Received: 23 March 2019 | Revised: 30 April 2019 | Accepted: 2 May 2019

DOI: 10.1002/ajh.25506

RESEARCH ARTICLE

Myelodysplasia as assessed by multiparameter flow cytometry refines prognostic stratification provided by genotypic risk in systemic mastocytosis

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Funding information
Associazione Italiana per la Ricerca sul Cancro, Grant/Award Number: 21267 Mynerva; Ministero della Salute, Grant/Award Numbers: GR-2013-02358238, GR-2016-02362631; AIRC

Abstract

Systemic mastocytosis (SM) is characterized by extreme heterogeneity of manifestations and prognosis. Several disease-related biomarkers, including clinical, hematological and molecular variables, have been correlated with prognosis. Although relevant, the mutation profile closely reflects the WHO classification that has per se prognostic value. High-risk mutations (HRM) are largely confined to advanced forms, and thus fail in providing information regarding progression and outcome in the not-advanced variants. In this work, we studied hematopoietic cells by multi-parameter flow cytometry (MFC) in order to highlight dysplastic traits that might provide insights into outcome. A score previously validated for myelodysplastic syndromes, with high reproducibility in standard diagnostics, was used. The application of an MFC score to a cohort of 71 SM cases, concurrently genotyped for configuring a HRM category, resulted in the identification of two separate patients' categories (MFC+ and MFC-) characterized by significantly different clinical and laboratory features at presentation. The extent of dysplasia by MFC tended to parallel WHO-category and genotype-related stratification. MFC+ patients had shorter survival compared to MFC- ones, for whom the incidence of progression and/or death was virtually null. Of note, MFC score remained prognostically informative in unadvanced subsets. Furthermore, the integration of MFC and HRM was an independent predictor for outcome, also overcoming WHO-categories in multivariate analysis for EFS. Our results support the use of MFC analysis in the evaluation of patients with SM, alone and in combination with HRM, for refinement of prognosis assessment.

1 | INTRODUCTION

Systemic mastocytosis (SM) presents unique clinical and biological features that led WHO to consider it as a separate entity in the updated 2016 Classification.[1] Nevertheless, SM has an extreme heterogeneity of disease manifestations, courses and prognoses. Selected clinical findings usually characterize aggressive vs indolent variants, a distinction that drives therapeutic decisions[2]; additional patient- and disease-related

variables, including advanced age, elevated beta-2-microglobulin and alkaline phosphatase (ALP) levels, correlate with survival.[2,3]

Systemic mastocytosis is associated with the driver *KIT* D816V mutation in more than 80% of cases, with rare patients harboring other activating *KIT* mutations. Mutations of genes usually associated with "high-risk" features in myeloid neoplasms[4,5] are harbored also by a proportion of SM patients, and provide clinically relevant information about disease course and prognosis. In particular, mutations in *SRSF2*, *ASXL1*, *RUNX1* and *CBL* were shown to be prognostically meaningful,[6-9] and their role was validated recently in our own cohort.[10] These mutations display a markedly different pattern of occurrence across clinical WHO variants. In indolent (ISM) and smoldering (SSM) systemic mastocytosis, relatively few patients harbor additional mutations, other than *KIT*D816V (14%), usually involving *TET2* and *DNMT3A*, commonly found also in clonal hemopoiesis of indeterminate potential (CHIP).[7] At the opposite, the majority of patients with advanced SM (including those with an associated hematological neoplasm, SM-AHN) harbor at least one "high-risk" (HR) mutation.[7] Further evidence suggests that multilineage involvement with *KIT* mutation affects the outcome of SM. In fact, in cases where *KIT* mutation is not restricted to the mast cell compartment, the disease usually presents a more aggressive course and a higher risk of progression.[11]

In advanced myeloid neoplasms and myelodysplastic syndromes (MDS), evidence of the involvement of hematopoiesis by neoplastic clone can be obtained through the analysis of myeloid dysplasia, by morphology evaluation of bone marrow (BM) cells. Beyond being key diagnostic criterion for recognizing different subtypes of MDS, myeloid dysplasia provides prognostic information also across non-MDS myeloid neoplasms, from acute myeloid leukemia[12,13] to eosinophilia-related disorders.[14] The relevance of this feature is also recognized in SM. In fact, the presence of hypercellularity or dysmyelopoiesis on morphologic assessment of BM is one of the findings used to define the smoldering variant. It is associated with an unfavorable outcome compared to indolent subset.[1,2,15-17] Multi-parameter flow cytometry (MFC) represents an effective tool for characterizing dysplastic features of hematopoiesis. It overcomes the operator-dependent variability in interpretation of dysplastic cell features by morphology only, and provides opportunity for quantification of the extent of deviation from normal.[18,19]

The aim of current study was to investigate MFC-assessed dysplasia in a cohort of patients with SM from our Center in order to correlate immunophenotypic data with clinical and biological characteristics, including genotype, and prognosis.

