PROFESSOR UMBERTO GIANELLI (Orcid ID : 0000-0003-3304-8417)

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PROGNOSTIC SIGNIFICANCE OF A COMPREHENSIVE HISTOLOGIC EVALUATION OF RETICULIN FIBROSIS, COLLAGEN DEPOSITION AND OSTEOSCLEROSIS IN PRIMARY MYELOFIBROSIS PATIENTS

Running title: Stromal changes in primary myelofibrosis.

Authors: Umberto Gianelli[1], Stefano Fiori[1], Daniele Cattaneo[2], Anna Bossi[3], Ivan Cortinovis[3], Arturo Bonometti[1], Giulia Ercoli[1], Cristina Bucelli[2], Nicola Orofino[2], Gaetano Bulfamante[4], Alessandra Iurlo[2].

Affiliations:

[1] Division of Pathology, Department of Pathophysiology and Transplantation, University of Milan, and IRCCS Ca' Granda - Maggiore Policlinico Hospital Foundation, Milan, Italy.

[2] Hematology Division, IRCCS Ca' Granda – Maggiore Policlinico Hospital Foundation, and Department of Oncology and Hemato-oncology, University of Milan, Milan, Italy.

[3] Laboratory G.A. Maccacaro, Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy.

[4] Division of Pathology, Department of Health Sciences, University of Milan and San Paolo Hospital, Milan, Italy.

Corresponding author:
Umberto Gianelli, MD,
Division of Pathology,
Department of Pathophysiology and Transplantation, University of Milan,
IRCCS Ca' Granda – Maggiore Policlinico Hospital Foundation,
Via Francesco Sforza 35, 20122 Milan, Italy.
Phone number: (+39) 02 55035875
Fax number: (+39) 02 55034608
e-mail: umberto.gianelli@unimi.it

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ABSTRACT

Aims: to evaluate whether a comprehensive histological evaluation of reticulin fibrosis, collagen deposition and osteosclerosis in bone marrow trephine biopsies (BMBs) of primary myelofibrosis (PMF) patients may have prognostic implications.

Methods: reticulin fibrosis, collagen deposition and osteosclerosis were graded from 0 to 3 in a series of 122 base-line BMBs. Then, we assigned to each case a comprehensive score (RCO score, ranging from 0 to 9) that allowed us to distinguish two groups of patients, with low-grade (RCO score 0-4) and high-grade (RCO score 5-9) stromal changes.

Results and discussion: of 122 patients, 88 displayed a low-grade and 34 a high-grade RCO score. The latter was more frequently associated with anemia, thrombocytopenia, peripheral blood blasts and increased lactate dehydrogenase levels. RCO score resulted strictly correlated with overall mortality (p=0.013) and International Prognostic Scoring System (IPSS) risk categories, and was able to discriminate the overall survival of both low- and high-grade patients (Log-Rank test: p<0.001). Moreover, it proved to be more accurate than the European Consensus on grading of bone marrow fibrosis (ECGMF grade) in identifying high-risk patients with poor prognosis.

Finally, a combined analysis of RCO scores and IPSS risk categories in an integrated clinical-pathological evaluation was able to increase the positive predictive value (PPV) for mortality in high-risk patients.

Conclusion: the comprehensive RCO score, obtained by histological evaluation of reticulin fibrosis, collagen deposition and osteosclerosis, resulted prognostically significant, more accurate than ECGMF grade in identifying high-risk patients, and improved PPV when applied in addition to IPSS.

Key words: primary myelofibrosis; bone marrow morphology; reticulin fibrosis; collagen deposition; osteosclerosis.

INTRODUCTION

Reticulin fibers are a physiological component of bone marrow stroma, and can be appreciated as a loose meshwork in histological sections stained with silver impregnation. Several reactive and neoplastic conditions elicit an increase of the normal meshwork, and an advanced degree of reticulin fibrosis is often accompanied by other stromal changes, such as collagen deposition, osteosclerosis and microvessel proliferation. These changes are not physiologically found in bone marrow, exception made for sporadic perivascular collagen fibers. The pathogenesis of bone marrow stromal changes is unclear, but evidences suggest that reticulin fibrosis and collagen deposition may differ in terms of reversibility and clinical implications [1,2].

Attempts to estimate the grade of bone marrow fibrosis have been carried out since the first grading system in 1971 [3]. The 2005 European Consensus on grading of bone marrow fibrosis (ECGMF) [4], included in the 2008 WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues [5] and confirmed, with minor changes, in the 2016 update of WHO classification [6], is the current grading system for bone marrow fibrosis. It focuses mainly on reticulin fibrosis, while collagen deposition and osteosclerosis are not evaluated separately, and lack a well-established grading system.

