Integration of multiparameter flow cytometry score improves prognostic stratification provided by standard models in primary myelofibrosis

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Abstract
Prognostic modeling in myelofibrosis (MF) has classically pursued the integration of informative clinical and hematological parameters to separate patients' categories with different outcomes. Modern stratification includes also genetic data from karyotype and mutations. However, some poorly standardized variables, as peripheral blood (PB) blast count by morphology, are still included. In this study, we used multiparameter flow cytometry (MFC) with the aim of improving performance of existing scores. We studied 363 MF patients with available MFC files for PB CD34+ cells count determination at diagnosis. We adapted Ogata score to MF context including 2 parameters: absolute CD34+ cells count (/μL) and granulocytes to lymphocytes SSC ratio. A score of 1 was attributed to above-threshold values of each parameter. Accordingly, patients were categorized as MFC low (score = 0, 62.0%), MFC int (score = 1, 29.5%), and MFC high (score = 2, 8.5%). MFClow had significantly longer median OS (not reached) compared to MFCint (55 months) and MFChigh (19 months). We integrated MFC into established models as a substitute of morphological PB blasts count. Patients were reclassified according to MFC-enhanced scores, and concordance (C-) indexes were compared. As regards IPSS, C-indexes were 0.67 and 0.74 for standard and MFC-enhanced model, respectively (Z score = 3.82; p = 0.0001). MFC-enhanced MIPSS70+ model in PMF patients yielded a C-index of 0.78, outperforming its standard counterpart (C-index 0.73; Z score = 2.88, p = 0.004). Our data suggest that the incorporation of MFC-derived parameters, easily attainable from standard assay used for CD34+ cells determination, might help to refine the current prognostic stratification models in myelofibrosis.
INTRODUCTION

According to 2016 World Health Organization (WHO) classification, primary myelofibrosis (PMF) is a hematopoietic stem-cell-derived clonal disease included in myeloproliferative neoplasms (MPNs) and associated with varying degrees of bone marrow (BM) fibrosis.\(^1\) PMF is subcategorized into prefibrotic and overt PMF depending on specific features.\(^2,3\) Furthermore, approximately 15% of patients with other MPNs can evolve to a secondary form of MF, defined as postessential thrombocythemia (PET) and postpolycythemia vera (PPV) MF, that show quite similar outcome and treatment.\(^4,5\)

The most frequent clinical manifestations include anemia, hepatosplenomegaly, and constitutional symptoms. In general, however, these disorders may be very heterogeneous in terms of symptom burden and time to disease progression, posing the need for a personalization of clinical workup and therapeutic approach. Allogeneic hematopoietic stem cells transplantation (HSCT) is still the only curative modality, but assigning a patient to the procedure is the most challenging decision from the clinician’s point of view due to its treatment-related toxicity.\(^6,7\) As a reference to facilitate relevant therapeutic choices, prognostic modeling in MF has pursued the identification of meaningful parameters and their integration to separate categories of patients with significantly different prognosis. The International Prognostic Scoring System (IPSS) was developed in 2009 and enlists easily attainable clinical and laboratory variables.\(^8\) More modern stratification models, such as the MPSS\(^2\) and the Oxford score for PMF, and the MYSEC-PM score for secondary forms of MF, have incorporated supplemental information based on genetics, from karyotype to driver and additional mutations.\(^9,10\)

Despite recent improvements, some poorly standardized parameters are still included in the main prognostic models: among these, peripheral blood (PB) blast count is based on morphologic assessment of PB smears and as such is largely operator-dependent, especially considering that the threshold for blast count is \(\geq 1\%\) to \(\geq 3\%\) in the different scores. Moreover, the definition of blast cell can be difficult to attribute in the case of immature stages of the monocytic lineage, further underlining the reliability of this variable that potentially affects the final categorization and decision-making of the patient. Although not to be considered its equivalent, the absolute number of circulating \(CD34^+\) cells correlated with PB blasts\(^8,11\) but was not systematically explored for its impact on prognosis; rather a threshold value for \(CD34^+\) cells count of \(\geq 15 \times 10^7/L\) was identified as highly associated to MF diagnosis.\(^11\)

Beyond providing quantitative enumeration of \(CD34^+\) cells, multiparameter flow cytometry (MFC) is a straightforward method to highlight dysplastic features, as extensively shown in the setting of myelodysplastic syndromes (MDS).\(^12,13\)\(^\) Moving from this rationale, we sought to use MFC with the aim of improving current prognostic stratification in patients affected by MF.

