Leukemia (2014) **28**, 1494–1500

www.nature.com/leu

OPEN

ORIGINAL ARTICLE

CALR and *ASXL1* mutations-based molecular prognostication in primary myelofibrosis: an international study of 570 patients

A Tefferi[1], P Guglielmelli[2], TL Lasho[1], G Rotunno[2], C Finke[1], C Mannarelli[2], AA Belachew[1], A Pancrazzi[2], EA Wassie[1], RP Ketterling[3], CA Hanson[4], A Pardanani[1] and AM Vannucchi[2]

Current prognostication in primary myelofibrosis (PMF) is based on the dynamic international prognostic scoring system (DIPSS)-plus, which employs clinical and cytogenetic variables. We recently reported DIPSS-plus independent prognostic significance for calreticulin (*CALR*) (favorable) and *ASXL1* (unfavorable) mutations. In the current study, 570 PMF patients were recruited for derivation ($n = 277$) and validation ($n = 293$) of a molecular prognostic model based on these two mutations. Survival was the longest in $CALR^+ ASXL1^-$ (median 10.4 years) and shortest in $CALR^- ASXL1^+$ patients (median, 2.3 years; hazard ratio (HR), 5.9; 95% confidence interval (CI), 3.5–10.0). $CALR^+ ASXL1^+$ and $CALR^- ASXL1^-$ patients had similar survival and were grouped together in an intermediate-risk category (median survival, 5.8 years; HR, 2.5; 95% CI, 1.5–4.0). The *CALR*/*ASXL1* mutations-based prognostic model was DIPSS-plus independent ($P < 0.0001$) and effective in identifying low-/intermediate-1-risk patients with shorter (median, 4 years) or longer (median 20 years) survival and high-/intermediate-2-risk patients with shorter (median, 2.3 years) survival. Multivariable analysis distinguished $CALR^- ASXL1^+$ mutational status as the most significant risk factor for survival: HR 3.7 vs 2.8 for age >65 years vs 2.7 for unfavorable karyotype. These observations signify immediate clinical relevance and warrant i) *CALR* and *ASXL1* mutation determination in all patients with PMF and ii) molecular revision of DIPSS-plus.

Leukemia (2014) **28,** 1494–1500; doi:10.1038/leu.2014.57

INTRODUCTION

Karyotype and somatic mutations have a major part in disease prognostication and management of patients with myeloid malignancies. For example, the presence of unfavorable karyotype is prognostically detrimental in both acute myeloid leukemia (AML) and chronic myeloid neoplasms and is often an indication for treatment with allogeneic stem cell transplant (ASCT). The latter is also the preferred treatment of choice in AML associated with *FLT3*-ITD, whereas chemotherapy alone might be adequate for AML patients expressing *NPM1* mutations without *FLT3*-ITD.[1] Similarly, the prognostic relevance of mutations in myelodysplastic syndromes[2] and primary myelofibrosis (PMF)[3] has been recognized but not yet implemented in clinical practice.

Somatic mutations have now been incorporated into formal diagnostic criteria in myeloproliferative neoplasms (MPNs).[4] *JAK2* or *MPL* mutations are found in 50–70% of patients with PMF or essential thrombocythemia (ET) and calreticulin (*CALR*) mutations account for the majority of the remaining cases;[5,6] in strictly World Health Organization-defined disease, *CALR* mutations were seen in 49% of ET and 74% of PMF patients not expressing mutant *JAK2* or *MPL*.[7,8] In ET, *CALR* mutations correlated with male sex, younger age, lower leukocyte count, lower hemoglobin level and higher platelet count[8] and in PMF with younger age, higher platelet count and lower incidences of anemia, leukocytosis and spliceosome mutations.[7] Furthermore, *CALR* mutations in ET were associated with longer thrombosis-free survival[8,9] and in PMF with longer overall survival.[7]

Before the discovery of *CALR* mutations in ET and PMF, we had identified mutant *ASXL1* as dynamic international prognostic scoring system (DIPSS)-plus[10] and IPSS[11] independent risk factor for survival in PMF.[3] More recently, we discovered the prognostic synergism between *CALR* and *ASXL1* mutations in PMF and highlighted the inferior survival associated with '$CALR^- ASXL1^+$' mutational status.[7] The main objective of the current study was to further explore the prognostic interaction between *CALR* and *ASXL1* mutations in PMF with the intent to derive (using a patient cohort from the Mayo Clinic, USA) and validate (using a patient cohort from the University of Florence, Italy) a molecular prognostic model, in the context both DIPSS-plus and IPSS.

