



Journal of The Ferrata Storti Foundation

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Haematologica 2020 [Epub ahead of print]

Citation: Mattia Schino, Vincenzo Fiorentino, Elena Rossi, Silvia Betti, Monica Di Cecca, Valentina Ranucci, Patrizia Chiusolo, Maurizio Martini, Valerio De Stefano, and Luigi Maria Larocca. Bone marrow megakaryocytic activation predicts fibrotic evolution of Philadelphia-negative myeloproliferative neoplasms.

Haematologica. 2020; 105:xxx

doi:10.3324/haematol.2020.264143

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Article

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

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Running heads: Megakaryocytic activation and fibrotic evolution of MPNs

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Word count: Abstract: 201; Text: 3125; References: 32; Figure 2; Table 4; Supplementary Figures 3; Supplementary Table 1

Abstract

Philadelphia-negative chronic myeloproliferative neoplasms (MPNs) have been traditionally considered as indistinctly slowly progressing conditions; recent evidence proves that a subset of cases have a rapid evolution, so that MPNs' 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) clusters formation and evidence of arrangement of collagen fibers around the perimeter of MK. We retrospectively analyzed the bone marrow biopsy of two MPNs cohorts of patients with polycythemia (PV) (n=64) and non-PV patients [including essential thrombocythemia (ET), and early/prefibrotic primary myelofibrosis (PMF)] (n=222).

M-ACT showed a significant correlation with splenomegaly, white blood cell (WBC) count, and LDH 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 ($P < .0001$). Interestingly, M-ACT was not found in the subgroup of ET patients.

In conclusion, M-ACT can be helpful in the differential diagnosis of MPNs and can represent a new morphologic parameter with a predictive value for progression of MPNs.

Key words: MPNs, Megakaryocytic activation, PV, PMF

Introduction

Philadelphia-negative chronic myeloproliferative neoplasms (MPNs) represent a group of hematological disorders that originate 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 WHO 2017 classification, MPNs can be divided into three main sets: Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF), whose early stages' differential diagnosis is often challenging.¹

While MPNs 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 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 MPNs' 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 did not consider morphological and phenotypical features, except bone marrow (BM) fibrosis grade in the MIPSS70 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 bone marrow (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 2-4 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 Catholic University 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 (V.D.S. and E.R. as senior members).

All 286 cases were sorted out up 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 bone marrow fibrosis. Accordingly, patients with diagnosis of overt PMF or secondary myelofibrosis were excluded. Furthermore, BM biopsies were revised by two skilled pathologists (L.M.R. and M.M.) 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 LDH increase (i.e. LDH serum levels ≥ 250 UI/L), palpable splenomegaly, leukocytosis (i.e. WBC $\geq 11 \times 10^9$ /L), high hemoglobin level (i.e. Hgb > 16 g/dL for woman and Hgb > 16.5 g/dL for man) and thrombocytosis (i.e. PLT $\geq 450 \times 10^9$ /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 PV cohort (64 patients), in Table 2 and Table 1S for non-PV cohort [including 199 early/prefibrotic PMF patients (Table 2) and 23 ET patients (Table 1S)].

Bone marrow biopsy analysis and M-ACT histological parameters

All the 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-labelled clean container filled with 10% natural buffered formalin at pH 7.6 for 12 hours for fixation, 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 included in paraffin. Sections, 3-5 μm thick, were taken from each block for staining with 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 (L.M.L. and M.M.), 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-PVs group, respectively.

In the definition of M-ACT the following parameters were examined in detail (as shown in Figure 1): (1) MK emperipolesis, (2) MK clustering and (3) peri-MK fibrosis.

- 1) MK emperipolesis was defined as the presence of one or more leukocyte or a precursor of hematopoiesis within the cytoplasm of at least 30% MKs in the specimen.
- 2) MK clustering was defined as an aggregation of 3 or more megakaryocytes in close contact with each other and at least 25% of MKs are distributed in clusters in the specimen.

- 3) peri-MK fibrosis was used to define the arrangement of collagen fibers around the perimeter of the vast majority of MKs, 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.

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 chi-square statistic, using the Fisher's exact test. To evaluate the agreement between the two pathologists about the presence or absence of M-ACT in bone marrow biopsies, we calculated the inter-rater agreement (Kappa) using MedCalc software.

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 PV cohort

Twenty-six out of the 64 PVs did not meet histological criteria for M-ACT (40%), versus 38 who did (60%). In PV cohort, M-ACT showed a significant correlation with one clinical parameter, i.e. palpable splenomegaly ($P=.001$), and with hematologic parameters, like platelet count ($P=.0001$), LDH serum levels ($P=.002$) and WBC count ($P=.001$). On the other hand, no significant correlation was found between M-ACT and age ($P=1.00$), gender ($P=1.00$), A/V thrombosis ($P=.43$), while major bleeding ($P=.06$), Hgb level ($P=.06$) and JAK2V617F burden $>50\%$ ($P=.059$) showed a certain associative trend (Table 1).