2 | METHODS

2.1 | Patients

The enrollment criteria for our study were a diagnosis of SM, according to WHO criteria, and the availability of: i) Flow Cytometry Standard (FCS) files at diagnosis; ii) genotypic characterization for *KIT* and selected additional mutations using NGS-target sequencing approach (as specified below); iii) written informed consent. After approval from the institutional review board, we interrogated our database in order to identify eligible patients. Additional cases fulfilling above criteria were referred from other centers, specifically Verona (n, 12), Roma (n, 4) and Milano (n, 1). For these cases, FCS files were sent to our Center and analyzed centrally for the study's purposes. Mutation analysis by NGS was performed in our center for all samples.

2.2 | Multi-parameter flow cytometry

In order to assess dysplasia by MFC, we used a previously validated score adopted for MDS[20-22] that has high reproducibility and is of easy attainability in standard diagnostic procedures. Bone marrow samples were handled according to diagnostic standard for SM diagnosis.[23] Briefly, fresh BM aspirate was stained for surface markers using a stain-lyse-and-then-wash procedure. In acquisition phase, two modalities were allowed: i) a one-step acquisition of at least 5×10^5 total BM cells; ii) a two-step acquisition, first of at least 1×10^5 total BM cells, followed by a second-step with live-gate on mast cells (identified by CD117 intensity and scatter properties). Infinicyt software (Cytognos SL, Salamanca, Spain) was used for data analysis. First, MFC files were reviewed for basic standard requirements, operationally defined as follows: i) a minimum set of monoclonal antibodies in 4-8-color combination, the minimum allowed combination including anti-CD45, CD34, CD117, CD25, HLA-DR; ii) a cell viability of at least 75% by forward (FSC) and side scatter (SSC) properties; iii) a maximum value of 30 for coefficient of variation (percentage) of time parameter in lymphocytes. Then, after those eligibility criteria had been verified, an analytical strategy was applied to obtain MFC score as previously described.[22] Briefly, the following major BM cell compartments were identified based on FSC and SSC characteristics and their reactivity for CD45 and CD34: i) myeloid $CD34^+/CD117^+$ fraction (myeloblast-related cluster); (ii) $CD34^+$ B-cell progenitors, featured by HLA-DR-high/CD117- profile with low SSC; (iii) maturing granulocytic compartment, selected on the basis of $CD45^+$dim/CD34- with high SSC; iv) mature lymphocytes, defined by their typical CD45-high expression and low SSC signal.

The four parameters provided by the score were: a) percentage of cells in the myeloblast-related cluster among all nucleated cells; b) percentage of B-progenitor cells among all $CD34^+$ cells; c) the lymphocyte to myeloblast CD45 MFI ratio; d) the granulocyte to lymphocyte SSC peak channel ratio. A score of one point was attributed for each variable according to previously defined and validated criteria.[22] The BM samples from 20 healthy donors were studied in parallel as a control.

2.3 | Molecular genetics

The c*KIT* mutation was evaluated by reverse transcriptase polymerase chain reaction (RT-PCR), as reported. Next-Generation deep amplicon sequencing with Ion Torrent platform (ThermoFisher Scientific, Waltham, Massachusetts, USA) was performed to investigate a custom panel of 20 candidate genes: *KIT*, *ETNK1*, *JAK2*, *MPL*, *CALR*, *EZH2*, *ASXL1*, *IDH1*, *IDH2*, *SRSF2*, *CBL*, *DNMT3A*, *KRAS*, *NRAS*, *RUNX1*, *SF3B1*, *TET2*, *TP53*, *U2AF1* and *IKZF1*. For genes with known mutational hotspots, only those regions were amplified, otherwise all coding exons were sequenced. Sequence alignment and filtering was performed using NextGENe

version 2.4.2.1 (SoftGenetics Pennsylvania, US) (details are reported in Supplemental Method section).

2.4 | Statistical analysis

Pair-wise 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. Survival was estimated with the Kaplan Meier method, and long-term outcomes were compared with the log-rank test. Events affecting the event-free survival were defined as death or disease progression (defined as change from indolent/smoldering SM to aggressive SM or mast cell leukemia, or acquisition of AHN). The Cox proportional hazard model was applied to estimate hazard ratios with 95% confidence intervals (CI) for all survival outcomes (OS and EFS), both in univariate and multivariate analysis. All *P* values were two-sided, and a 5% significance level was fixed. Data were processed using R software (http://cran.r-project.org).

3 | RESULTS

We included seventy-one patients, first referred from 2008 to 2018 with a diagnosis of SM, confirmed according to 2016 WHO criteria. This was for those whom mutation information and FCS files at diagnosis were available; information was last updated in December 2018. The characteristics of patients are summarized in Table 1. Fifty-seven of 71 patients (80.3%) were diagnosed with non-advanced forms, 52 (73.2%) indolent (ISM) and five (7.0%) smoldering (SSM). The 14 advanced cases included five aggressive variants (ASM, 7.0%), 6 SM-AHN (8.5%) and three mast cell leukemias (MCL, 4.2%). Among SM-AHN, one patient was diagnosed with MDS with multilineage dysplasia, four with myelodysplastic/myeloproliferative neoplasms (one unclassifiable and three refractory anemias with ring sideroblasts associated with thrombocytosis RARS-t), and one with acute myeloid leukemia.