MATERIALS AND METHODS

The current study was approved by the institutional review boards of the Mayo Clinic, Rochester, MN, USA and University of Florence, Florence, Italy. All patients provided informed written consent for study sample collection as well as permission for its use in research. Inclusion to the current study required availability of archived peripheral blood or bone marrow sample collected at the time of diagnosis or first referral; a total of 277 patients from the Mayo Clinic (the Mayo cohort) and 293 from the University of Florence (the Florence cohort) met these stipulations. The diagnoses of PMF and leukemic transformation were according to World Health Organization criteria.[12] Unfavorable karyotype designation and DIPSS-plus or IPSS risk categorization were as previously described.[10,11,13]

We used published methods to screen for *CALR*, *JAK2*, *MPL*, *ASXL1*, *EZH2*, *IDH1*, *IDH2* and spliceosome (*SRSF2*, *SF3B1*and *U2AF1*) mutations.[7,8,14–18] All statistical analyses considered clinical and laboratory parameters obtained at time of diagnosis (Florence cohort) or first referral (Mayo cohort). Differences in the distribution of continuous variables between categories were analyzed by either Mann–Whitney (for comparison of two groups) or Kruskal–Wallis (comparison of three or more groups) test.

[1]Division of Hematology, Department of Medicine, Mayo Clinic, Rochester, MN, USA; [2]Laboratorio Congiunto MMPC, Department of experimental and clinical medicine, University of Florence, Florence, Italy; [3]Division of Cytogenetics, Department of Medicine, Mayo Clinic, Rochester, MN, USA and [4]Division of Hematopathology, Department of Medicine, Mayo Clinic, Rochester, MN, USA. Correspondence: Proffesor A Tefferi, Division of Hematology, Department of Medicine, Mayo Clinic, 200 First Street, SW, Rochester, MN 55905, USA.
E-mail: tefferi.ayalew@mayo.edu
Received 8 January 2014; accepted 30 January 2014; accepted article preview online 5 February 2014; advance online publication, 28 February 2014

Patient groups with nominal variables were compared by chi-square test. Overall survival analysis was considered from the date of diagnosis (Florence cohort) or first referral (Mayo cohort) to date of death (uncensored) or last contact (censored). Date of leukemic transformation replaced date of death, as the uncensored variable, for estimating leukemia-free survival. Overall and leukemia-free survival curves were prepared by the Kaplan–Meier method and compared by the log-rank test. Cox proportional hazard regression model was used for multivariable analysis. *P*-values <0.05 were considered significant. The Stat View (SAS Institute, Cary, NC, USA) statistical package was used for all calculations for the Mayo cohort and SPSS software was used for the Florence cohort.[8]

RESULTS

A total of 570 study patients were recruited from two centers: 277 from the Mayo Clinic (derivation cohort) and 293 from the University of Florence (validation cohort). Tables 1 and 2 summarize the presenting clinical and laboratory characteristics of the two patient cohorts, respectively.

Mayo Clinic patients (derivation cohort)

Phenotypic correlates. The information in Table 1 confirms our previous observations regarding the phenotypic correlates of *CALR* mutations in PMF, including younger age, higher platelet count, lower leukocyte count, lower frequency of anemia, lower DIPSS-plus risk distribution and lower incidence of spliceosome mutations.[7] The presence of *ASXL1* mutations in *CALR*-mutated patients was associated with the male gender ($P=0.03$), lower platelet count ($P=0.03$), lower hemoglobin level ($P=0.01$), higher transfusion requirement ($P=0.04$), higher circulating blast percentage ($P=0.02$), higher incidence of constitutional symptoms ($P=0.007$) and higher DIPSS-plus risk distribution ($P=0.04$). In other words, the *CALR* phenotype in PMF appeared to be adversely affected by the presence of *ASXL1* mutations.