The grade of bone marrow fibrosis according to ECGMF (ECGMF grade) bears clinical implications, especially in myeloid neoplasms. It has predictive value on response to therapy and outcome in *BCR-ABL1*-positive chronic myeloid leukemia [7], and correlates with transfusion requirement and prognosis in myelodysplastic syndromes [8,9]. Among *BCR-ABL1*-negative myeloproliferative neoplasms (MPNs), cases of polycythemia vera with an initial grade of bone marrow fibrosis (MF-1) show an inferior myelofibrosis-free survival, while advanced fibrosis (MF-2 or MF-3) is more frequently associated with a complex karyotype [10,11]. In primary myelofibrosis (PMF), the ECGMF grade is associated with relevant hematological findings, and its prognostic role is proved in comparison with other prognostic scoring systems [12,13]. Moreover, a more accurate stratification of PMF patients is achieved when considering the ECGMF grade along with International Prognostic Scoring System (IPSS) risk categories [14]. Patients with a high ECGMF grade (MF-2 or MF-3) display features indicative of an advanced disease, such as anemia, leukopenia, thrombocytopenia, constitutional symptoms, larger splenomegaly and high IPSS risk categories. They are also more likely to have additional somatic mutations in *ASXL1* and *EZH2* genes, which are connected to adverse prognosis, and tend to have a significant reduction of overall survival when compared to early PMF patients [15].

Other stromal changes as collagen deposition and osteosclerosis are gaining attention, especially in the context of Janus kinase (*JAK*) inhibitors therapy [16-23] and hematopoietic stem cell transplantation (HSCT) [24]. Therefore, a careful histological examination is crucial for PMF patients, both at the first biopsy and during follow-up. A new grading system of bone marrow stromal changes has been recently proposed by Kvasnicka *et al.*, and proved to be reproducible. It evaluates separately reticulin fibrosis, collagen deposition and osteosclerosis, with specific recommendations to improve the staining quality and laboratory standards [25].

In this study, we hypothesize that a comprehensive assessment of reticulin fibrosis, collagen deposition and osteosclerosis may have prognostic implication and allow a better stratification of PMF patients. To verify the hypothesis, reticulin fibrosis, collagen deposition and osteosclerosis were first evaluated separately, according to the grading system proposed by Kvasnicka *et al.* [25]. Then, for each case, the evaluated stromal changes were summarized in a comprehensive score (RCO score). Finally, the results obtained with RCO score were compared to those obtained with ECGMF grade and IPSS risk categories.

MATERIALS AND METHODS

Patients

The study involved 122 consecutive PMF patients diagnosed between 1998 and 2015 (median follow up: 4.7 years, range: 1.4 months - 13.6 years) at the Hematology Division of the Fondazione IRCCS Ca' Granda — Ospedale Maggiore Policlinico of Milan. Inclusion criteria were the trephine biopsy length (more than 1 cm) and the availability of complete clinical history, laboratory and follow-up data, and the patient's informed consent. All cases met the 2008 WHO criteria for PMF, and biopsies were performed at base-line observation: in particular, no patient received prior cytoreductive therapy.

The cases were reviewed by two experienced pathologists (UG, SF) and classified, according to the 2016 updated WHO criteria, as prePMF and overt PMF [6].

Bone Marrow Evaluation

Formalin-fixed, paraffin-embedded bone marrow trephine biopsies were cut at a 5-micron interval and the resulting sections were stained with Hematoxilin-Eosin, Giemsa, Gomori's silver impregnation and Masson's trichrome. The grade of bone marrow stromal changes was assessed according to the new grading system [25].

We decided not to evaluate microvessel proliferation, another stromal alteration that occurs along with the increase of bone marrow fibrosis [26], since it lacks a well-established grading system.

Perivascular reticulin and collagen fibers were considered as internal quality controls, and fibers density was evaluated only in hematopoietic areas. In case of heterogeneous patterns, the final grade was considered the highest in at least 30% of bone marrow area.

