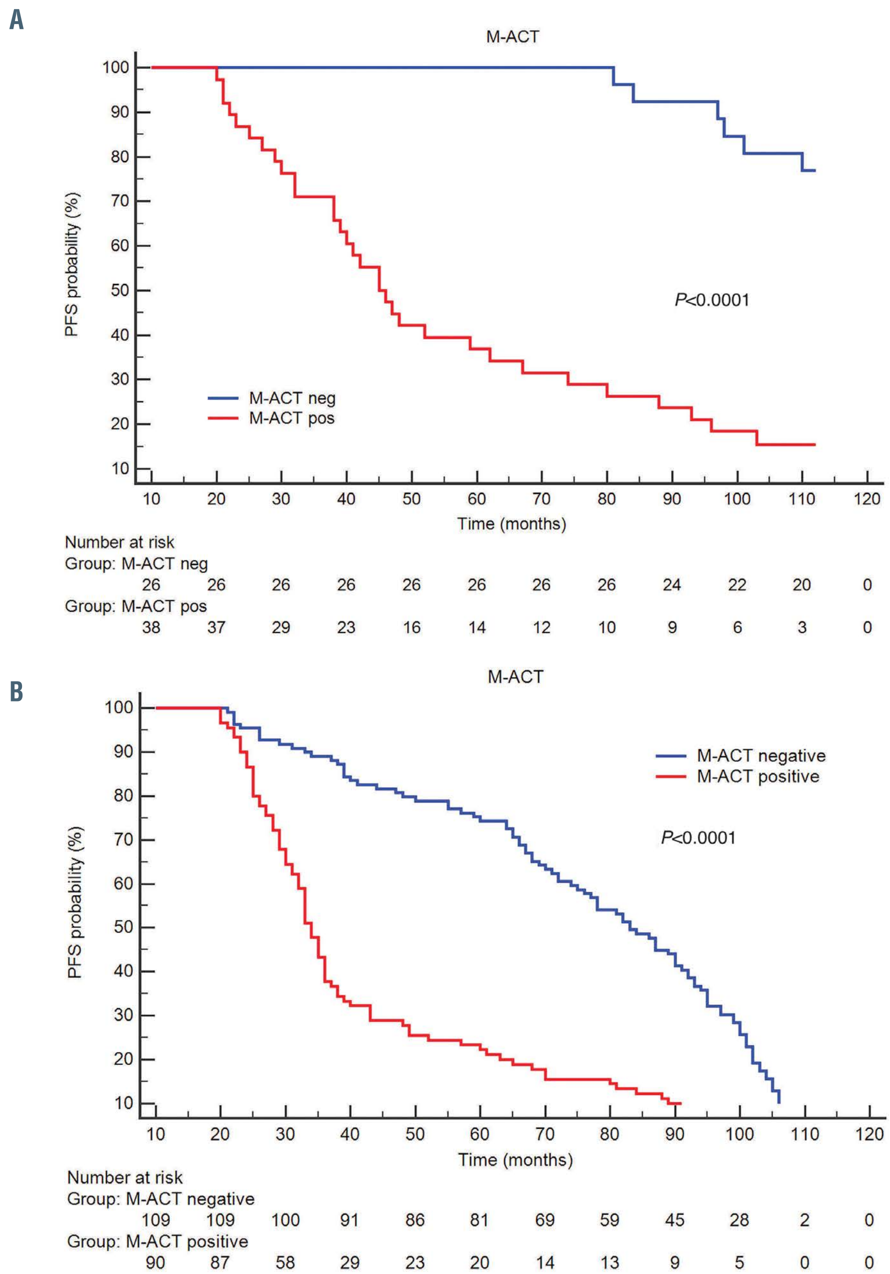

Figure 2. Kaplan-Meier curves for progression-free survival of polycythemia vera and of early/prefibrotic primary myelofibrosis patients. (A) Kaplan-Meier curve for progression-free survival (PFS) of polycythemia vera (PV) patients stratified by megakaryocytic activation (M-ACT); (B) Kaplan-Meier curve for PFS of early/prefibrotic primary myelofibrosis (PMF) patients by M-ACT. M-ACT positive patients (red-line) was significantly associated to a worse PFS (P<0.0001) with respect to those without M-ACT (blue-line) in both groups.

(P=0.1024) and A/V thrombosis (P=0.4216) did not show significant correlation with PFS.

Multivariate analysis of PFS, including M-ACT status, *JAK2* status, WBC count and history of major bleeding, showed that the presence of M-ACT and the *JAK2* V617F allele burden were the only significant predictors (for M-ACT status, P<0.0001, HR 10.4180, 95% CI: 4.0978-26.4858; for *JAK2* V617F allele burden ≥50%, P=0.0105, HR 0.0105, 95% CI: 1.2855-6.6460; Table 3).