Prognostic evaluation of CALR, ASXL1 and other relevant mutations. In multivariable analysis that included all prognostically relevant mutations,[3] absence of *CALR* (hazard ratio (HR), 2.6; 95% confidence interval (CI), 1.8–3.9), presence of *ASXL1* (HR, 2.0; 95% CI, 1.5–2.7) and presence of *SRSF2* (HR, 1.7; 95% CI, 1.1–2.8) mutations were significantly associated with shortened survival; *EZH2* ($P=0.07$) or *IDH* ($P=0.48$) mutations were not significant. Absence of *CALR* (HR, 2.3; 95% CI, 1.5–3.5) and presence of *ASXL1* (HR, 1.5; 95% CI, 1.1–2.1) mutations remained significant when DIPSS-plus risk stratification was added into the multivariable model. *CALR* and *ASXL1* mutational status were then considered together to further enhance their prognostic contribution; the longest survival was seen in $CALR^{+}ASXL1^{-}$ patients (median, 10.4 years), which was significantly better than that of $CALR^{-}ASXL1^{+}$

Table 1. Clinical and laboratory features of 277 patients with primary myelofibrosis (Mayo cohort), stratified by the presence or absence of CALR and ASXL1 mutations

Variables	*All patients* (n = *277*)	*CALR − ASXL1 +* (n = *62; 22%*)	*CALR − ASXL1 −* (n = *146; 53%*)	*CALR + ASXL1 +* (n = *23; 8%*)	*CALR + ASXL1 −* (n = *46; 17%*)	P-*value*
Age in years, median (range)	64 (32–87)	68 (39–81)	65 (35–87)	57 (42–70)	56 (32–82)	<0.0001
Age >65 years, *n* (%)	119 (43%)	40 (64.5%)	65 (44.5%)	4 (17.4%)	10 (21.7%)	<0.0001
Males (%)	177 (64.6%)	44 (71%)	89 (61%)	19 (82.6%)	25 (54.3%)	0.06
Hemoglobin, g/dl; median (range)	10.4 (5.8–16.1)	10.0 (6.6–16.1)	10.3 (5.8–16.0)	10.6 (6.5–12.9)	11.3 (8.1–14.3)	0.01
Leukocytes, $\times 10^9$/l; median (range)	9.0 (1.0–218)	11.9 (1.9–146)	9.4 (1.0–218)	8.0 (1.8–26.5)	7.7 (3.5–44.0)	0.03
Platelets, $\times 10^9$/l; median (range)	240 (11–2466)	211 (11–1288)	208 (12–2466)	275 (76–563)	415 (57–1493)	<0.0001
Circulating blast %; median (range)	1 (0–15)	1 (0–11)	0 (0–14)	1 (0–15)	1 (0–12)	0.03
DIPSS-plus[a] *risk group*						
Low	34 (12.2%)	4 (6.5%)	14 (9.6%)	2 (8.7%)	14 (30.4%)	<0.0001