Reticulin fibrosis was evaluated on Gomori's silver impregnation stained slides and scored as: MF-0, scattered linear reticulin with no intersections (crossovers), corresponding to normal bone marrow; MF-1, loose network of reticulin with many intersections, especially in perivascular areas; MF-2, diffuse and dense increase in reticulin

with extensive intersections, occasionally with focal bundles of thick fibers mostly consistent with collagen and/or focal osteosclerosis; MF-3, diffuse and dense increase in reticulin with extensive intersections and coarse bundles of thick fibers mostly consistent with collagen, usually associated with osteosclerosis. Collagen deposition was evaluated on Masson's trichrome stained slides and scored as: Co-0, perivascular collagen only (normal); Co-1, focal paratrabecular and/or central collagen deposition without connecting meshwork; Co-2, paratrabecular and/or central deposition of collagen with focally connecting meshwork or generalized paratrabecular apposition of collagen; Co-3, diffuse (complete) connecting meshwork of collagen. Osteosclerosis was evaluated on Hematoxilin-Eosin stained slides and scored as: Ost-0, regular bone trabeculae (distinct paratrabecular borders); Ost-1, focal budding, hooks, spikes or paratrabecular apposition of new bone; Ost-2, diffuse paratrabecular new bone formation with thickening of trabeculae, occasionally with focal interconnections; Ost-3, extensive interconnecting meshwork of new bone with overall effacement of marrow spaces. Examples of the evaluated morphological changes are reported in Figure 1.

A comprehensive score (RCO – Reticulin, Collagen, Osteosclerosis – score), obtained by summing the grade of each morphological parameter (grade of reticulin fibrosis + grade of collagen deposition + grade of osteosclerosis), ranging from 0 to 9, was assigned to each case. To simplify clinical applicability, the cases were divided into two groups: RCO low-grade (RCO score ranging from 0 to 4) and RCO high-grade (RCO score ranging from 5 to 9). We chose to set a cut-off of 5 for RCO high-grade cases because it implied, in our series, at least two high-grade (grade 2 or 3) stromal changes.

Statistical Analysis

Continuous variables were expressed as median and range, and categorical variables as absolute frequency and percentage. A logistic model was used to assess association between covariates and mortality. Kaplan-Meier survival analysis was used to evaluate different groups of ECGMF grade, RCO score and IPSS in relation to time.

Statistical significance was set at a level of p <0.05. Sidak correction was used for multiple testing. Given m different null hypotheses and a familywise α of 0.05, each null hypothesis with a p-value lower than $1-(1-\alpha)^{1/m}$ was rejected.

Data processing and all the statistical analyses were performed using the SAS software (version 9.2; SAS Institute, Cary, NC, USA).

RESULTS

Base-line characteristics of the patients

The study involved a consecutive series of 122 PMF patients diagnosed according to the 2008 WHO criteria and revised according to the 2016 update of WHO classification; 69 (57%) were revised as prePMF (ECGMF grade: MF-0 or MF-1) and 53 (43%) as overt PMF (ECGMF grade: MF-2 or MF-3).

The series included 56 males and 66 females (M/F = 85%) with a median age at diagnosis of 68 years (range 30–85). Anemia (hemoglobin <10 g/dL) was found in 20 patients (16%), leukocytosis (leukocytes >25 $x10^9$/L) in 4 patients (3%), and thrombocytopenia (platelets <100 $x10^9$/L) in 7 patients (6%). The main clinical data are summarized in Table 1.

*JAK2*V617F mutation was detected in 81 cases (66%). Among the remaining 41, three carried the *MPL*W515L mutation, one the *MPL*W515R and 27 a mutation in exon 9 of *CALR* gene. In the latter group, 15 cases showed a type 1 mutation (del52bp), nine a type 2 mutation (ins5bp), and three other variants (del4bp, del19bp and del5ins12bp). The remaining 10 patients were “triple-negative” (i.e., with no *JAK2*, *CALR* or *MPL* mutations).

According to IPSS, 38 cases were stratified as low risk, 51 as intermediate-1 risk, 21 as intermediate-2 risk, and 12 as high risk.

By the time of the analysis, 21 patients (17%) had died; leukemic evolution occurred in 14 patients (11.5%), and thrombo-hemorrhagic events in 25 (20.5%).

Morphological evaluation of Reticulin, Collagen, Osteosclerosis and RCO score

The histologic analysis of bone marrow stromal changes, according to the grading system proposed by Kvasnicka *et al.* [25], gave the following results: reticulin fibrosis, MF-0=9, MF-1=60, MF-2=31 and MF-3=22; collagen deposition, Co-0=64, Co-1=23, Co-2=21 and Co-3=14; osteosclerosis, Ost-0=72, Ost-1=24, Ost-2=19 and Ost-3=7. Stromal changes evolved harmonically for the three morphological features, with few exceptions. Four out of 60 cases with MF-1 reticulin fibrosis displayed Ost-2 osteosclerosis (7%) (Figure 2). Moreover, of 31 cases with MF-2 reticulin fibrosis, two were associated with a Co-3 collagen deposition and seven with a Ost-2 osteosclerosis. The details of stromal changes evaluation are listed in Table 2.