We found that patients with M-ACT had a significant lower progression-free survival (PFS) than those without M-ACT (Table 1; Figure 2 panel A, for PFS: median PFS for M-ACT positive patients 58 months versus median PFS for M-ACT negative patients 108 months, $P<.0001$, HR 6.81, 95% CI from 3.48 to 13.32). Moreover, JAK2V617F allele burden $\geq 50\%$ and history of major bleeding had a significant correlation with a worse PFS ($P=.0225$ and $P=.0174$, respectively, Figure S1), while WBC count $>11 \times 10^9/l$ showed a certain trend toward significance ($P=.0823$, Figure S1). Conversely, age ($P=.3718$), gender ($P=.3645$), LDH serum level ($P=.1305$), PLT count ($P=.5643$), Hgb level ($P=.1024$) and A/V thrombosis ($P=.4216$) 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 JAK2V617F allele burden were the only significant predictors (for M-ACT status, $P<.0001$, HR 10.4180, 95% CI: from 4.0978 to 26.4858; for JAK2V617F allele burden $\geq 50\%$, $P=.0105$, HR 0.0105, 95% CI: from 1.2855 to 6.6460; Table 3).

Megakaryocytic activation in non-PV MPNs 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 ($P=.001$) and history of major bleeding ($P=.001$),

and with hematologic parameters, like platelet count (P=.0001), LDH serum levels (P=.003), WBC count (P=.002), presence of CALR mutations (P=.001; Table 2). Notably, we found a significant association between M-ACT and CALR type 1 mutation (P=.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 gender, being M-ACT more prevalent in females (P=.05), and with JAK2V617F allele burden \geq 50% (P=.04). On the contrary, no significant correlation was found between M-ACT and age (P=1.00), Hgb level (P=.43), MPL mutations (P=1.00) and A/V thrombosis (P=.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 versus median PFS for M-ACT negative patients 77 months, P<.0001, HR 3.17, 95% CI from 2.27 to 4.44). Moreover, male gender, CALR type 1 mutations, WBC count $>11\times 10^9/l$, presence of palpable splenomegaly, PLT $\geq 600\times 10^9/l$ and LDH ≥ 250 U/l had a significant correlation with a worse PFS (P=.0187, P<.0001, P<.0001, P<.0001, P<.0001 and P=.0025, respectively, Figure 2S). Conversely, age (P=.8831), major bleeding (P=.7244), JAK2 V617F allele burden \geq 50% (P=.3459), Hgb level (P=.5234), MPL mutations (P=.2268) and A/V thrombosis (P=.2003) did not show significant correlation with PFS.

Multivariate analysis of PFS, including M-ACT status, CALR status, WBC count, gender, LDH serum level, splenomegaly, and platelet count, showed that the presence of M-ACT and CALR type 1 mutation, WBC count $>11\times 10^9/l$ and male gender were the significant predictors (for M-ACT status, P<.0001, HR 2.1510, 95% CI: from 1.5598 to 2.9661; for CALR status, P=.0285, HR 1.446, 95% CI: from 1.0395 to 2.0124; for WBC count, P=.0211, HR 1.5425, 95% CI: from 1.0673 to 2.2294; for gender, P=.0074, HR 1.5024, 95% CI: from 1.1153 to 2.0240; Table 4).

In the non-PV MPNs 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 (Figure 3S; $P < .0001$) and without M-ACT (Figure 3S; $P < .0001$).

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 out of 85 (24.7%) triple negative patients *vs* 90 out of 222 (40.5%); $P = .0115$], 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 versus median PFS for M-ACT negative triple-negative patients 79 months, $P < .0023$, HR 2.76, 95% CI 1.44 to 5.32; data not shown).

Discussion

For about two decades, one of the most important problems in the treatment of patients with MPNs 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 order to identify the fraction of MPNs 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 monocentric study, we propose a novel morphological parameter, defined as M-ACT, as a new possible predictive marker of fibrotic evolution among Philadelphia-negative MPNs. 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 MPNs' bone marrow biopsies at diagnosis, extensive evidences 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 $\geq 50\%$, as already reported;²⁶ in early/prefibrotic PMF patients' PFS was influenced by the presence of the CALR type 1 mutation, WBC count $>11 \times 10^9/l$ and male gender, in agreement with previous reports.²⁷

Moreover, patients with M-ACT had a significant correlation with a worse PFS and with an overt-myelofibrotic bone marrow failure, in both PVs and early/prefibrotic PMF ($P < .0001$). 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 MPNs, a central role seems to be played by megakaryocytes. Patients with MPNs and fibrotic evolution showed a significant increased number of megakaryocytes 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 MPNs have shown that megakaryocytes expand excessively, are immature and show delayed apoptosis owing to increased expression of the anti-apoptotic factor Bcl-xL.²⁹ Moreover, mice with a megakaryocyte-specific deficiency of the transcription factor-encoding gene GATA1 show elevated numbers of immature megakaryocytes in the bone marrow 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, megakaryocytes from individuals with PMF secrete increased levels of the fibrotic cytokines such as TGF- β , compared to megakaryocytes 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 megakaryocyte 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 megakaryocytes in the bone marrow fibrotic evolution of patients with MPNs.