Intermediate-1	103 (37.2%)	22 (35.5%)	59 (40.4%)	10 (43.5%)	12 (26.1%)	
Intermediate-2	54 (19.5%)	4 (6.5%)	30 (20.5%)	5 (21.7%)	15 (32.6%)	
High	86 (31%)	32 (51.5%)	43 (29.5%)	6 (26.1%)	5 (10.9%)	
Constitutional symptoms, *n* (%)	94 (33.9%)	33 (53.2%)	44 (30.1%)	10 (43.5%)	7 (15.2%)	0.0002
Circulating blasts ⩾1%, *n* (%)	128 (46.2%)	26 (41.9%)	77 (52%)	4 (17.4%)	22 (47.8%)	0.02
Hemoglobin <10 g/dl, *n* (%)	131 (47.3%)	35 (56.4%)	76 (52%)	10 (43.5%)	10 (21.7%)	0.001
Transfusion requiring, *n* (%)	88 (31.8%)	26 (41.9%)	53 (36.3%)	5 (21.7%)	4 (8.7%)	0.0008
Leukocytes $>25\times10^9$/l, *n* (%)	43 (15.5%)	18 (29.0%)	19 (13%)	2 (8.7%)	4 (8.7%)	0.008
Leukocytes $>10\times10^9$/l, *n* (%)	119 (43%)	34 (54.8%)	66 (45.2%)	8 (34.8%)	11 (23.9%)	0.01
Platelets $<100\times10^9$/l, *n* (%)	57 (20.6%)	19 (30.6%)	32 (21.9%)	1 (4.3%)	5 (10.9%)	0.02
Platelets $>450\times10^9$/l, *n* (%)	51 (18.4%)	10 (16.4%)	19 (13%)	3 (13%)	19 (41.3%)	0.0002
IDH1/2-mutated, *n* (%) 'N' evaluable = 266	13 (4.9%)	4 (6.7%)	4 (2.9%)	1 (4.5%)	4 (8.7%)	0.39
SF3B1-mutated, *n* (%) 'N' evaluable = 254	18 (7.1%)	2 (3.3%)	15 (11.5%)	0 (0%)	1 (2.4%)	0.04
U2AF1-mutated; *n* (%) 'N' evaluable = 245	39 (15.9%)	18 (30.5%)	20 (15.5%)	0 (0%)	1 (2.6%)	0.0004
SRSF2-mutated, *n* (%) 'N' evaluable = 266	30 (11.3%)	11 (18.0%)	17 (12.4%)	0 (0%)	2 (4.3%)	0.05
U2AF1/SRSF2/SF3B1 mutated 'N' evaluable = 240; *n(%)*	81 (33.7%)	29 (49.1%)	48 (38.1%)	0 (0%)	4 (10.2%)	<0.0001
EZH2-mutated, *n* (%) 'N' evaluable = 251	14 (5.6%)	4 (6.7%)	6 (4.7%)	1 (5%)	3 (7%)	0.9
Cytogenetic categories, n *(%)* 'N' *evaluable = 274*						
Normal	177 (64.6%)	49 (80.3%)	82 (56.6%)	16 (69.6%)	30 (66.7%)	0.01
Abnormal	97 (35/4%)	12 (19.7%)	63 (43.4%)	7 (30.4%)	15 (33.3%)	
Cytogenetic categories, n *(%)* 'N' *evaluable = 274*						0.62
Favorable	248 (90.5%)	57 (93.4%)	129 (89%)	20 (87%)	42 (93.3%)	
Unfavorable	26 (9.5%)	4 (6.6%)	16 (11%)	3 (13%)	3(6.7%)	
Deaths, *n* (%)	189 (68%)	57 (92%)	101 (69%)	12 (52%)	19 (41%)	<0.0001
Documented leukemic transformations, *n* (%)	31 (11%)	9 (15%)	16 (11%)	1 (4%)	5 (11%)	0.7