METHODS

2.1 Patients

The development of our study involved two stages: a pilot phase and an expansion phase. In the pilot phase, we investigated a large number of phenotypic parameters in a prospective series of patients, referred with a clinical suspicion of MPN and undergoing a comprehensive diagnostic workup. Based on the selection of a critical number of parameters on this pilot patient set, we interrogated our database to include a large expansion cohort. The enrollment criteria for expansion phase were a diagnosis of MF, revised according to 2016 WHO criteria in case of PMF, or the IWG-MRT criteria for secondary forms of MF, and the availability of i) Flow Cytometry Standard (FCS) files for \(CD34^+\) cell count in PB carried out at diagnosis or during follow-up and ii) written informed consent. The study was approved by local institutional review board (IRB; project MYNERVA, approval 14 560) and performed in accordance with the Declaration of Helsinki. After approval by the IRB, we interrogated our database to identify eligible patients.

2.2 Multiparameter flow cytometry

In the pilot cohort, different bone marrow cell compartments were investigated through a panel of 28 monoclonal antibodies (MoAb) on EDTA-anticoagulated BM withdrawn at MPN diagnosis. Precursor and mature compartments (B-, neutrophilic, monocytic, erythroid, plasmacytoid dendritic cells, basophils) were identified by specific features, that is, \(CD34, CD45,\) and side scatter (SSC) signal. Multilineage dysplasia was assessed by 2 phenotypic scores: i) one validated by European Leukemia Net (ELN) in MDS\(^14,15\) (Ogata score) and adapted to MF to include 3 parameters and ii) the second one, adapted from Matarraz et al\(^16\) (Salamanca score) to include 63 parameters (Data S1).

Based on the preliminary data from this pilot patient set, we expanded our study by applying the adapted Ogata score to FCS files obtained at the time of routine \(CD34^+\) quantification on PB, determined within 1 year from MF diagnosis, that were retrieved from our database. PB samples were handled according to diagnostic standard for \(CD34^+\) cell count.\(^17\) Briefly, fresh EDTA-anticoagulated PB was stained for surface markers using a stain-lyse-and-no-wash procedure in tubes designed for determining absolute counts of leucocytes in single platform as being provided with a lyophilized pellet containing a known number of fluorescent beads (Becton Dickinson Trucount, San Jose, CA). The following antibody combination was used: CD45-FITC/ CD34-PE/7AAD. In acquisition phase, a stopping gate was set on \(CD34^+\) acquisition gate to get a total number of events sufficient to contain \(\geq 100 CD34^+\) cells. In the case of hypocellular samples, twenty \(CD34^+\) cells were considered adequate, provided they were well-clustered and backward checked in all colors to exclude artifacts.

PB FCS files were reanalyzed in each patient by Infinicyt software (Cytognos SL, Salamanca, Spain) to obtain the adapted MFC score as follows. Three cell compartments were identified on PB based on FSC...
and SSC characteristics and their reactivity for CD45 and CD34. These subsets were: i) CD34+ cells, featured by CD45−dim/SSC intermediate; ii) maturing granulocytic compartment, selected based on CD45−dim/CD34+ with high SSC; and iii) mature lymphocytes, defined by their typical CD45−high expression and low SSC signal. The three parameters investigated for their prognostic significance were: a) absolute count (μL) of CD34+ cells; b) the lymphocyte to myeloblast CD45 mean florescence intensity (MFI) ratio; and c) the granulocyte to lymphocytes SSC peak channel ratio. MFC methods are described in detail in Data S2.

2.3 | Mutational and cytogenetic analyses

Mutation analysis was performed on DNA from PB or BM cells. JAK2 V617F and MPL W515 mutation were detected by real-time polymerase chain reaction or high-resolution melting analysis.18 CALR mutations were identified by capillary electrophoresis and bidirectional sequencing and classified as type 1 or type 2 like.19 Next generation sequencing was used to detect mutations in selected myeloid genes, including EZH2, ASXL1, IDH1/IDH2, and SRSF2, previously shown to be prognostically informative in PMF10,18; a high molecular risk (HMR) category was defined by the presence of one or more of these mutations (additional information is provided in the Data S3). Cytogenetic analysis and reporting were performed according to the International System for Human Cytogenetic Nomenclature criteria20 using standardized techniques.