Mortality, as a dependent variable in a logistic model, was associated with the grade of reticulin fibrosis and collagen deposition, but not with osteosclerosis. In fact, the risk of death was 3.3 ($IC_{95\%}$ 1.3-9.0) folds greater in the advanced grade of reticulin fibrosis (grade 2-3 *vs* 0-1) and 2.7 ($IC_{95\%}$ 1.2-6.3) folds greater in the advanced grade of collagen deposition (grade 2-3 *vs* 0-1).

A comprehensive score (RCO – Reticulin, Collagen, Osteosclerosis – score), obtained by summing the grade of each morphological parameter (grade of reticulin fibrosis + grade of collagen deposition + grade of osteosclerosis), and thus ranging from 0 to 9, was assigned to each case.

RCO score 1 was the most frequent in our series: it was found in 51 patients (41.8%), 50 of which showed a MF-1/Co-0/Ost-0 combination. RCO score 4 was the second most frequent and found in 14 patients (11.4%). It was ascribable to a MF-2/Co-1/Ost-1 combination in 11 out of 14 patients (78.5%).

RCO scores and individual grades of reticulin fibrosis, collagen deposition and osteosclerosis are reported in Table 3.

Prognostic significance of RCO score

To simplify the clinical applicability, we divided the patients in two groups, according to their RCO score: RCO low-grade (RCO score 0-4) and RCO high-grade (RCO score 5-9). Eighty-eight (72%) out of 122 patients were placed in the RCO low-grade group and 34 (28%) in the RCO high-grade group.

Patients in the RCO high-grade group displayed laboratory features indicative of an advanced disease: they had a higher percentage of anemia, thrombocytopenia, leukocytosis, peripheral blood blasts and increased lactate dehydrogenase (LDH) levels.

Clinical features according to RCO grade group are summarized in Table 4.

RCO score resulted strictly associated (p<0.001) with IPSS risk categories and overall survival (RCO low-grade: 10 dead out of 88 *vs* RCO high-grade: 11 dead patients out of 34; p=0.0059). On the contrary, it was not associated with the percentage of leukemic transformation (p=0.18) or thrombo-hemorrhagic events (p=0.76).

RCO score was able to discriminate the overall survival (OS) of patients: the 75th percentile OS in the RCO score low-grade group was 12.5 years, against 3.5 years in the RCO score high-grade group (Log-Rank test: p<0.001).

We compared the OS curves obtained with RCO score to those obtained with ECGMF grade: RCO score turned out to be more accurate in identifying high risk patients with poor prognosis (Figure 3A).

The RCO low-grade group included 11 overt PMF patients (MF-2) with RCO score 4. These patients did not seem to differ in terms of OS when compared to prePMF (MF-0 or MF-1) RCO low-grade patients (Figure 3A). We compared clinical and pathological features of prePMF and overt PMF patients in the RCO low-grade group. As seen in Table 5, no significant difference was found.

We matched the OS curves obtained with RCO score to those obtained with IPSS risk categories: the two systems showed comparable results, even if IPSS was slightly better in differentiating high risk patients after 60 months of follow-up (Figure 3B).

Then we investigated if a combined evaluation of RCO score and IPSS risk categories could allow a more accurate stratification. For this purpose, each patient was assigned to a further risk subgroup, created by an

integrated estimation of IPSS risk categories and RCO score: *Low-risk* (IPSS low/intermediate-1 risk + RCO low-grade), *Moderate-risk* (IPSS intermediate-2/high-risk + RCO low-grade or IPSS low/intermediate-1 risk + RCO high-grade), and *High-risk* (IPSS intermediate-2/high risk + RCO high-grade). RCO score proved to add prognostic information to IPSS risk categories: in fact, the combined evaluation was more accurate than IPSS alone. For instance, the median survival of intermediate-2/high-risk IPSS patients declined from 9.7 years to 4.9 years if a high-grade RCO score was associated. A significant difference was also found in OS curves obtained with RCO score and IPSS together ($p<0.001$). These results are summarized in Figure 3C.

The combined evaluation provided an increase in positive predictive value (PPV) for mortality in the *High-risk* group: PPV raised from 32% (when considering RCO score alone) and 36% (when considering IPSS alone), to 42% when considering RCO score and IPSS together. The results are listed in Table 6.

Finally, we compared OS curves resulting from two different combined analyses: IPSS + RCO score *vs* IPSS + ECGMF grade. The former combination was more accurate in discriminating the OS of *High-risk* patients, especially after 60 months of follow-up (Figure 3D).