Abbreviation: DIPSS, Dynamic International Prognostic Scoring System. The presence of 0, 1, '2 or 3' and ⩾4 adverse factors defines low, intermediate-1, intermediate-2 and high-risk disease. [a]DIPSS-plus, Dynamic International Prognostic Scoring System-plus (reference in text): DIPSS-plus uses eight independent predictors of inferior survival: age >65 years, hemoglobin <10 g/dl, leukocytes $>25\times10^9$/l, circulating blasts ⩾1%, constitutional symptoms, red cell transfusion dependency, platelet count $<100\times10^9$/l and unfavorable karyotype (that is, complex karyotype or sole or two abnormalities that include +8, −7/7q-, i(17q), inv(3), -5/5q-, 12p- or 11q23 rearrangement).

Table 2. Clinical and laboratory features of 293 patients with primary myelofibrosis (Florence cohort), stratified by the presence or absence of CALR and ASXL1 mutations

Variables	*All patients (n = 293)*	*CALR − ASXL1 + (n = 46; 16%)*	*CALR − ASXL1 − (n = 191; 65%)*	*CALR + ASXL1 + (n = 11; 4%)*	*CALR + ASXL1 − (n = 45; 15%)*	P-*value*
Age in years, median (range)	61.8 (14–90)	66.6 (14–88)	62.4 (18–90)	60.2 (39–69)	48 (16–83)	<0.0001
Age >65 years, *n* (%)	120 (41)	24 (52.2)	87 (45.5)	1 (9.1)	8 (17.8)	<0.0001
Males (%)	180 (61.4)	33 (71.7)	112 (58.6)	9 (81.8)	26 (57.8)	0.181
Hemoglobin, g/dl, median (range)	11.8 (5.0–17.0)	12.5 (5.5–17.0)	10.3 (5.0–15.3)	11.2 (9.3–15.6)	11.7 (7.1–15.5)	0.001
Leukocytes, $\times 10^9$/l, median (range)	9.1 (1.9–106.1)	8.9 (2.0–90.8)	9.1 (1.9–106.1)	6.4 (2.2–12.7)	9.5 (3.5–24.4)	0.340
Platelets, $\times 10^9$/l, median (range)	328 (19–3279)	244 (25–2926)	325 (19–3279)	387 (46–775)	700 (123–1563)	<0.0001
Circulating blast %, mean (s.d.)	0.6 ± 2.0	2.1 ± 3.7	0.3 ± 1.2	0.3 ± 0.5	0.8 ± 1.7	<0.0001
IPSS[a] *risk group*						
Low	100 (36.8)	9 (20.5)	65 (36.1)	5 (36.1)	21 (51.2)	0.001
Intermediate-1	80 (29.4)	7 (15.9)	63 (35.0)	1 (14.3)	9 (22.0)	
Intermediate-2	43 (15.8)	12 (27.3)	25 (13.9)	0	6 (14.6)	
High	49 (18.0)	16 (36.4)	27 (15.0)	1 (14.3)	5 (12.2)	
Constitutional symptoms, *n* (%)	95 (32.6)	18 (39.1)	60 (31.7)	3 (27.3)	14 (31.1)	0.767
Circulating blasts ⩾1%, *n* (%)	66 (22.5)	20 (43.5)	29 (15.2)	5 (45.5)	12 (26.7)	<0.0001
Hemoglobin <10 g/dl, *n* (%)	82 (28.0)	19 (41.3)	43 (22.5)	5 (45.5)	15 (33.3)	0.027
Transfusion requiring, *n* (%)	78 (26.6)	26 (56.5)	41 (21.5)	1 (9.0)	10 (22.2)	<0.0001
Leukocytes $>25 \times 10^9$/l, *n* (%)	48 (16.4)	13 (28.3)	26 (13.6)	4 (36.4)	5 (11.1)	0.019
Leukocytes $>10 \times 10^9$/l, *n* (%)	141 (48.1)	23 (50.0)	89 (46.9)	7 (63.6)	22 (48.9)	0.726
Platelets $<100 \times 10^9$/l, *n* (%)	51 (17.4)	5 (10.8)	34 (17.8)	6 (54.5)	6 (13.3)	0.006
Platelets $>450 \times 10^9$/l, *n* (%)	127 (43.3)	11 (23.9)	79 (39.8)	7 (63.6)	33 (73.3)	<0.0001
IDH1/2-mutated, *n* (%) *'N'* evaluable = 277	6 (2.2)	3 (7.0)	3 (1.6)	0	0	0.108
SRSF2-mutated, *n* (%) *'N'* evaluable = 279	26 (9.3)	6 (13.6)	18 (9.8)	1 (10.0)	1 (2.4)	0.345
EZH2-mutated, *n* (%) *'N'* evaluable = 292	17 (5.8)	7 (15.2)	7 (3.7)	1 (9.1)	2 (14.4)	0.025
Cytogenetic categories, n *(%) 'N' evaluable = 153*						
Normal	98 (64.1)	18 (62.1)	60 (61.2)	2 (50.0)	18 (81.8)	0.290
Abnormal	55 (35.9)	11 (37.9)	38 (38.8)	2 (50.0)	4 (18.2)	
Abnormal karyotypes, n *(%) 'N' evaluable = 55*						
Favorable	43 (78.2)	9 (81.8)	30 (79.0)	2 (100)	2 (50.0)	0.932
Unfavorable	12 (21.8)	2 (22.2)	8 (21.0)	0	2 (50.0)	
Acute leukemia, *n* (%)	35 (11.9)	8 (17.4)	21 (11.0)	4 (36.4)	2 (4.4)	0.018
Death, *n* (%)	81 (27.6)	24 (52.2)	47 (24.6)	4 (36.4)	6 (13.3)	<0.0001
Follow-up, months, median (range)	44.3 (3.9–333.7)	32.6 (4.7–275.6)	42.9 (3.9–333.7)	91.9 (10.6–286.3)	58.7 (5.6–331.6)	<0.0001