3.1 | Mutation analysis

Overall, 68 of 71 patients (95.8%) were positive for the *KIT*D816V mutation; no other *KIT* mutation was found in the remaining three patients by NGS whole gene sequencing. As regards additional myeloid mutations, we found 42 mutations in 23 patients (Figure 1). Mutation details are reported in Supplement data (Table S1). Based on their relative prognostic significance,[5-7] we distinguished high-risk (HR) mutations (namely, those involving *ASXL1*, *SRSF2*, *RUNX1*, *CBL*, *NRAS*, *EZH2*), from those occurring in other genes whose prognostic value has not been validated. HR mutations were observed in 12 patients, of whom 10 were advanced SM forms, specifically two ASM, 5 AHN and 3 MCL. Of the remainder, one was a case of ISM harboring a mutation of *SRSF2*, and one a case of SSM harboring a *CBL* mutation. Ten out of 12 HRM+ patients exhibited the S/A/R (*SRSF2*, *ASXL1*, *RUNX1*) mutant profile.[8]

The median age at diagnosis was higher for patients bearing HRM (70 y, range 57 y-81 y) compared to the WT group (48 y, range 17 y-

TABLE 1 Clinical and laboratory features of patients according to multiparameter flow cytometry score

Variables	Overall [n = 71]	MFC− (score 0-1) [n = 50]	MFC+ (score 2-4) [n = 21]	*P* value
Median age (range)	51 (17-81)	48 (17-76)	66 (35-81)	.0004
Leukocyte count × 10^9/L, median (range)	7.2 (3.0-33.1)	7.0 (3.0-12.1)	8.6 (4.2-33.1)	.124
Hemoglobin, g/dL, median (range)	13.8 (6.4-17.4)	14.3 (6.4-17.4)	12.7 (8.1-15.6)	.0012
Platelet count × 10^9/L, median (range)	270 (70-979)	297 (89-395)	215 (10-979)	.0707
Serum tryptase ng/mL; median (range)	37 (3.0-2000)	33 (3.0-505)	64 (8.0-2000)	.0306
WHO category; n (%)				
Indolent	52 (73.2)	42 (84.0)	10 (47.6)	.0003
Smoldering	5 (7.05)	3 (6.0)	2 (9.5)	
Associated hematologic neoplasm	6 (8.5)	3 (6.0)	3 (14.3)	
Aggressive	5 (7.05)	2 (4.0)	3 (14.3)	
Mast cell leukemia	3 (4.2)	0	3 (14.3)	
Genotype				
Wild type	48 (67.6)	42 (84.0)	6 (28.6)	<.0001
Non-high risk	11 (15.5)	5 (10.0)	6 (28.6)	
High risk	12 (16.9)	3 (6.0)	9 (42.8)	

Note: P value, in the comparison of MFC− and MFC+. *P* values associated with significance (ie, <.05) are highlighted by bold.
Abbreviation: MFC, multi-parameter flow cytometry.

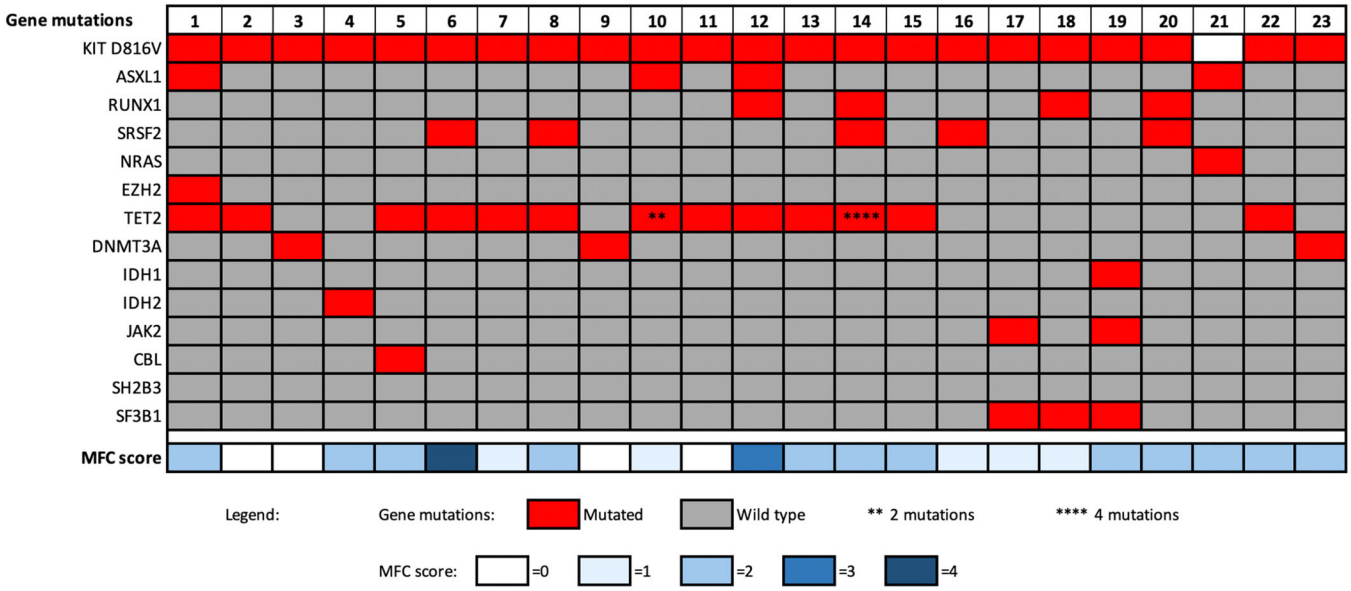

FIGURE 1 Pattern of *KIT* and additional gene mutations. The gene mutations are represented for the 23 patients who harbored at least one additional mutation to *KIT*; the remaining 45 who were positive for *KIT*D816V only are not represented. Mutated and wild-type status are depicted by red and gray, respectively; the number of asterisks corresponds to the number of mutations in case of multiple mutations. The MFC score is graphically expressed by the color code of the blue scale at the bottom of the figure: the higher the score, the darker the shade of blue. MFC, multi-parameter flow cytometry