Megakaryocytic activation in non-polycythemia vera myeloproliferative neoplasm cohort

One hundred and nine of 199 early/prefibrotic PMF patients did not meet histological criteria for M-ACT (55%) *versus* 90 who did (45%). In this cohort, M-ACT showed a strong correlation with clinical parameters, such as palpable splenomegaly (P=0.001) and history of major bleeding (P=0.001), and with hematologic parameters, like platelet count (P=0.0001), LDH serum levels (P=0.003), WBC count (P=0.002), presence of *CALR* mutations (P=0.001; Table 2). Notably, we found a significant association between M-ACT and *CALR* type 1 mutation (P=0.0001) while we did not find a significant correlation between M-ACT and *CALR* type 2 mutation (P=1.0). We found a significant yet milder correlation with sex, with M-ACT being more prevalent in females (P=0.05), and with *JAK2* V617F allele burden≥50% (P=0.04). On the contrary, no significant correlation was found between

M-ACT and age (P=1.00), Hgb level (P=0.43), *MPL* mutations (P=1.00) and A/V thrombosis (P=0.42; Table 1).

Similarly to what happened in the PV cohort, when we correlated M-ACT status with PFS, we found that patients with early/prefibrotic PMF and with M-ACT had a significant lower PFS than those without M-ACT (Table 1; Figure 2 panel B, for PFS: median PFS for M-ACT positive patients 44 months *vs.* median PFS for M-ACT negative patients 77 months, P<0.0001, HR 3.17, 95% CI: 2.27-4.44). Moreover, male sex, *CALR* type 1 mutations, WBC count >11x10^9/L, presence of palpable splenomegaly, PLT≥600x10^9/L and LDH ≥250 U/L had a significant correlation with a worse PFS (P=0.0187, P<0.0001, P<0.0001, P<0.0001, P<0.0001 and P=0.0025, respectively, *Online Supplementary Figure 2S*). Conversely, age (P=0.8831), major bleeding (P=0.7244), *JAK2* V617F allele burden≥ 50% (P=0.3459), Hgb level (P=0.5234), *MPL* mutations (P=0.2268) and A/V thrombosis (P=0.2003) did not show significant correlation with PFS.

Multivariate analysis of PFS, including M-ACT status, *CALR* status, WBC count, sex, LDH serous level, splenomegaly, and platelet count, showed that the presence of M-ACT and *CALR* type 1 mutation, WBC count >11x10^9/L and male sex were the significant predictors (for M-ACT status, P<0.0001, HR 2.1510, 95% CI: 1.5598-2.9661; for *CALR* status, P=0.0285, HR 1.446, 95% CI: 1.0395-2.0124; for WBC count, P=0.0211, HR 1.5425, 95% CI: 1.0673-2.2294; for sex, P=0.0074, HR 1.5024, 95% CI: 1.1153-2.0240; Table 4).

In the non-PV MPN cohort, we also analyzed a small subgroup of 23 ET patients. We did not find M-ACT in any of the ET BM biopsies performed at the time of the diagnosis. The ET patients had a better PFS in comparison to patients with early/prefibrotic PMF either with M-ACT (*Online Supplementary Figure S3*; P<0.0001) and without M-ACT (*Online Supplementary Figure S3*; P<0.0001).

Interestingly, we found that the incidence of M-ACT among triple-negative patients in the non-PV cohort was significantly lower in respect to patients with a driver gene mutation (21 of 85 [24.7%] triple negative patients *vs.* 90 of 222 [40.5%]; P=0.0115), and that triple-negative patients with M-ACT also had a significant lower PFS than those without M-ACT (median PFS for M-ACT positive triple-negative patients 56 months *vs.* median PFS for M-ACT negative triple-negative patients 79 months, P<0.0023, HR 2.76, 95% CI: 1.44-5.32; *data not shown*).

Discussion

For about two decades, one of the most important problems in the treatment of patients with MPN has been the identification of biological and non-biological factors that could represent a determinant key to the prediction of prognosis. Accordingly, several prognostic scores have succeeded over time, mainly based on clinical, hematological and molecular parameters, in identifying the fraction of MPN patients that could have a high risk of developing a leukemic transformation or a bone marrow fibrotic failure. However, none of these models take the morphological parameters into factual consideration, while these parameters play an important role in the diagnostic phase.

In this retrospective and single-center study, we propose a novel morphological parameter, defined as M-ACT, as a new possible predictive marker of fibrotic evolution among Philadelphia-negative MPN. Furthermore, this new parameter seems to be useful to supplement WHO 2017 classification criteria in the differential diagnosis of the MPN subtype between ET and early/prefibrotic PMF.

In our study, carried out on a large cohort of MPN BM biopsies at diagnosis, extensive evidence support this statement. In fact, in univariate analysis M-ACT correlates with relevant MPN clinical and hematologic parameters (see Table 1 and 2), such as palpable splenomegaly, WBC or PLT count, and LDH levels, but also with molecular profiles defined by the *JAK2* V617F allele burden and *CALR* mutations (especially the *CALR* type 1 mutation).