DISCUSSION

We hypothesized that a comprehensive histologic evaluation of reticulin fibrosis, collagen deposition and osteosclerosis, according to the grading system proposed by Kvasnicka *et al.* [25] may have prognostic implication and may allow a better stratification of PMF patients.

To verify the hypothesis, we assessed reticulin fibrosis, collagen deposition and osteosclerosis in base-line bone marrow trephine biopsies of 122 consecutive PMF patients, assigning a grade from 0 to 3 to each parameter. A comprehensive score (RCO score, obtained by summing the individual grade of Reticulin fibrosis, Collagen deposition and Osteosclerosis) for each case enabled us to identify two groups of patients characterized by low-grade (RCO score 0-4) and high-grade (RCO score 5-9) bone marrow stromal changes, and by different clinical features. In fact, patients in the RCO high-grade group more frequently displayed laboratory features indicative of an advanced disease, such as anemia, thrombocytopenia, peripheral blood blasts and increased LDH levels.

RCO score resulted strictly associated with OS and IPSS risk categories. OS curves according to RCO score were significantly different for low- and high-grade groups: the 75th percentile value of 12.5 years for RCO low-grade patients decreased to 3.5 years for RCO high-grade patients. Moreover, RCO score resulted more accurate than the current grading system, based on the ECGMF grade, in identifying high risk patients with poor prognosis. Interestingly, the subset of RCO low-grade patients with overt PMF (ECGMF grade MF-2) according to the 2016 update of WHO classification displayed an OS curve comparable to RCO low-grade patients with prePMF (ECGMF grade MF-0 or MF-1), and did not show any significant clinical and pathological differences. This finding furtherly supports the value of a comprehensive histologic evaluation of bone marrow stromal changes in stratifying PMF patients.

Finally, a combined assessment of RCO score and IPSS risk categories increased PPV for mortality. In fact, for *High-risk* patients (RCO high-grade and IPSS intermediate-2/high risk), PPV raised from 32% (when considering RCO score alone) and 36% (when considering IPSS alone), to 42% when considering RCO score and IPSS together. This means that the application of the RCO along with the IPSS risk category allows a better stratification of PMF patients.

To our knowledge, this is the first study that specifically investigates the prognostic significance of a comprehensive histologic evaluation of reticulin fibrosis, collagen deposition and osteosclerosis in a series of PMF patients.

The ECGMF grade is known to play a major prognostic role in PMF patients. For example, when our group examined the prognostic significance of ECGMF grade in PMF patients and its relationship with IPSS risk categories [13,14], the two systems resulted independently able to predict survival at a multivariate analysis. Additionally, a more accurate prognostication was achieved when the two systems were used together. More recently, a work by Guglielmelli *et al.* [15] on a large series of PMF patients confirmed our results, disclosing that a high ECGMF grade (MF-2 or MF-3) is associated with cytopenias, circulating blasts, larger splenomegaly and a higher IPSS risk category. These patients were also enriched in prognostically unfavorable mutations, in particular *ASXL1* and *EZH2*. These data suggest that a high ECGMF grade represents an adverse prognostic

factor, and that it can provide supplementary information in lower IPSS risk categories, a group of patients where clear indications for treatment are still missing.

Other stromal changes, as collagen deposition and osteosclerosis, remained under-recognized, although commonly found in association with advanced bone marrow fibrosis in *BCR-ABL1*-negative MPNs [27,28]. A grading system for reticulin fibrosis, collagen deposition and osteosclerosis was recently proposed by Kvasnicka *et al.* [25]. It proved to be reproducible in a multicenter study that involved 11 haematopathologists in a blinded assessment. The overall inter-rater reliability ranged between 0.898 and 0.926 for all three analyzed parameters (reticulin fibrosis, collagen deposition and osteosclerosis). An additional issue of that work consists in the identification of specific problems and pitfalls in the interpretation of each single morphological parameter (for instance, biopsy length, presence of crushing artefacts and squeezing, thickness of sections and quality of staining).

Collagen deposition and osteosclerosis are gaining attention as indicators of therapy response, in the background of *JAK* inhibitors treatment and HSCT. In fact, a stabilization or regression of reticulin fibrosis, collagen deposition and osteosclerosis is described after prolonged therapy with *JAK* inhibitors [18-23]. While a prompt reduction in reticulin fibrosis is also reported after HSCT, the complete regression of collagen deposition or osteosclerosis is unclear [24]. Hence, a reproducible grading system for collagen deposition and osteosclerosis is necessary for a supervision of response to therapy in these patients.