78 y, $P < .0001$). A non-significant trend for age emerged when comparing WT to non-HRM patients (56 y, range 22 y-73 y, $P = .196$), that is consistent with the age-related occurrence of these mutations.

3.2 | Immunophenotypic data and correlation with clinical-genetic characteristics

The median MFC score in the entire cohort was 1 (range 0-4). The distribution of phenotypic abnormalities in the overall cohort, and according to clinical variant, are detailed in Table S2. The most frequently observed abnormality was a reduction of $CD34^+$ B-cell progenitors (observed in 29 of 71 patients); this abnormality resulted significantly associated with advanced forms (85.7% vs 29.8%; $P = .0002$). We thereby grouped patients according to their MFC score: 50 (70.4%) patients displayed a score ≤1 (MFC-), and 21 (29.6%) a MFC score >1 (MFC+).

Some statistically significant differences emerged from the comparison of the two groups regarding clinical features. In fact, MFC- patients were younger, had lower leukocyte counts, higher hemoglobin levels, and lower tryptase levels (Table 1). Furthermore, we observed that advanced forms were mainly included in the MFC+ category (42.9% vs 10%; $P = .0001$). In more detail, 90% of indolent and smoldering patients were comprised within the MCF- category, while 43% of MFC+ cases were aggressive variants ($P = .0003$; Table 1). The score of MFC progressively increased from ISM (median score = 0) to AHN (median score = 1.5) to ASM/MCL (median score = 2) ($P < .0001$; Figure 2A). Similar findings concerned genotypic categories: 42 of 51 MFC- cases (82.4%) had wild type status for all non-*KIT* genes analyzed, while the majority (15 of 21, 71.4%) of MFC+ patients harbored at least 1 mutation, almost half of which were HRM-type (Table 1). The MFC+ group included 31 mutations of a total of 42 (73.8%), despite MFC+ patients being about one-third only of the whole cohort.

Of interest, the extent of dysplasia assessed by MFC tended to parallel the genotypic features: the median MFC score was 0 (range 0-3) in the WT group vs a median of 2 (range 0-2) in patients with additional, non-HMR mutations and a median of 2 (range 1-4) in patients with HMR features ($P < .0001$; Figure 2B).

3.3 | Prognostic analysis

We then investigated the prognostic relevance of MFC-assessed dysplasia by comparing the outcome of patients according to MFC-defined groups. The MFC+ patients had shorter OS and EFS compared to MFC- ones, for whom the incidence of progression and/or death was virtually null (Figure 3A,B). By splitting MFC- patients in 2 subcategories by their actual score equal to zero or one point, we found that the outcome was substantially superimposable, but clearly separated from that of MFC+ cases (Figures S1 and S2).

Consistent with previous findings,[6-8] including our own study,[10] the occurrence of HRM mutations allowed clearly identifying patients with dismal OS and EFS (Figures S3 and S4).

Based on the above findings that MFC and HRM showed better performance in identifying patients featured by favorable and dismal prognosis, respectively, we asked whether combination of these two variables improved outcome prediction. Our analysis showed that this was indeed the case. As depicted in Figure 3C,D, patients with MFC- and no HRM had the best outcome, in terms of both OS and EFS. Patents with HRM had the worst OS and EFS; patients who were HRM- but MCF+ had an intermediate behavior. The limited number