2.4 | Risk stratification

Patients were annotated for the main risk stratification models; specifically, all patients were classified according to IPSS,8 patients with PMF also according to MIPSS70+,21 patients with secondary MF also according to MIPSS70+, and according to MYSEC-PM.22

2.5 | Statistical analysis

Pairwise comparison between patients’ characteristics was performed using the Mann–Whitney test or the Kruskal-Wallis test for continuous variables and the Pearson’s chi-squared test or the Fisher’s exact test for categorical variables. Each phenotypic parameter was initially considered as a continuous variable and tested for its impact on prognosis by ROC curve, in order to select the optimal thresholds by setting the occurrence of death as the endpoint; Youden test was used for evaluating the performance of single cutoffs. Survival was calculated as the interval between diagnosis or referral and death or last follow-up with the Kaplan–Meier method, and long-term outcomes were compared with the log-rank test. The Cox proportional hazard model was applied to estimate hazard ratios with 95% confidence intervals (CI) for overall survival (OS) and leukemia-free survival (LFS) both in univariate and multivariate analysis. All p-values were two-sided, and a 5% significance level was set. Comparison among the distinct preexisting scoring systems and the proposed scores was done through the Harrell’s concordance index (C-index) and 95% CIs, to evaluate the ability of the individual prognostic classifications to predict outcome. Data were processed using R software version 4.1.1 (http://cran.r-project.org).

3 | RESULTS

3.1 | Patients—pilot cohort

Fifty-two consecutive patients with MPN diagnosis were enrolled, including 31 MF (7 pre-PMF, 14 overt PMF, 10 post-PV/ET MF); and a control cohort of 13 ET, 5 PV, 1 MPN-unclassifiable and 2 MDS/MPN. Median age was 59 years (range 24–83). Nineteen MF patients (out of 31, 63.3%) had JAK2 V617F, 9 (29.0%) CALR, 2 (6.4%) MPL W515L mutation. Eleven patients (35.5%) harbored at least 1 HMR mutation. According to MIPSS70, 8 (25.8%), 12 (38.7%), and 11 (33.5%) were low, intermediate, and high risk, respectively. When considering MFC, MF patients ranked higher values for both the Ogata and Salamanca scores. Furthermore, Salamanca score values had a trend to correlate with WHO variant (Figure 1A) and stratification according to MIPSS70 in MF (Figure 1B), with median value of 7.75, 13.25, and 15.5 in low-, intermediate-, and high-risk category, respectively (KW test p = 0.06). We sought for correlations with baseline characteristics, and separated MF cases according to values ≥ or < to the median of Salamanca (13.5) and Ogata score (2). As regards the Salamanca score, patients with higher values were featured by significant differences in hemoglobin (11.9 vs. 13.7 g/dL; p = 0.015), platelet count (330 vs. 558 x 109/L; p = 0.004), LDH (380 vs. 275 U/L; p = 0.034), and circulating CD34+ cells (60.4 vs. 5.3/μL; p = 0.007). Regarding the Ogata score, higher values correlated with lower hemoglobin (11.1 vs. 12.7 g/dL; p = 0.041) and higher CD34+ cells (104.3 vs. 10.3/μL; p = 0.0042), while there were no significant differences for platelets or LDH. In a preliminary prognostic analysis, we observed an impact by categorization below/above the median value according to both score models either on OS or LFS (Figure S1–S2).