In conclusion, our study documents that the histologic evaluation of reticulin fibrosis, collagen deposition and osteosclerosis in a comprehensive RCO score is prognostically significant, more accurate than ECGMF grade in identifying high risk patients with poor prognosis, and improves PPV for mortality when applied in addition to IPSS risk categories. Still, two important limitations of our study consist in its retrospective nature and the relatively low number of examined patients and clinical events. For this reason, prospective studies with a larger number of patients are needed to confirm the prognostic value of the RCO score, to evaluate its relationship with the presence of adverse somatic mutations, such as *ASXL1* and *EZH2*, and its relevance in management of PMF patients at diagnosis and after therapy.

Authors' contributions

UG was responsible for the integrity of the work as a whole.

UG, SF, DC and AI designed the study, analyzed the data and wrote the manuscript.

AB and IC performed statistical analyses.

UG, SF, ArBo and GE revised bone marrow biopsies.

AI, DC, CB and NO provided clinical data and followed the patients.

GB revised the paper.

UG and AI approved the final version of the manuscript.

Ethics statement

This study has been performed according to the Declaration of Helsinki.

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FIGURES LEGEND

Figure 1. Examples of different grades of bone marrow stromal changes: first row, reticulin fibrosis (MF-0, MF-1, MF-2, and MF-3; Gomori's silver impregnation, 20x); second row, collagen deposition (Co-0, Co-1, Co-2, and Co-3; Masson's trichrome staining, 20x); third row, osteosclerosis (Ost-0, Ost-1, Ost-2, and Ost-3; Hematoxilin-Eosin staining, 20x).

Figure 2. Example of a case with discordant stromal features. A loose interconnecting reticulin meshwork, grade MF-1 (A, Gomori's silver impregnation, 20x) was associated with focal paratrabecular collagen fibers, grade Co-1 (B, Masson's trichrome, 20x) and with a diffuse thickening of bone trabeculae, with hooks, spikes and focal interconnections, grade Ost-2 (C, Hematoxilin-eosin, 10x). The case was included in low-grade RCO score group (RCO score = 4).

Figure 3A. Overall survival curves of the 122 PMF patients stratified according to the ECGMF grade (grey line) *vs* the RCO score (black line). RCO low-grade (score 0-4) and RCO high-grade (score 5-9).

Figure 3B. Overall survival curves of the 122 PMF patients stratified according to IPSS (grey line) *vs* RCO score (black line). RCO low-grade (score 0-4) and RCO high-grade (score 5-9).

Figure 3C. Overall survival curves of the 122 PMF patients stratified according to IPSS alone (grey lines) *vs* IPSS + RCO (black lines). *Low-risk* (IPSS low/intermediate-1 risk + RCO low-grade), *Moderate-risk* (IPSS intermediate-2/high risk + RCO low-grade or IPSS low/intermediate-1 risk + RCO high-grade) and *High-risk* (IPSS intermediate-2/high risk + RCO high-grade score).

Figure 3D. Overall survival curves of the 122 PMF patients stratified according to the combined evaluation of IPSS + ECGMF grade (grey lines) *vs* IPSS + RCO (black lines). IPSS + ECGMF: *Low-risk* (IPSS low/intermediate-1 risk + ECGMF MF-0/MF-1), *Moderate-risk* (IPSS low/intermediate-1 risk + ECGMF: MF-2/MF-3 or IPSS intermediate-2/high risk + ECGMF MF-0/MF-1) and *High-risk* (IPSS: intermediate-2/high risk + ECGMF MF-2/MF-3).

IPSS + RCO: *Low-risk* (IPSS low/intermediate-1 risk + RCO low-grade), *Moderate-risk* (IPSS intermediate-2/high risk + RCO low-grade or IPSS low/intermediate-1 risk + RCO high-grade) and *High-risk* (IPSS intermediate-2/high risk + RCO high-grade).

Table 1. Base-line clinical features of the 122 PMF patients according to the 2016 update of WHO classification.