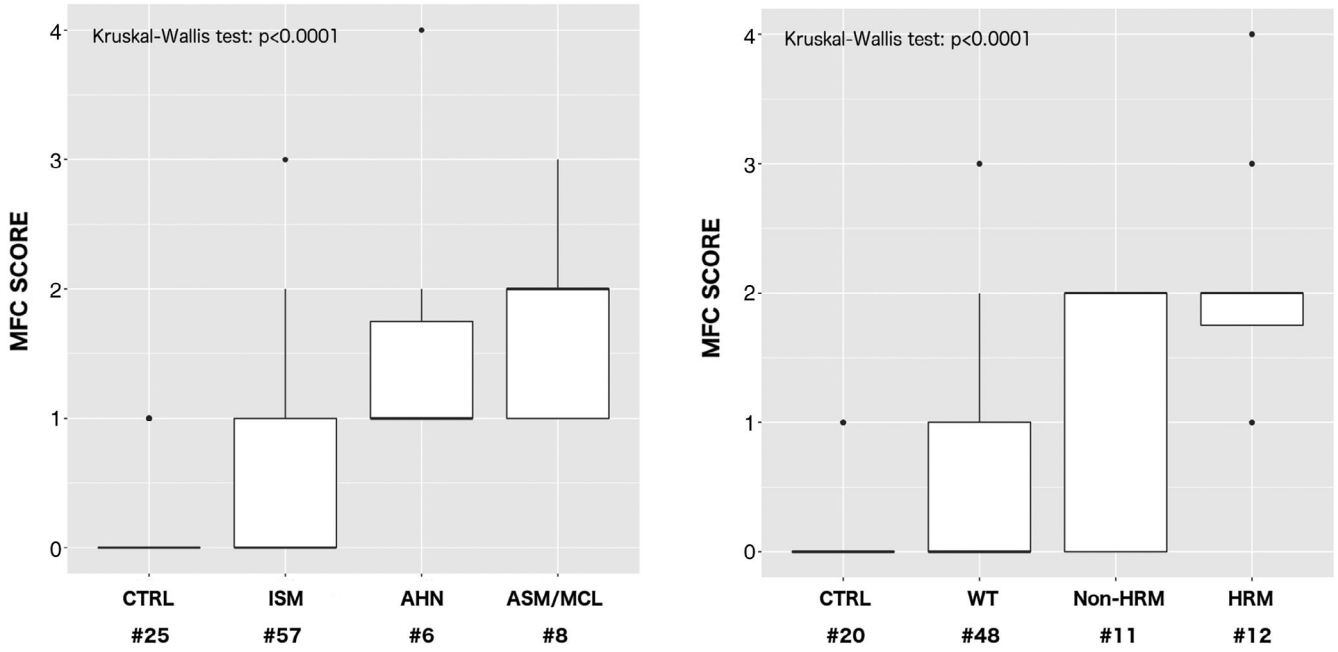

FIGURE 2 Distribution of MFC values according to WHO categories (A) and genotypic profile (B). Dots correspond to individual MFC values, the distribution of which is depicted also by box plots. Panel A: patients are grouped according to WHO clinical variants as indolent (ISM), mastocytosis with an associated hematological neoplasm (AHN), aggressive systemic mastocytosis or mast cell leukemia (ASM/MCL). Panel B: patients are grouped according to genotype as wild type for any studied gene (WT), bearing at least one high-risk mutation (HRM), and bearing at least one non-high-risk mutation (Non-HRM). In both panels, a group of healthy donors as control reference (CTRL) is presented. MFC, multi-parameter flow cytometry

of cases (n = 3) without MFC abnormalities, did not allow us to address the potential value of a phenotypic profile within the category of patients lacking HR mutations.

The fact that HR mutations almost exclusively occur in advanced variants largely prevents any meaningful use of this parameter for prediction of disease progression in patients with non-advanced SM. Conversely, we found that the presence of MFC-assessed dysplasia deserved a prognostic relevance in this subgroup; in fact, EFS resulted significantly shorter in MFC+ compared to MFC- patients [median EFS 117.0 (95% CI 8.9-225.0) vs not reached, $P = .0011$] (Figures S5 and S6).

Finally, we carried out multivariable analysis for OS and EFS, including age and WHO variant, in two steps: first by including MFC and HRM as separate variables, then by combining them for the above specified 3-tier stratification (Table S3). As regarded OS, the WHO category remained the only significant variable (HR 4.42, 95%CI 1.62-12.03; $P = .0035$). Conversely, regarding EFS, the combination of MFC and HRM performed better than other parameters, and overcame the value of WHO variant, remaining the only statistically significant variable (HR 8.54, 95% CI 2.32-31.39; $P = .001$).

4 | DISCUSSION

Current paradigm of treatment of SM envisions a conservative approach for indolent forms, where life expectancy is not impaired significantly. Cytoreductive treatment is indicated for advanced variants, where the need to control manifestations of myeloproliferation, and to prevent damage to target organs, overtake the potential side effects of therapies. Although the clinical manifestations of advanced SM often mandate early initiation of treatment, in other cases the distinction between mediator-related signs/symptoms and "true" organ damage may be difficult. In this context, the risk on one side is to over treat with cytoreductive drugs, on the other to overlook subtle disease-related issues that might deserve a timely therapeutic intervention to avoid further damage. Therefore, several potential biomarkers of disease-related damage in SM, including clinical, hematologic and molecular variables, have been explored, with the aim to find biomarkers that might help in clinical decisions. The integration of such variables recently led to the development of two prognostic models by Tefferi and coworkers at Mayo Clinic. One was based upon clinical parameters only, the second including clinical and genomic findings.[24] These models were shown to perform accurately and were validated in an independent patients' set from our database.[10]

It is reasonable to argue that, among the different variables shown to impact survival, those that are based on acquired molecular abnormalities that drive the clonal proliferation, and/or contribute to clonal progression, are the most objective and pathogenetically relevant. However, at large variance with other myeloid neoplasms, the pattern of the mutation profile in SM tends to closely reflect the classification of the different WHO-defined clinical entities, a distinction that has prognostic value per se. In fact, HRM are largely confined to WHO-defined advanced forms, where they may contribute to further stratify prognostic categories of patients,[25] but fail in providing relevant information regarding progression and outcome in the unadvanced