3.2 | Patients—expansion cohort

Based on the above data, in particular, the prognostic insight yielded by the simplest of the two scores (i.e., Ogata score), we explored its adaption to MFC determination of CD34+ cells on PB. After interrogating our database for that purpose, we recruited 378 patients, referred from 1995 to 2020, with a diagnosis of MF, for whom FCS files at diagnosis were available; information was last updated in December 2020. The median percentage of CD34+ cells on WBC was 0.05% (range 0.00%–35.25%). Fifteen patients showed a CD34+ cell count higher than 5% of WBC, a finding consistent with accelerated or blast phase upfront and were thus excluded from further
analyses. The characteristics of the remaining 363 patients are summarized in Table 1: 223 (61.4%) were diagnosed with PMF, 86 were prefibrotic (23.7%), and 137 overt (37.7%). One-hundred-forty (28.6%) patients had secondary MF, of whom 65 (17.9%) PET and 75 (20.7%) PPV. Median age was 54.6 years (range 19–87). A driver mutation was found in 347 (95.6%) patients: JAK2V617F in 245 (67.5%), CALR in 75, 50 type 1 (13.8%), and 25 type 2 (6.9%) and MPL in 18 (5.0%). Ten patients harbored two concomitant driver mutations: one case each for JAK2/CALR (type 1) and MPL/CALR (type 1), whereas 8 cases had JAK2/MPL double mutant. Sixteen (4.4%) patients were triple-negative. HMR mutations were present in 105 (28.9%) cases.

### 3.3 Immunophenotypic data and correlation with clinical-genetic characteristics

The median absolute value of circulating CD34+ cells was 21.77 × 10^9/L (range 0.0–4431). In 24 patients, CD34+ cells were not detectable with a sensitivity of 10^{-4}. The median ratio of CD45 expression by CD34+ cells upon lymphocytes was 5.24 (range 1.85–11.21). The median ratio of SSC signal between neutrophils and lymphocytes was 7.02 (3.42–11.75).

In order to explore the impact on prognosis of each phenotypic parameter and to define optimal cutoff, we used ROC analysis with death event as endpoint (Figure S3). The area under the curve (AUC) for CD45 ratio was 0.45 (95% C.I. 0.38–0.52, p = 0.16); based on its low performance, such parameter was excluded from further analyses. SSC ratio showed an AUC of 0.31 (95% C.I. 0.25–0.37, p < 0.001) and the best cutoff was 6.0. The AUC for CD34+ cell count was 0.70 (95% C.I. 0.64–0.76, p < 0.001) with the optimal cutoff set at 100 × 10^9/L cells. We confirmed the significance of these two parameters and their relative thresholds on OS in univariate analysis by Kaplan–Meier curves (Figure S4). By including the two parameters in a Cox regression for OS, both SSC ratio (HR 0.29, p < 0.001) and CD34+ cell count (HR 3.18, p < 0.001) maintained an independent value. We thus elaborated a MFC model by attributing a score of 1 for each above-threshold parameter and thereby grouped patients as MFC_{low} (score = 0, n = 225, 62.0%), MFC_{int} (score = 1, n = 107, 29.5%), or MFC_{high} (score = 2, n = 31, 8.5%).

Some clinical features showed an association with the three MFC categories: in fact, MFC_{high} patients were the oldest, had the highest values of leucocyte count, lower levels of Hb and platelet count, higher JAK2V617F variant allele frequency (VAF), were less likely diagnosed with pre-fibrotic PMF and showed the highest incidence of HMR mutations, with consistent trends for MFC_{int} and MFC_{low}.