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 bone marrow fibrosis. However, the argument is debated, especially considering 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 bone marrow failure, could also select those patients that would benefit from precocious treatment.³²

Our analysis not only supports the role of the megakaryocytes and its 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 megakaryocyte, should be a future therapeutic strategy to be investigated.³⁰

M-ACT is also a very useful morphological parameter in the diagnostic phase of MPNs. 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 though 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 on the bone marrow 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, so that the estimate of the time of progression to overt myelofibrosis and the estimate of PFS based on the patient chart review can lack of accuracy. Moreover, the team physician was unchanged over years and the criteria for 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 monocentric study need confirmation in other independent MPN patient cohorts, and M-ACT should be validated as a prognostic tool.

List of abbreviations

MPNs: chronic myeloproliferative neoplasms

PV: Polycythemia Vera

ET: Essential Thrombocythemia

PMF: Primary Myelofibrosis

M-ACT: Megakaryocytic Activation

MK: megakaryocytes

PFS: progression free survival

OS: overall survival

Declarations

Ethics approval and consent to participate

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.

Acknowledgment

We thank Dr. Sara Capodimonti and Dr. Tonia Cenci for their technical support.

Founding

This study was supported by Università Cattolica del Sacro Cuore, Fondi d'Ateneo, Linea D1 (2018 and 2019; MM and LML).

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

L.M.L., M.S., V.F. and M.M. were the principal authors and the main contributors in writing the manuscript. E.R., S.B., V.R., M.D.C. and P.C. analyzed and interpreted the patient data. M.M., L.M.L., M.S. and V.F. performed the biopsies analysis. L.M.L. and V.D.S. read and corrected the manuscript. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table 1. Correlation between M-ACT and the main PV patient's clinical and molecular features.

PVs	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]	.059
Secondary MF-progression	41 (64.1%)	6 (9.4%)	32 (50%)	2.87 [0.332- 1.591]	.0001
Time-progression ²	78.3 (26-116)	95.5 (72-116)	44.7 (26-48)	2.78 [0.036- 2.589]	.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]	.06
LDH ⁴	351.7 (210-522)	241.3 (210- 380)	410.5 (238- 522)	1.54 [0.025- 3.532]	.002
Palpable splenomegaly	36 (56.2%)	16 (25.0%)	20 (31.3%)	1.83 [0.179- 3.795]	.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]	.001
PLT ⁶	590.8 (380-851)	507.2 (380- 623)	720.4 (430- 851)	2.39 [0.082- 2.629]	.0001
A/V thrombosis	24 (37.5%)	8 (12.5%)	16 (25.0%)	1.02 [0.882 – 1.069]	.43
Major bleeding	12 (18.7%)	1 (1.6%)	11 (17.2%)	1.54 [0.332- 1.591]	.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

¹In years

²In months

³(g/dL)

⁴(U/L)

⁵(x 10⁹ /L)

⁶(x 10⁹ /L)

Table 2. Correlation between M-ACT and the main early/prefibrotic PMF 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 [0.678-1.036]	1.00
Male	91 (45.7%)	57 (28.6%)	34 (17.1%)		
Female	108 (54.3%)	52 (26.7%)	56 (28.1%)	1.04 [0.872-1.055]	.05
JAK2-RT > 50%	37 (18.6%)	26 (13.1%)	11 (5.5%)	1.27 [0.073-2.879]	.04
CALR mut.	60 (30.1%)	19 (9.5%)	41 (20.6%)	2.14 [0.301-1.902]	.001
CALR type 1	46 (23.1%)	11 (5.5%)	35 (17.6%)	3.01 [1.527-3.812]	.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]	.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]	.43
LDH serum levels ⁴	402.7 (205-612)	230.3 (205-390)	407.9 (288-612)	1.54 [0.045-3.235]	.003
Palpable splenomegaly	111 (55.8%)	49 (24.6%)	62 (31.1%)	1.82 [0.1797-3.759]	.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]	.002
PLT ⁶	620.1 (366-861)	520.6 (366-650)	710.3 (490-861)	2.46 [0.087-2.279]	.0001
A/V thrombosis	74 (37.2%)	30 (15.1%)	44 (22.1%)	1.03 [0.243-1.912]	.001
Major bleeding	30 (15.1%)	14 (7.0%)	16 (8.0%)	1.00 [0.928-1.079]	.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]

¹In years

²In months

³(g/dL)

⁴(UI/L)

⁵(x 10⁹ /L)

⁶(x 10⁹ /L)

Table 3. PV cohort: PFS multivariate analysis.