Abbreviation: IPSS, International Prognostic Scoring System. [a]IPSS, International Prognostic Scoring System (reference in text): IPSS uses five independent predictors of inferior survival: age >65 years, hemoglobin <10 g/dl, leukocytes $>25 \times 109$/l, circulating blasts ⩾1%, constitutional symptoms. The presence of 0, 1, 2 and ⩾3 adverse factors defines low, intermediate-1, intermediate-2 and high-risk disease.

patients (median, 2.3 years; HR, 5.9; 95% CI, 3.5–10.0) and *CALR*$^-$*ASXL1*$^-$ patients (median, 5.4 years; HR, 2.3, 95% CI, 1.6–4.2). The difference in survival between *CALR*$^+$*ASXL1*$^-$ and *CALR*$^+$*ASXL1*$^+$ patients (median, 7.8 years; HR, 1.7; 95% CI, 0.9–3.6) was of borderline significance ($P = 0.13$). On the other hand, survival was not significantly different between patients with *CALR*$^+$*ASXL1*$^+$ and *CALR*$^-$*ASXL1*$^-$ mutational status ($P = 0.2$).

Construction of a prognostic model based on CALR and ASXL1 mutational status. Based on the above-stated observations, we constructed a three-tier molecular risk stratification on the basis of *CALR*/*ASXL1* mutational status: low risk included *CALR*$^+$*ASXL1*$^-$ patients (median survival, 10.4 years); high risk included *CALR*$^-$*ASXL1*$^+$ patients (median survival, 2.3 years; HR, 5.9; 95% CI, 3.5–10.0); and intermediate risk included either *CALR*$^+$*ASXL1*$^+$ or *CALR*$^-$*ASXL1* (median survival, 5.8 years; HR, 2.5; 95% CI, 1.5–4.0). This *CALR*/*ASXL1* mutations-based prognostic model (Figure 1) was DIPSS-plus independent ($P < 0.0001$) and highly effective in identifying short- (median survival, 4 years; HR, 10.3; 95% CI, 3.3–31.9) and long-term (median survival, 20 years) survivors with DIPSS-plus low-/intermediate-1-risk disease (Figure 2) and short-term survivors with high-/intermediate-2-risk disease (median survival, 2.0 years; HR 2.0; 95% CI, 1.1–3.7) (Figure 3). Conversely, DIPSS-plus risk stratification showed added value in molecularly low ($P = 0.0005$) or intermediate ($P < 0.0001$) risk disease but not in high-risk disease ($P = 0.21$).

Multivariable analysis that included the *CALR*/*ASXL1* mutations-based molecular risk stratification along with all eight DIPSS-plus risk factors distinguished *CALR*$^-$*ASXL1*$^+$ mutational status as the most significant risk factor for survival (HR, 3.7) followed by age >65 years (HR, 2.8) and unfavorable karyotype (HR, 2.7). In this model, constitutional symptoms and circulating blast percentage lost their significance, whereas anemia, leukocytosis and thrombocytopenia remained significant. *CALR*$^-$*ASXL1*$^+$ mutational status was also associated with inferior leukemia-free survival ($P = 0.04$; HR, 3.3; 95% CI, 1.1–10.0) and its significance was reduced to borderline status ($P = 0.06$) during multivariable analysis that included unfavorable karyotype and platelet count $<100 \times 10^9$/l, which are previously recognized risk factors for leukemic transformation.[10]

University of Florence patients (validation cohort)

The phenotypic correlates of *CALR* mutations in the Florence cohort (Table 2) were mostly similar to those noted in the Mayo cohort (Table 1), but also showed some differences. As was the case in the Mayo cohort, *CALR*-mutated Florence patients were younger and displayed higher platelet count and lower IPSS risk scores, whereas the associations with leukocyte count, hemoglobin level and constitutional symptoms were less evident. As was