![Figure 1](https://example.com/figure1.png)
| Variables | Overall | MFC<sub>low</sub> (score = 0) | MFC<sub>int</sub> (score = 1) | MFC<sub>high</sub> (score = 2) | p   |
|-----------|---------|--------------------------|--------------------------|--------------------------|-----|
| N (%)     | 363 (100) | 225 (62.0) | 107 (29.5) | 31 (8.5) |     |
| Median age (range) | 59 (19–87) | 63 (32–85) | 66 (42–86) |     | 0.0152 |
| Leukocyte count × 10⁹/l, median (range) | 9.05 (0.6–90.0) | 8.0 (0.6–60.0) | 11.9 (1.5–85.8) | 16.1 (4.5–90) | <0.0001 |
| Hemoglobin, g/dl, median (range) | 124 (4.7–18.0) | 129 (4.7–17.5) | 122 (7.4–18.0) | 11.0 (7.7–16.3) | 0.0045 |
| Platelet count × 10⁹/l, median (range) | 399 (14–1800) | 428 (25–1800) | 347 (14–1660) | 254 (16–729) | <0.0001 |
| WHO category; n (%) | | | | |     |
| Prefibrotic PMF | 86 (23.7) | 67 (29.8) | 17 (15.9) | 2 (6.5) | 0.0051 |
| Overt PMF | 137 (37.7) | 78 (34.7) | 42 (39.2) | 17 (54.8) |     |
| PPV/PET MF | 140 (38.6) | 80 (35.5) | 48 (44.9) | 12 (38.7) |     |
| Driver gene mutations; n (%) | | | | |     |
| JAK2 V617F | 254 (69.7) | 154 (68.4) | 76 (71.0) | 24 (77.4) | 0.77 |
| CALR | 77 (21.2) | 52 (23.0) | 21 (19.6) | 4 (12.9) |     |
| MPL | 26 (7.2) | 18 (8.0) | 7 (6.5) | 1 (3.2) |     |
| Triple negative | 16 (4.4) | 9 (4.0) | 5 (4.8) | 2 (6.5) |     |
| JAK2 V617 allele burden, median (range) | 47 (0–100) | 42 (0–100) | 50 (1–98) | 60 (21–98) | 0.0156 |
| High molecular risk positive, n (%) | 105 (28.9) | 47 (20.1) | 38 (35.5) | 20 (64.5) | <0.0001 |
| TPSS model (n = 363) | 363 | 215 | 84 | 64 |     |
| Low | 118 (32.5) | 86 (40.0) | 16 (19.0) | 16 (25.0) | <0.0001 |
| Intermediate-1 | 127 (35.0) | 81 (37.7) | 25 (29.8) | 21 (32.8) |     |
| Intermediate-2 | 66 (18.2) | 31 (14.4) | 19 (22.6) | 16 (25.0) |     |
| High | 52 (14.3) | 17 (7.9) | 24 (28.6) | 11 (17.2) |     |
| MIPSS70+ model (n = 201) | 201 | 133 | 41 | 27 | <0.0001 |
| Low | 89 (44.3) | 77 (57.9) | 4 (9.8) | 8 (29.65) |     |
| Intermediate | 21 (10.4) | 17 (12.8) | 2 (4.9) | 2 (7.4) |     |
| High | 58 (28.9) | 30 (22.5) | 19 (46.3) | 9 (33.3) |     |
| Very high | 33 (16.4) | 9 (6.8) | 16 (39.0) | 8 (29.65) |     |
| MYSEC-PM model (n = 140) | 140 | 68 | 41 | 31 | 0.0397 |
| Low | 22 (15.7) | 17 (25.0) | 4 (9.8) | 1 (3.2) |     |
| Intermediate | 86 (61.4) | 39 (57.4) | 25 (7.0) | 22 (71.0) |     |
| High | 32 (22.9) | 12 (17.6) | 12 (29.2) | 8 (25.8) |     |

Note: p value, in the comparison of MFC-related categories (Chi-square test for categorical variables, Kruskal-Wallis test for comparisons of medians). As regards stratification according to prognostic models, the percentages are expressed upon the relative number of patients considered for each subset. p values associated with significance (i.e., <0.05) are highlighted by bold.

Abbreviations: MFC, multiparameter flow cytometry; PET, postessential thrombocythemia; PMF, primary myelofibrosis; PPV, postpolycythemia vera; WHO, World Health Organization.
categories (Table 1). Furthermore, prognostic stratification according to the main prognostic models (IPSS, MIPSS70+ , MYSEC-PM) reflected the distribution of MFC-related groups (Table 1). No significant differences were observed regarding the frequency of driver gene mutations.

### 3.4 Prognostic analysis of MFC model

We then investigated the prognostic relevance of MFC model by comparing the outcome of patients according to MFC-defined groups: MFC_{low} category had significantly longer OS (median not reached) compared to MFC_{int} (55 months, HR 2.90, p < 0.0001) and MFC_{high} (19 months, HR 11.91, p < 0.0001) ones (Figure S5). This finding did not change substantially when censoring at the date of transplant the patients that received allogeneic HSCT (n = 16) (data not shown). The impact of MFC model on survival estimates was shown to be highly significant in the three diagnostic categories, although in secondary MF, there was no separation of MFC_{low} and MFC_{int} groups (Figure S6). Then, in order to investigate the independent significance of MFC score on OS, we performed Cox regression multivariate analysis including main prognostic models (IPSS, MIPSS70+, MYSEC, HMR). MFC category maintained an independent value in all analyses, proving to be superior to MYSEC-PM and HMR in relative.
multivariate models (Table S1). Noteworthy, we observed that the MFChigh group was characterized by a particularly short time to progression to blast phase (median 42 months, Figure S7).