<i>Covariate</i>	<i>b</i>	<i>SE</i>	<i>Wald</i>	<i>P</i>	<i>Exp(b)</i>	<i>95% CI of Exp(b)</i>
<i>Major bleeding</i>	-0.1562	0.3890	0.1614	0.6879	0.8554	0.3991 to 1.8333
<i>JAK2 status</i>	1.0726	0.4191	6.5500	0.0105	2.9229	1.2855 to 6.6460
<i>M-ACT</i>	2.3435	0.4761	24.2335	<0.0001	10.4180	4.0978 to 26.4858
<i>WBC</i>	-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 \geq 50% *vs* JAK2 burden<50% (JAK2 status); Megakaryocyte activation *vs* no megakaryocyte activation (M-ACT); WBC \geq 11.0x10⁹/l *vs* WBC<11.0x10⁹/l

Table 4. Early/prefibrotic PMF subset: PFS multivariate analysis.

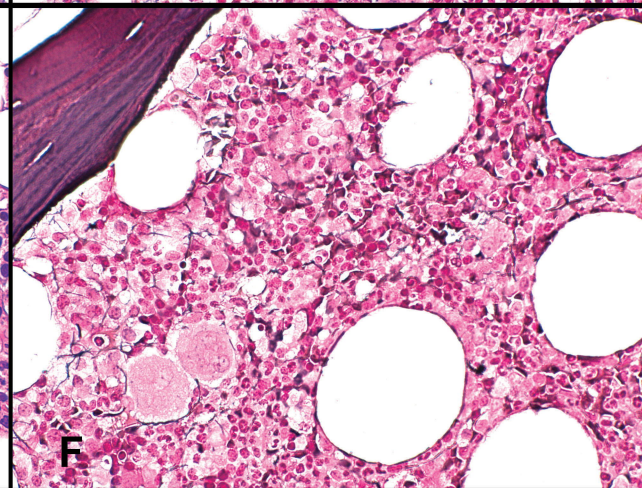
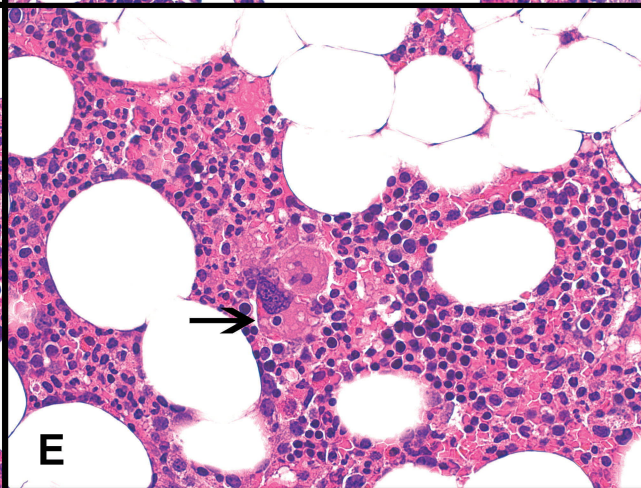
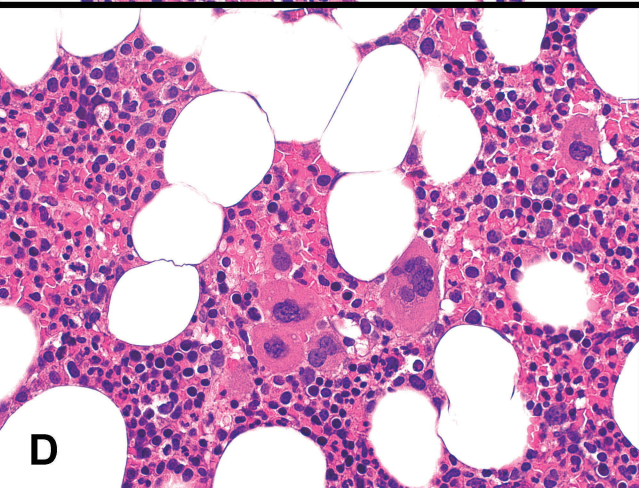
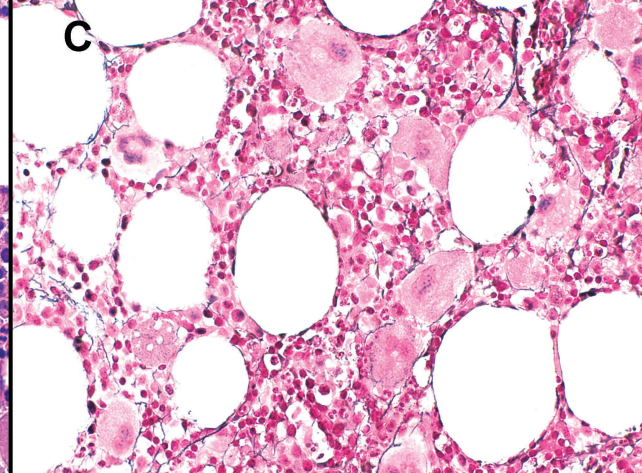
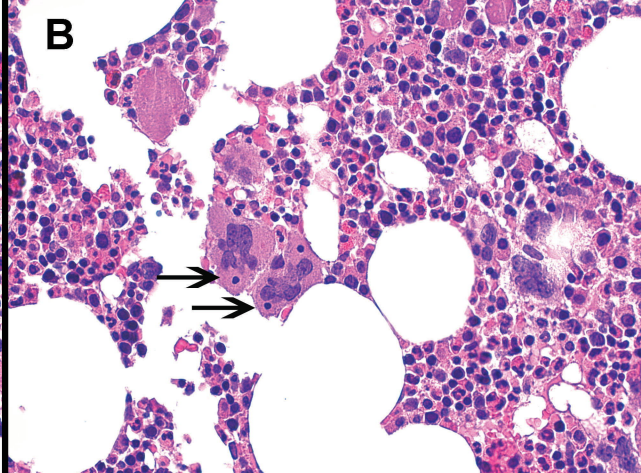
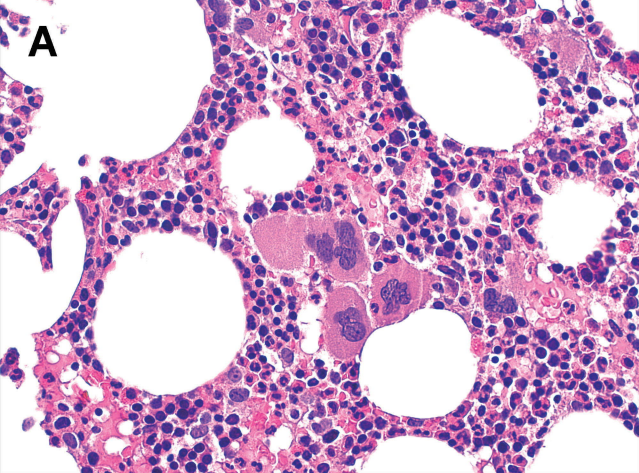
<i>Covariate</i>	<i>b</i>	<i>SE</i>	<i>Wald</i>	<i>P</i>	<i>Exp(b)</i>	<i>95% CI of Exp(b)</i>
<i>WBC</i>	0.4334	0.1879	5.3207	0.0211	1.5425	1.0673 to 2.2294
<i>M-ACT</i>	0.7659	0.1640	21.8235	<0.0001	2.1510	1.5598 to 2.9661
<i>CALR status</i>	0.3690	0.1685	4.7956	0.0285	1.4463	1.0395 to 2.0124
<i>Gender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