	prePMF	Overt PMF	Totals	*p* value (**)
No. of patients (%)	69 *(56.6)*	53 *(43.4)*	122	
Median age at diagnosis, years (range)	66 (30-84)	69 (37-85)	68 (30-85)	0.058
M/F, ratio	31/38 (0.82)	25/28 (0.89)	56/66 (0.85)	0.805
Hb level (g/dL), median (range)	13.7 (6.8-17.6)	11.5 (8.1-15.9)	12.8 (6.8-17.6)	0.0001
Hb level <10 g/dL, no. of patients *(%)*	5 *(7.2)*	15 *(28.3)*	20 *(16.3)*	0.0018
PLT count (x10^9/L), median (range)	726 (54-1695)	424 (38-1685)	681 (38-1695)	<0.0001
PLT count <100 x10^9/L, no. of patients *(%)*	1 *(1.4)*	6 *(11.3)*	7 *(5.7)*	0.042
WBC count (x10^9/L), median (range)	9 (3.3-22.1)	10.3 (1.9-64.3)	9.5 (1.9-64.3)	0.057
WBC count >25 x10^9/L, no. of patients *(%)*	0	4 *(7.5)*	4 *(3.3)*	0.033
Circulating blasts ≥1%, no. of patients *(%)*	1 *(1.5)*	19 *(35.8)*	20 *(16.4)*	<0.0001
LDH (IU/l)*, median (range)	441 (172-1293)	853 (174-2640)	518 (172-2640)	<0.0001
No. of deaths *(%)*	6 *(8.7)*	15 *(28.3)*	21 *(17.2)*	0.005
No. of leukemic evolutions *(%)*	5 *(7.2)*	9 *(17.0)*	14 *(11.5)*	0.150
No. of thrombo-hemorrhagic events *(%)*	13 *(18.8)*	12 *(22.6)*	25 *(20.5)*	0.655
JAK2 mutated, no. of patients *(%)*	51 *(73.9)*	30 *(56.6)*	81 *(66.3)*	0.045
CALR mutated, no. of patients *(%)*	13 (18.8)	14 (26.4)	27 (22.1)	0.045
Type 1 mutation	6 *(8.7)*	9 *(16.9)*	15 *(12.3)*	
Type 2 mutation	5 *(7.2)*	4 *(7.5)*	9 *(7.4)*	
Other mutations	2 *(2.9)*	1 *(1.9)*	3 *(2.5)*	
MPL mutated, no. of patients *(%)*	1 *(1.4)*	3 *(5.7)*	4 *(3.3)*	0.316
Triple negative, no. of patients *(%)*	4 *(5.8)*	6 *(11.3)*	10 *(8.2)*	0.270
IPSS, no. of patients *(%)*				
Low risk	31 (*44.9*)	7 (*13.2*)	38 (*31.1*)	
Intermediate-1 risk	34 (*49.3*)	17 (*32.1*)	51 (*41.8*)	<0.0001
Intermediate-2 risk	3 (*4.3*)	18 (*34.0*)	21 (*17.2*)	
High risk	1 (*1.4*)	11 (*20.7*)	12 (*9.8*)	

(*) LDH normal values: 135-214 IU/l.

(**) Given m (=18) different null hypotheses and a familywise α of 0.05, each null hypotheses that has a *p*-value lower than $1-(1-\alpha)^{1/m}$ (=0.0028) is rejected.

Table 2. Details of stromal changes evaluation in 122 PMF patients.

		Collagen deposition				Osteosclerosis			
Reticulin fibrosis	**Totals**	Co-0	Co-1	Co-2	Co-3	Ost-0	Ost-1	Ost-2	Ost-3
MF-0	**9**	9	0	0	0	8	1	0	0
MF-1	**60**	54	6	0	0	56	0	4	0
MF-2	**31**	0	17	12	**2**	8	16	7	0
MF-3	**22**	1	0	9	12	0	7	8	7
Totals	**122**	**64**	**23**	**21**	**14**	**72**	**24**	**19**	**7**

Table 3. Frequency distribution of RCO scores and percentage of patients with high-grade (grade 2 or 3) reticulin fibrosis, collagen deposition and osteosclerosis.

RCO score	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Frequency	8	51	6	9	14	7	8	9	4	6
MF-2 or MF-3 (%)				55.6	100.0	100.0	100.0	100.0	100.0	100.0
Co-2 or Co-3 (%)					21.5	71.4	100.0	100.0	100.0	100.0
Ost-2 or Ost-3 (%)						28.6	50.0	66.7	100.0	100.0

Table 4. Base-line clinical features of the 122 PMF patients according to RCO score.