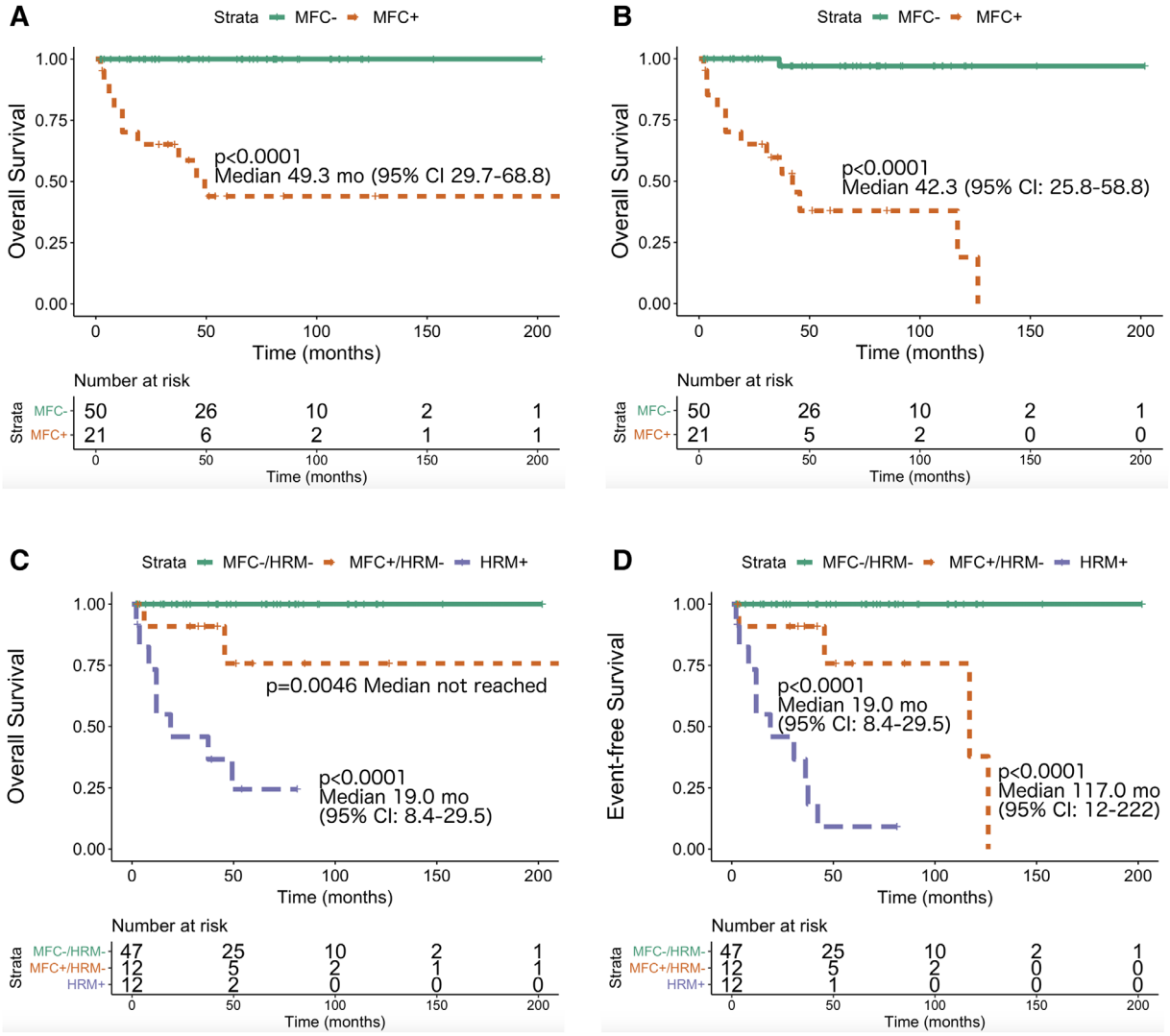

FIGURE 3 Overall (OS) and event-free (EFS) survival according to MFC score (A-B) and to the combination of MFC and genotypic profile (C-D). Overall (A) and event-free (B) survival according to MFC-assessed score: equal to or less than one phenotypic abnormality (MFC-) compared to more than two (MFC+). In panels C-D, patients were stratified in three tiers based on the combination of MFC and genotype: MFC-score 0-1 and no-HRM (green solid line) vs MFC-score 2-4 and no-HRM (red dashed line) vs HRM+ (blue dashed line); overall survival (panel C) and event-free (panel D) survival. *P* values for comparisons were obtained by the log-rank test. MFC, multi-parameter flow cytometry

variants. Therefore, improving the capacity to predict outcomes in patients diagnosed with unadvanced forms of SM, represents an unmet clinical need.