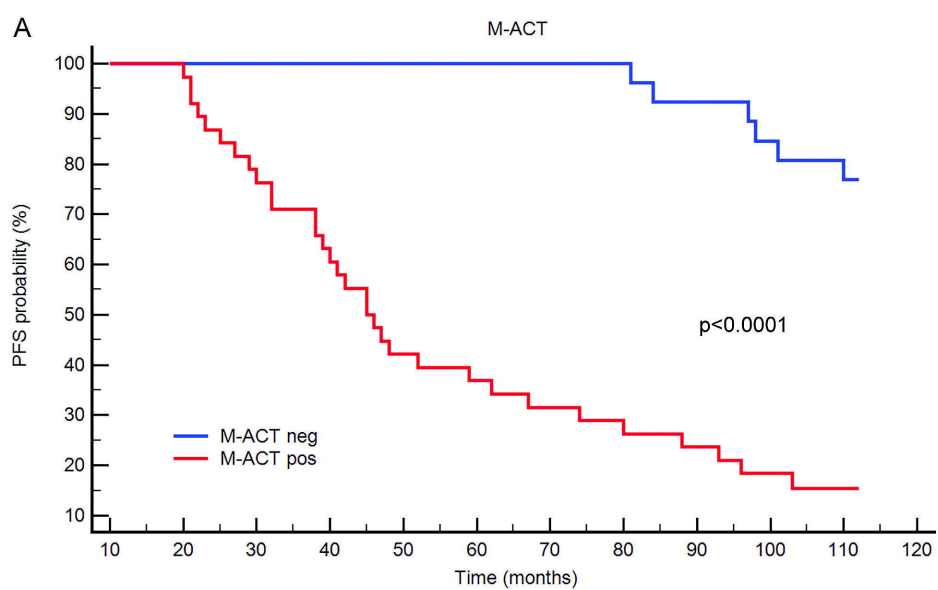
b= coefficient estimates; SE=standard error for coefficient estimates b; Exp(b)= Hazard Ratio value; 95% CI of Exp(b)= 95% confidence interval of Hazard Ratio. CALR mutations *vs* no CALR wt (CALR); Gender male *vs* Gender female (Gender); Megakaryocyte activation *vs* no megakaryocyte activation (M-ACT); WBC \geq 11.0x10⁹/l *vs* WBC<11.0x10⁹/l; LDH \geq 250 UI/l *vs* LDH<250 UI/l; Palpable splenomegaly *vs* no palpable splenomegaly; PLT \geq 450x10⁹/l *vs* PLT<450x10⁹/l.