### 3.5 | Integration of MFC score in established prognostic models

Moving from the independent significance of MFC score, we sought to integrate it as a parameter into well-established and widely adopted prognostic stratification models. Since the MFC score includes a standardized modality of PB precursor cell quantification, we hypothesized that MFC score could effectively substitute the assessment of peripheral blasts by morphology, a parameter that is included in all prognostic models although with different cutoffs. To this end, we stratified the patients from our cohort according to IPSS (overall series), MIPSS70+ (PMF), and MYSEC-PM (PET/PPV MF), each of them in their standard and MFC-enhanced version. In the latter one, we removed the parameter of PB blasts from scoring and then attributed a score of 0, 1, or 2 to MFClow, MFCint, and MFChigh, respectively, to reclassify the patients according to the new calculation.

As regards IPSS, 109 out of 363 (30.0%) patients changed their prognostic category from standard to MFC-enhanced model (Figure 2A), most of them (89, corresponding to 81.7% of transitioned patients) being escalated to a higher risk tier. Restricting the analysis to PMF patients with all data available for MIPSS70+- stratification (201 out of 223 PMF patients), 26 out of 201 (12.9%) were assigned to a different risk group based on MFC score (Figure 2B). This proportion remained substantially superimposable also when focusing the analysis to overt PMF only (15 out of 128, 11.7%). As regards secondary MF, in the comparison with the MYSEC-PM score, 33 out of 140 (23.6%) cases moved to a different category.

### 3.6 | Performance of MFC-enhanced stratification as compared to standard models

Once the patients were reclassified according to the MFC-enhanced scores, we carried out a Kaplan–Meier survival analysis, calculated the concordance (C-) index for each model, and compared the performance of the standard versions with the relative MFC-enhanced ones (Figure 3). As regards IPSS, the C-index was 0.67 [standard error (SE) 0.02] and 0.74 (SE 0.02) for standard and MFC-enhanced model, respectively. The superiority of MFC-enhanced model was statistically significant with a Z score equal to −3.82 (p = 0.0001) (Figure 3A–B). In PMF patients, MFC-enhanced MIPSS70+ model yielded a C-index of 0.79 (SE 0.02) thus outperforming its standard counterpart (C-index

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**FIGURE 3** Overall survival (OS) according to standard (A)–(C) and MFC-enhanced (B)–(D) risk stratification. The performance of standard IPSS (A) and MYPSS70+ (C) prognostic models were compared to the relative MFC-enhanced version (B)–(D). Median OS, hazard ratio (HR) and 95% confidence intervals (CI) were calculated with respect to lower risk tier and reported for each category. Concordance (C-) index was reported for each model and the results from the comparison of standard versus MFC-enhanced stratification were expressed by Z-score and p values at the right top part of (B) and (D) panels [Color figure can be viewed at wileyonlinelibrary.com]
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Our data are consistent with the relevant role of MFC in assessing myeloid dysplasia, with relative implications in different hematological conditions. Especially in the setting of MDS and chronic myelomonocytic leukemia (CMML) and genetically undefined acute myeloid leukemia. Furthermore, spotting phenotypic abnormalities on myeloid cell compartments has been correlated to clonal hemopoiesis of indeterminate potential (CHIP), either in AML upon remission or in patients with untreated multiple myelomas. As a whole, MFC can provide quick and meaningful insights into the extent of clonal involve-

Our study has some acknowledgeable limitations, primarily related to the retrospective design, spanning a relatively large enrol-

In conclusion, our data support the adoption of MFC-integrating prognostic models, that showed superior performance in estimating outcome compared to original models. The implementation of MFC, standardized and obtainable from standard testing, might offer a further step toward tailoring therapeutic approaches upon individual disease-associated risk.
CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available on request from the corresponding author.

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How to cite this article: Mannelli F, Bencini S, Coltro G, et al. Integration of multiparameter flow cytometry score improves prognostic stratification provided by standard models in primary myelofibrosis. Am J Hematol. 2022;97(7):846-855. doi:10.1002/ajh.26548