To address this issue, we investigated whether analysis of dysplastic traits of different cellular compartments of hematopoietic cells, performed with an immunophenotypic approach, might provide clues to outcome in patients with SM. Aberrant expression of CD2, CD25 and CD30 on neoplastic mast cells represents useful diagnostic information, but does not add to prognosis assessment. On the other hand, data from the REMA Spanish group indicate that multi-lineage vs uni-lineage involvement by *KIT* mutation has prognostic relevance, since it is associated with dismal outcome.[11] Furthermore, an immature phenotypic profile of mast cells represents an excellent surrogate marker for a *KIT*-mutated status across hematopoiesis.[26] However, these approaches are technically demanding and require ad hoc sorting facilities and expertise, therefore they cannot be implemented in a routine diagnostic process. On the other hand, characterization of dysplastic features of BM cells has been largely explored in the settings of MDS. This results in a clinically validated score[20-22] that relies on few core parameters. It is normalized on internal reference (ie, lymphocytes), and as such may be relatively easy to derive from standard MFC approaches that are employed at the time of diagnosis.

In the present study, we applied the MFC score to a cohort of 71 SM cases that were concurrently genotyped for non-*KIT* additional somatic mutations configuring a HRM category, as previously reported. Our data indicate that MFC score allows to identify two separate patients' categories. They are characterized by significantly different clinical and laboratory features at disease presentation, that overall resulted closely congruent with the distribution of WHO-categories (Table 1). On the other hand, when we grouped together

patients according to their WHO-clinical variant and genotype, we observed progressively higher MFC score points from non-aggressive to advanced forms (Figure 2A,B). While such a correlation could have been reasonably predicted based on the established association of advanced forms with HRM, we were intrigued to observe that patients devoid of high-risk mutations, therefore predicted to have better prognosis, yet showed higher median MFC values than patients without any non-*KIT* additional mutation (Figure 2B). We interpreted this finding as the capability of MFC to provide evidence of a latent underlying BM clonal disorder, consistent with previous observations about phenotypic aberrancies in individuals with cytopenias of undetermined significance.[27]

Indeed, we found that MFC-defined categories correlated with significantly different outcomes, as assessed by both overall and event-free survival. In particular, in the absence of phenotypic abnormalities (MCF-), no events of progression to advanced forms or deaths were observed (Figure 3A,B). Confirming previous reports, patients in the HRM category had dismal outcomes (Figure 3C,D illustrating OS and EFS, respectively) as compared to patients without HRM. Remarkably, among the latter, those presenting with an MCF+ score had significantly shorter OS and EFS compared to MCF- (Figure 3C, D), indicating that the MFC score might refine prognostic accuracy in cases where the mutation profile is silent. Of importance, the prognostic value of MFC remained valid also when applied specifically to unadvanced forms (Figures S5 and S6), especially regarding EFS (P = .001) with a trend to reduced OS (P = .07). Finally, we observed that the integration of the two parameters (MFC and HRM) behaved as an independent predictor for EFS and OS, also overcoming WHO-categories in multivariate analysis specifically addressing EFS (Table 3).

Overall, our results support a step-wise application of the above prognostic variables that can be easily collected at the time of SM diagnostic pathway. A high-risk genotype points to patients with unfavorable outcomes that have to be considered candidates for intensive treatment modalities, including inclusion in clinical trials. On the other hand, for patients lacking HR mutations, the evaluation of MFC is easily attainable from standard testing used for diagnostic purposes. It might contribute to identifying those patients with very favorable outcomes, from others where close monitoring is indicated, due to the potentially progressive nature of the disease itself. On that view, MFC abnormalities might also contribute to refine SSM definition, specifically when relying on morphologic dysplasia to meet B-findings.

We acknowledge that a pitfall of our study is represented by small sample size, especially when we focused the analysis on patients' subgroups; as such our data do not allow drawing definitive conclusions but need to be confirmed in larger studies.

ACKNOWLEDGMENTS

This work was supported by AIRC 5x1000 call "Metastatic disease: the key unmet need in oncology" to MYNERVA project, #21267 (MYeloid NEoplasms Research Venture AIRC). A detailed description of the MYNERVA project is available at http://www.progettoagimm.it. Supported also by a grant from Ministero della Salute GR-2013-02358238 to FM and Ministero della Salute GR-2016-02362631 to LP.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