In PV patients the PFS was influenced at the multivariate analysis by the *JAK2* V617F allele burden >50%, as already reported;[26] in early/prefibrotic PMF patients' PFS was influenced by the presence of the *CALR* type 1 mutation, WBC count >11x10^9/L and male sex, in agreement with previous reports.[27]

Moreover, patients with M-ACT had a significant correlation with a worse PFS and with an overt-myelofibrotic BM failure, in both PV and early/prefibrotic PMF (P<0.0001). This last result is also confirmed at multivariate analysis. Interestingly, PV and early/prefibrotic PMF patients with this parameter showed a rapid clinical progression before the end of the 5-year follow-up, suggesting that M-ACT could be an early predictive marker capable of precociously identifying patients that need a closer follow-up.

Numerous scientific papers have highlighted that in the evolution towards myelofibrosis of MPN, a central role seems to be played by MK. Patients with MPN and fibrotic evolution showed a significantly increased number of MK with an abnormal nuclear/cytoplasmic ratio and a reduced polyploid state, often organized in clusters.[27,28] Experiments using *in vitro* cultures of CD34$^+$ hematopoietic stem cells of patients with fibrotic MPN have shown that MK expand excessively, are immature and show delayed apoptosis owing to increased expression of the anti-apoptotic factor BCL-XL.[29] Moreover, mice with a MK-specific deficiency of the transcription factor–encoding gene *GATA1* show elevated numbers of immature MK in the BM and an increased and pathologic neutrophil emperipolesis that may represent one of the mechanisms leading to myelofibrosis by releasing fibrogenic MK cytokines and neutrophil proteases in the microenvironment of *in vivo* experiments.[14,16] Finally, MK from individuals with PMF secrete increased levels of the fibrotic cytokines such as TGF-β, compared to MK from healthy individuals, and the extracellular matrix (ECM) microenvironment, especially the fibronectin component, is able to sustain progenitor cell proliferation and megakaryopoiesis in a TPO-independent manner.[16,30,31] These pro-fibrotic cytokines would presumably act mainly in the microenvironment near to those MK clusters which are, in turn, their main producers. Furthermore, the criteria defining the megakaryocytic activation could represent the morphological counterpart of what is postulated by *in vitro* and *in vivo* studies regarding the role of MK in the BM fibrotic evolution of patients with MPN.

Recent evidence has suggested that treating patients with early-stage MF may lead to better outcomes with a less severe splenomegaly, a lower incidence of cytopenia, and less-severe BM fibrosis. However, the argument is debated, especially considerating the adverse events of the *JAK2* inhibitor treatment (ruxolitinib). M-ACT parameter, as an early predictive marker capable of precociously identifying patients with an overt-myelofibrotic BM failure, could also select those patients that would benefit from precocious treatment.[32]

Our analysis not only supports the role of the MK and their activation in the evolution of PV and early/prefibrotic PMF, but also seems to suggest that, for the treatment of this neoplasia, as well as the inhibition of specific mutations, which may partially alter the natural history of the disease, the blockage of the fibrotic evolution and therefore of its main key-player, the MK, should be a future therapeutic strategy to be investigated.[30]

M-ACT is also a very useful morphological parameter in the diagnostic phase of MPN. In fact, none of the ET patients showed M-ACT, which when present, identifies only an early/prefibrotic PMF.

Interestingly the M-ACT showed a significant lower incidence in triple-negative patients in comparison to those with a driver gene mutation (24.7% *vs.* 40.5%) reinforcing the idea of a more indolent disease for this subgroup while maintaining its predictive role for the fibrotic evolution also in triple-negative patients.

In addition, M-ACT parameter evaluation represents an easily executable analysis with a high agreement index between pathologists. Moreover, the search for M-ACT in BM biopsies in the diagnostic phase of MPN patients can be performed widely without the need for further analysis such as immunohistochemistry or molecular analysis.

The main limitation of our study is the retrospective design, hence the time estimate of progression to overt myelofibrosis and the estimate of PFS based on the patient chart review can lack of accuracy. Moreover, the team physician remained the same over years and the criteria for the diagnosing progression to overt myelofibrosis were aligned with those of the IWG-MRT consensus.[21]

Although we analyzed a large cohort, the results of this single-center study need confirmation in other independent MPN patient cohorts, and M-ACT should be validated as a prognostic tool.

Disclosures

The authors have no conflict of interest to disclose. The study was carried out in accordance with the Declaration of Helsinki and the consent for retrospective analysis of all clinical data, according to the Ethical Committee of the Catholic University School of Medicine, was obtained by all the patients at the hospital admission. The report does not present identifying images or other personal or clinical details of participants that compromise anonymity.

Contributions

LML, MS, VF and MM were the principal authors and the main contributors in writing the manuscript; ER, SB, VR, MDC and PC analyzed and interpreted the patient data; MM, LML, MS and VF performed the biopsies analysis; LML and VDS read and corrected the manuscript. All authors read and approved the final manuscript.

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