Figure legend

Figure 1. Representative images of Megakaryocytic activation (M-ACT) in PV (panels A-C) and in early/prefibrotic PMF (panels D-F). Panels A and D: the panels show the emperipolesis, defined as the presence of at least one leukocyte (indicated by black arrows) or a precursor of hematopoiesis within the cytoplasm of a MK (400X magnification). Panels B and E: the panels show MK clustering, defined as an aggregation of 3 or more megakaryocytes in close contact with each other (200X and 400X magnification for panel B and E, respectively). Panels C and F: the panels show the peri-MK fibrosis, defined as the arrangement of collagen fibers around the perimeter of activated MKs (400X and 200X magnification for panel C and F, respectively).

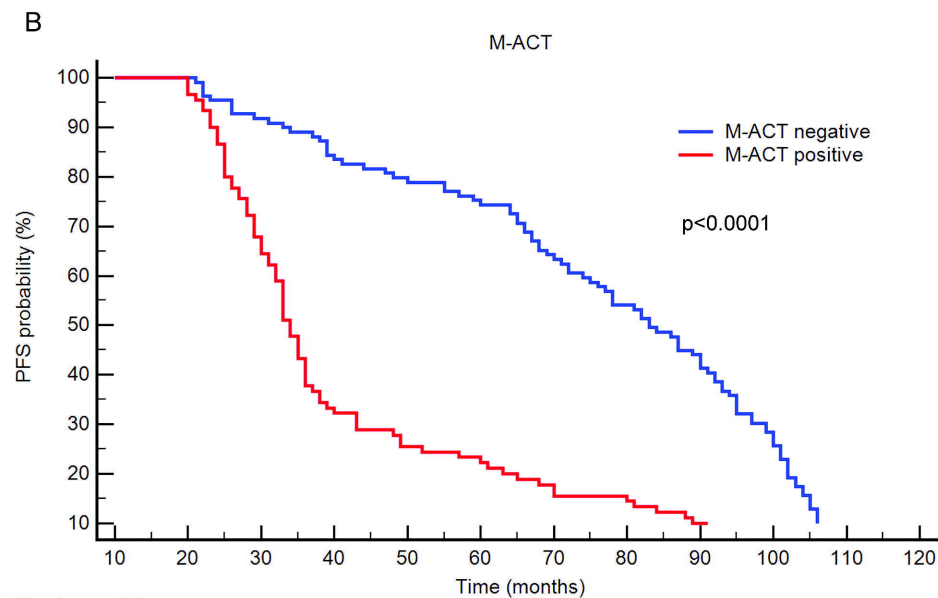
Figure 2. Kaplan-Meier curves for PFS of PV and of early/prefibrotic PMF patients. Panel A shows the Kaplan-Meier curve for PFS of PV patients stratified by M-ACT. Panel B shows the Kaplan-Meier curve for PFS of early/prefibrotic PMF patients by M-ACT. M-ACT positive patients (red-line) was significantly associated to a worse PFS ($p < 0.0001$) respect to those without M-ACT (blue-line) in both groups.





Number at risk

Time (months)	10	20	30	40	50	60	70	80	90	100	110	120
Group: M-ACT neg	26	26	26	26	26	26	26	26	24	22	20	0
Group: M-ACT pos	38	37	29	23	16	14	12	10	9	6	3	0



Number at risk

Time (months)	10	20	30	40	50	60	70	80	90	100	110	120
Group: M-ACT negative	109	109	100	91	86	81	69	59	45	28	2	0
Group: M-ACT positive	90	87	58	29	23	20	14	13	9	5	0	0

Supplemental data

Table 1S

ET	Overall (n=23)
Age ¹	61.4 (42-75)
Male	9 (39.1%)
Female	14 (60.9%)
JAK2-RT > 50%	7(30.4%)
CALR mut.	2 (8.7%)
CALR type 1	1 (4.3%)
CALR type 2	1 (4.3%)
MPL mut.	0 (0%)
Hgb ³	14.3 (13.8-15.7)
LDH serum levels ⁴	245.1 (180-322)
Palpable splenomegaly	4 (17.4%)
WBC ⁵	9.8(7.1-11.8)
PLT ⁶	615.3 (453-811)
A/V thrombosis	6 (26.1%)
Major bleeding	3 (13.0%)

¹In years

²In months

³(g/dL)

⁴(UI/L)

⁵(x 10⁹ /L)

⁶(x 10⁹ /L)

Supplementary figure legend

Figure 1S

Kaplan-Meier curves for PFS of PV patients stratified for JAK2 status, bleeding and WBC count. Patients with JAK2 burden $\geq 50\%$ and history of bleeding (red line) had a significant correlation with a worse PFS (for JAK2 status, $P=.0225$, HR 2.1274, 95% CI from 1.1124 to 4.0683; for bleeding, $P=.0174$, HR 2.9615, 95% CI from 1.2104 to 7.2458) respect to those with JAK2 burden $< 50\%$ and without history of bleeding (blue-line). Patients with WBC count $\geq 11 \times 10^9/l$ (red line) showed a certain trend toward significance with a worse PFS ($P=.0823$; HR 0.5171, 95% CI from 0.2458 to 1.0880) respect to those with WBC count $< 11 \times 10^9/l$ (blue-line).