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REFERENCES

1. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127(20):2391-2405.
2. Valent P, Akin C, Metcalfe DD. Mastocytosis: 2016 updated WHO classification and novel emerging treatment concepts. *Blood*. 2016; 129(11):1420-1427.
3. Pieri L, Bonadonna P, Elena C, et al. Clinical presentation and management practice of systemic mastocytosis. A survey on 460 Italian patients. *Am J Hematol*. 2016;91(7):692-699.
4. Vannucchi A, Lasho T, Guglielmelli P, et al. Mutations and prognosis in primary myelofibrosis. *Leukemia*. 2013;27(9):1861-1869.
5. Guglielmelli P, Lasho TL, Rotunno G, et al. MIPSS70: mutation-enhanced international prognostic score system for transplantation-age patients with primary myelofibrosis. *J Clin Oncol*. 2017;36(4): 310-318.
6. Schwaab J, Schnittger S, Sotlar K, et al. Comprehensive mutational profiling in advanced systemic mastocytosis. *Blood*. 2013;122(14): 2460-2466.
7. Pardanani A, Lasho T, Elala Y, et al. Next-generation sequencing in systemic mastocytosis: derivation of a mutation-augmented clinical prognostic model for survival. *Am J Hematol*. 2016;91(9):888-893.
8. Jawhar M, Schwaab J, Schnittger S, et al. Additional mutations in SRSF2, ASXL1 and/or RUNX1 identify a high-risk group of patients with KIT D816V+ advanced systemic mastocytosis. *Leukemia*. 2016; 30(1):136-143.
9. Pardanani AD, Lasho TL, Finke C, et al. ASXL1 and CBL mutations are independently predictive of inferior survival in advanced systemic mastocytosis. *Br J Haematol*. 2016;175(3):534-536.
10. Mannelli F, Gesullo F, Rotunno G, et al. Validation of the Mayo alliance prognostic system for mastocytosis. *Blood Cancer J*. 2019;9(2):18.
11. Garcia-Montero AC, Jara-Acevedo M, Teodosio C, et al. KIT mutation in mast cells and other bone marrow hematopoietic cell lineages in systemic mast cell disorders: a prospective study of the Spanish Network on Mastocytosis (REMA) in a series of 113 patients. *Blood*. 2006;108(7):2366-2372.
12. Rozman M, Navarro J-T, Arenillas L, et al. Multilineage dysplasia is associated with a poorer prognosis in patients with de novo acute myeloid leukemia with intermediate-risk cytogenetics and wild-type NPM1. *Ann Hematol*. 2014;93(10):1695-1703.
13. Weinberg OK, Seetharam M, Ren L, et al. Clinical characterization of acute myeloid leukemia with myelodysplasia-related changes as defined by the 2008 WHO classification system. *Blood*. 2009;113(9): 1906-1908.
14. Wang SA, Hasserjian RP, Tam W, et al. Bone marrow morphology is a strong discriminator between chronic eosinophilic leukemia, not otherwise specified and reactive idiopathic hypereosinophilic syndrome. *Haematologica*. 2017;102(8):1352-1360.

15. Tefferi A, Shah S, Reichard KK, Hanson CA, Pardanani A. Smoldering mastocytosis: survival comparisons with indolent and aggressive mastocytosis. *Am J Hematol*. 2019;94(1):E1-E2.
16. Valent P, Horny H-P, Escribano L, et al. Diagnostic criteria and classification of mastocytosis: a consensus proposal. *Leuk Res*. 2001;25(7):603-625.
17. Valent P, Akin C, Sperr WR, Horny H-PP, Metcalfe DD. Smouldering mastocytosis: a novel subtype of systemic mastocytosis with slow progression. *Int Arch Allergy Immunol*. 2002;127(2):137-139.
18. Matarraz S, López A, Barrena S, et al. Bone marrow cells from myelodysplastic syndromes show altered immunophenotypic profiles that may contribute to the diagnosis and prognostic stratification of the disease: a pilot study on a series of 56 patients. *Cytometry B Clin Cytom*. 2010;78(3):154-168.
19. van de Loosdrecht AA, Ireland R, Kern W, et al. Rationale for the clinical application of flow cytometry in patients with myelodysplastic syndromes: position paper of an international consortium and the European LeukemiaNet Working Group. *Leuk Lymphoma*. 2012;54(3):472-475.
20. Satoh C, Dan K, Yamashita T, Jo R, Tamura H, Ogata K. Flow cytometric parameters with little interexaminer variability for diagnosing low-grade myelodysplastic syndromes. *Leuk Res*. 2008;32(5):699-707.
21. Ogata K, Porta MG, Malcovati L, et al. Diagnostic utility of flow cytometry in low-grade myelodysplastic syndromes: a prospective validation study. *Haematologica*. 2009;94(8):1066-1074.
22. Porta MG, Picone C, Pascutto C, et al. Multicenter validation of a reproducible flow cytometric score for the diagnosis of low-grade myelodysplastic syndromes: results of a European LeukemiaNET study. *Haematologica*. 2012;97(8):1209-1217.
23. Escribano L, Diaz-Agustin B, López A, et al. Immunophenotypic analysis of mast cells in mastocytosis: when and how to do it. Proposals of the Spanish network on Mastocytosis (REMA). *Cytometry B Clin Cytom*. 2004;58B(1):1-8.
24. Pardanani A, Shah S, Mannelli F, et al. Mayo alliance prognostic system for mastocytosis: clinical and hybrid clinical-molecular models. *Blood Adv*. 2018;2(21):2964-2972.
25. Jawhar M, Schwaab J, Meggendorfer M, et al. The clinical and molecular diversity of mast cell leukemia with or without associated hematologic neoplasm. *Haematologica*. 2017;102(6):1035-1043.
26. Teodosio C, García-Montero A, Jara-Acevedo M, et al. An immature immunophenotype of bone marrow mast cells predicts for multilineage D816V KIT mutation in systemic mastocytosis. *Leukemia*. 2011;26(5):951-958.
27. Kern W, Haferlach C, Schnittger S, Alpermann T, Haferlach T. Serial assessment of suspected myelodysplastic syndromes: significance of flow cytometric findings validated by cytomorphology, cytogenetics, and molecular genetics. *Haematologica*. 2013;98(2):201-207.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Mannelli F, Gesullo F, Rotunno G, et al. Myelodysplasia as assessed by multiparameter flow cytometry refines prognostic stratification provided by genotypic risk in systemic mastocytosis. *Am J Hematol*. 2019; 1–8. https://doi.org/10.1002/ajh.25506