	RCO score		
	Low-grade	**High-grade**	***p* value (**)**
No. of patients *(%)*	88 *(72.1)*	34 *(27.9)*	
Median age at diagnosis, years (range)	68 (30-85)	69 (37-85)	0.147
M/F, ratio	38/50 (0.76)	18/16 (1.13)	0.332
Hb level (g/dL), median (range)	13.3 (6.8-17.6)	10.7 (8.1-15.0)	<0.0001
Hb level <10 g/dL, no. of patients *(%)*	7 *(8.0)*	13 *(38.2)*	<0.0001
PLT count (x10^9/L), median (range)	715 (54-1695)	303 (38-1129)	<0.0001
PLT count <100 x10^9/L, no. of patients *(%)*	1 *(1.14)*	6 *(17.6)*	0.0018
WBC count (x10^9/L), median (range)	9.4 (3.3-22.1)	10.7 (1.9-64.3)	0.154
WBC count >25 x10^9/L, no. of patients *(%)*	0 *(0.0)*	4 *(11.8)*	0.0053
Circulating blasts ≥1%, no. of patients *(%)*	3 *(3.4)*	17 *(50.0)*	<0.0001
LDH (IU/l)*, median (range)	466 (172-1879)	907 (174-2640)	<0.0001
No. of deaths *(%)*	10 *(11.4)*	11 *(32.4)*	0.0059
No. of leukemic evolutions *(%)*	8 *(9.1)*	6 *(17.6)*	0.184
No. of thrombo-hemorrhagic events *(%)*	21 *(23.9)*	9 *(26.5)*	0.764
JAK2 mutated, no. of patients *(%)*	61 *(69.3)*	20 *(58.8)*	0.271
IPSS, no. of patients *(%)*			
Low risk	35 *(39.8)*	3 *(8.8)*	<0.0001
Intermediate-1 risk	44 *(50.0)*	7 *(20.6)*	
Intermediate-2 risk	8 *(9.1)*	13 *(38.2)*	
High risk	1 *(1.1)*	11 *(32.4)*	

(*) LDH normal values: 135-214 IU/l.

(**) Given m (=15) different null hypotheses and a familywise α of 0.05, each null hypothesis with a *p*-value lower than $1-(1-\alpha)^{1/m}$ (=0.0034) is rejected.

Table 5. Base-line clinical features of the 88 RCO low-grade patients according to the grade of reticulin fibrosis (ECGMF grade).

	Low-grade RCO score group		
	MF-0 + MF-1	**MF-2 + MF-3**	***p* value (**)**
No. of patients *(%)*	69 (78)	19 (22)	
Median age at diagnosis, years (range)	68 (35-84)	70 (39-89)	0.2425
M/F, ratio	31/38 (0.82)	7/12 (0.58)	0.607
Hb level (g/dL), median (range)	12.8 (6.8-17.6)	13.1 (9.5-15.9)	0.2781
Hb level <10 g/dL, no. of patients *(%)*	5 (7)	2 (10)	0.641
PLT count (x10^9/L), median (range)	681 (54-1695)	674 (116-1685)	0.3801
PLT count <100 x10^9/L, no. of patients *(%)*	1 (1)	0 (0.0)	1.000
WBC count (x10^9/L), median (range)	9.5 (3.3-22.1)	9.4 (5.55-20.7)	0.2798
WBC count >25 x10^9/L, no. of patients *(%)*	0 (0.0)	0 (0.0)	1.000
Circulating blasts ≥1%, no. of patients *(%)*	1 (1)	2 (10)	0.116
LDH (IU/l)*, median (range)	518 (172-1293)	533 (264-1879)	0.0034
No. of deaths *(%)*	6 (9)	4 (21)	0.213
No. of leukemic evolutions *(%)*	5 (8)	3 (16)	0.362
No. of thrombo-hemorrhagic events *(%)*	16 (23)	5 (26)	0.768
JAK2 mutated, no. of patients *(%)*	51 (74)	10 (52)	0.095
IPSS, no. of patients *(%)*			
Low risk	31 (45)	4 (21)	0.069
Intermediate-1 risk	34 (49)	10 (53)	
Intermediate-2 risk	3 (4)	5 (26)	
High risk	1 (1)	0 (0.0)	

(*) LDH normal values: 135-214 IU/l.

(**) Given m (=15) different null hypotheses and a familywise α of 0.05, each null hypothesis with a *p*-value lower than $1-(1-\alpha)^{1/m}$ (=0.0034) is rejected.

Table 6. Absolute mortality (and percentage) in relation to risk subgroups obtained with a combined evaluation of IPSS risk categories + RCO score.

	Dead		Alive**		
Risk subgroup*	**n**	**%**	**n**	**%**	**Total**
Low-risk	8	10.0	71	89.9	79
Moderate-risk	3	15.8	16	84.2	19
High-risk	10	41.7	14	58.3	24
Total	21	17.2	101	82.8	122

(*) *Low-risk* (IPSS low/intermediate-1 risk + RCO low-grade), *Moderate-risk* (IPSS intermediate-2/high risk + RCO low-grade or IPSS low/intermediate-1 risk + RCO high-grade) and *High-risk* (IPSS intermediate-2/high risk + RCO high-grade score).

(**) Alive or lost at follow-up.