Figure 2S

Kaplan-Meier curves for early/prefibrotic PMF stratified for CALR mutations (type 1 and type 2 mutation), PLT count, WBC count, splenomegaly, LDH level and gender. Patients with WBC count $\geq 11 \times 10^9/l$, CALR type 1 mutation, $PLT \geq 450 \times 10^9/l$, palpable splenomegaly, $LDH \geq 250 UI/l$ and female gender (red line) had a significant correlation with a worse PFS (for WBC count, $P < .0001$, HR 1.9458, 95% CI from 1.4395 to 2.6303; for PLT count, $P < .0001$, HR 1.9993, 95% CI from 1.4597 to 2.7384; for splenomegaly, $P < .0001$, HR 1.8993, 95% CI from 1.4132 to 2.5524; for LDH level, $P=.0025$, HR 1.5678, 95% CI from 1.1710 to 2.0991; for gender, $P=.0187$, HR 1.4125, 95% CI from 1.0592 to 1.8835; for CALR status; CALR type 1 mutation versus CALR wild type, $P < .0001$, HR 2.1476, 95% CI from 1.4040 to 3.2850) respect to those with WBC count $< 11 \times 10^9/l$, CALR wild-type, $PLT < 450 \times 10^9/l$, absence of palpable splenomegaly, $LDH < 250 UI/l$ and male gender (blue-line).

Figure 3S

Kaplan-Meier curves for PFS early/prefibrotic PMF and ET stratified for M-ACT parameter. ET patients that did not have a M-ACT in any case showed a significant correlation with a better PFS in comparison to both early/prefibrotic PMF with that without M-ACT ($P < .0001$).

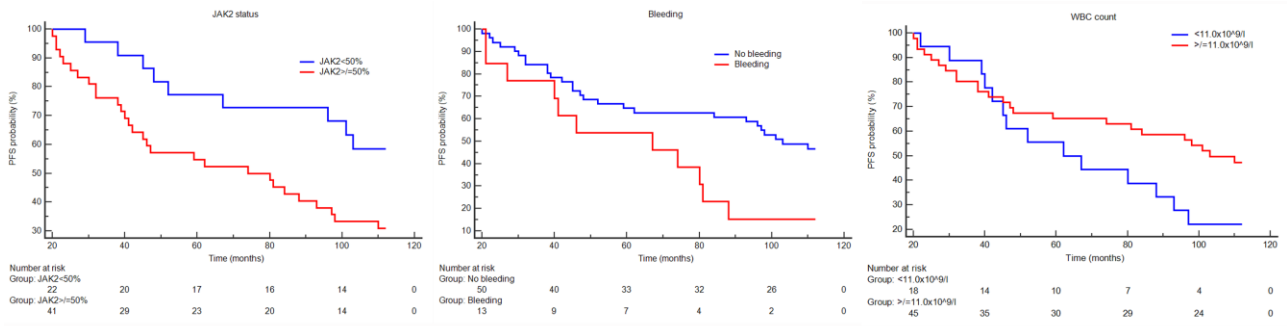


Figure 1S

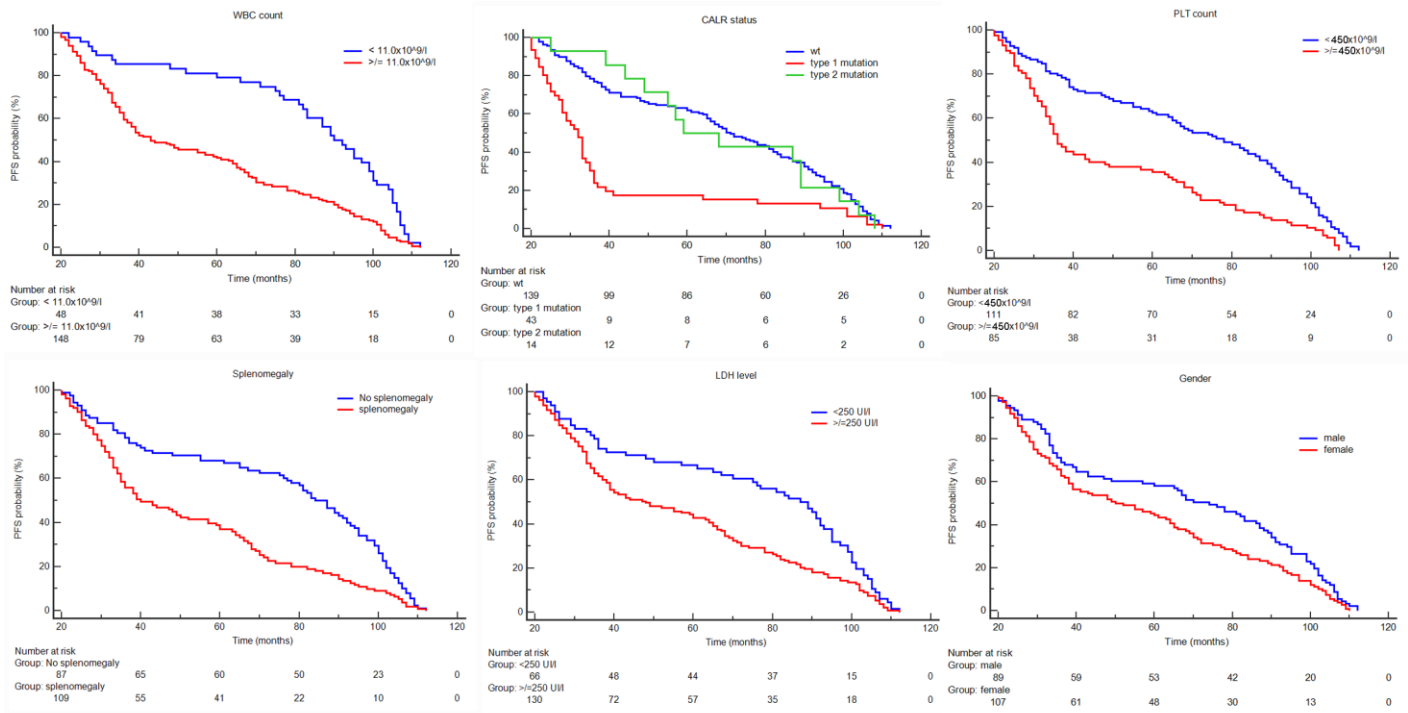
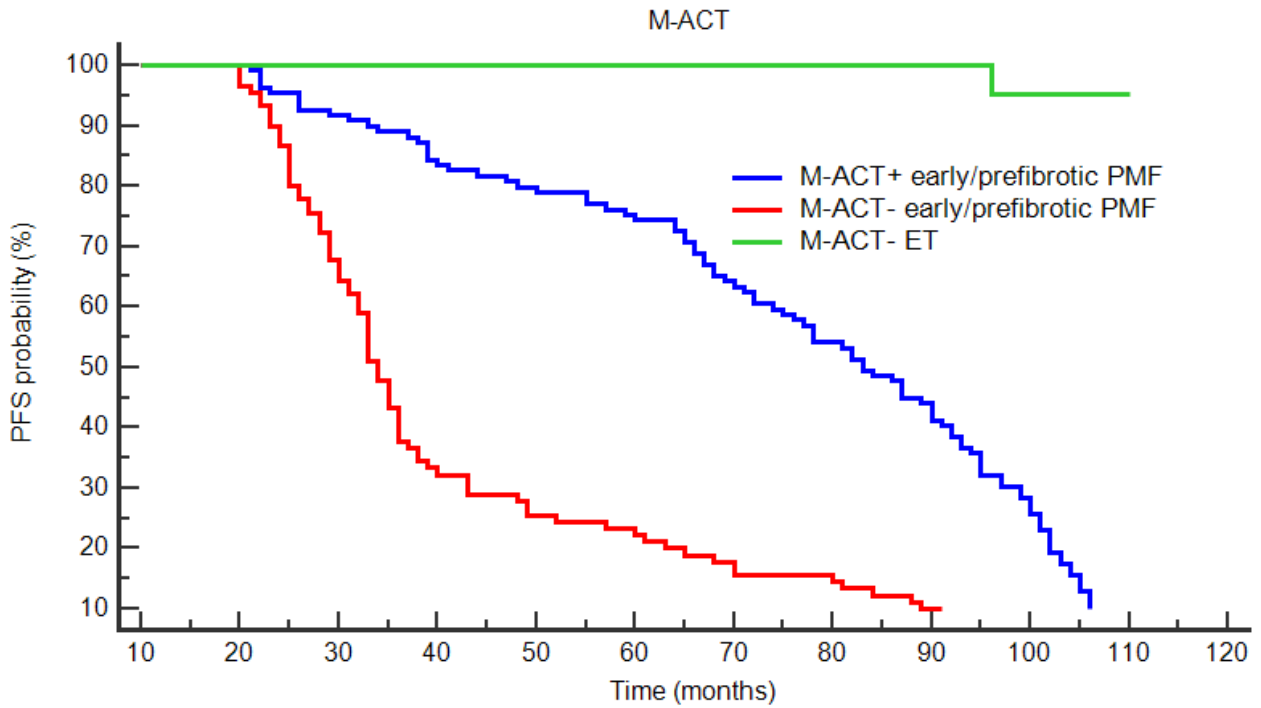


Figure 2S



Number at risk

Group: M-ACT+ early/prefibrotic PMF

109	109	100	91	86	81	69	59	45	28	2	0
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Group: M-ACT- early/prefibrotic PMF

90	87	58	29	23	20	14	13	9	5	0	0
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Group: M-ACT- ET

23	23	23	23	23	23	23	22	21	20	0	0
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Figure 3S