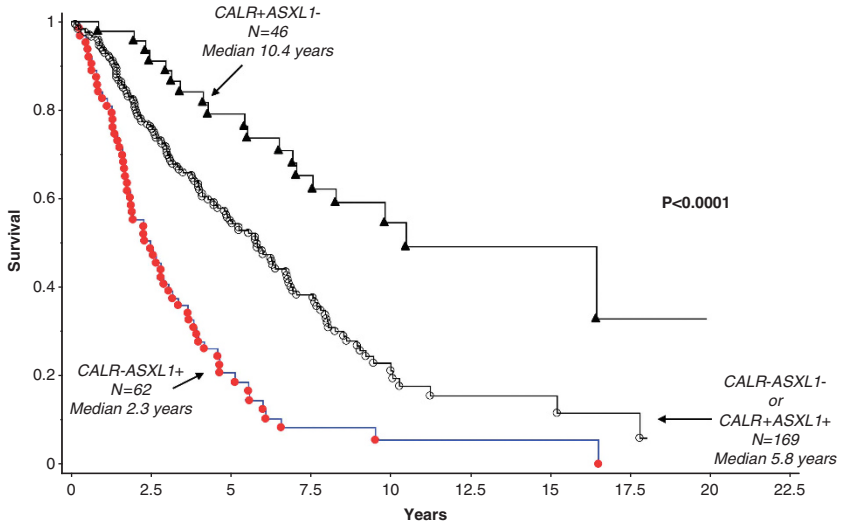

Figure 1. Kaplan–Meier estimates of overall survival in 277 Mayo Clinic patients with PMF, stratified by the presence or absence of *CALR* and *ASXL1* mutations.

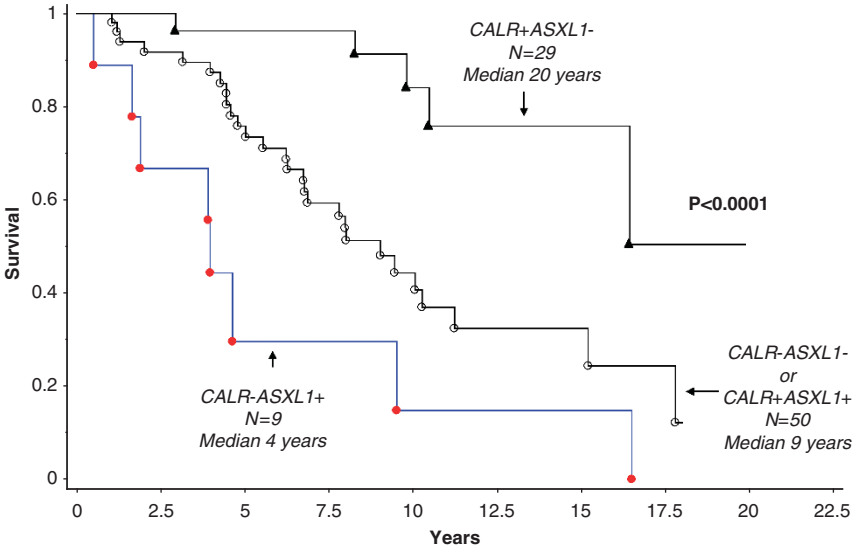

Figure 2. Kaplan–Meier estimates of overall survival in 88 Mayo Clinic patients with low- or intermediate-1-risk PMF, according to the DIPSS-plus,[10] stratified by the presence or absence of *CALR* and *ASXL1* mutations.

the case in the Mayo cohort, the presence of *ASXL1* mutations in *CALR*-mutated cases was detrimental and associated with higher rate of marked leukocytosis, circulating blast percentage and thrombocytopenia. In multivariable analysis that included IPSS risk scores, presence of mutant *CALR* had a favorable (HR, 0.5; 95% CI, 0.2–0.98) and mutant *ASXL1* an unfavorable (HR, 2.3; 95% CI, 1.4–3.8) impact on survival. A similar IPSS-inclusive multivariable analysis confirmed the independent prognostic value of the three-tier *CALR/ASXL1* risk stratification: HR (95% CI) were 6.4 (2.2–18.9) for $CALR^{-}ASXL1^{+}$ patients and 3.4 (1.2–9.4) for patients with $CALR^{+}ASXL1^{+}$ or $CALR^{-}ASXL1^{-}$ mutational status.

Application of the Mayo cohort-derived *CALR/ASXL1* mutations-based prognostic model was equally effective in delineating Florence patients with significant survival differences (Figure 4); median survivals were 'not reached' in low-risk patients ($CALR^{+}ASXL1^{-}$), 11.5 years (HR, 3.2; 95% CI, 1.3–8.1) in intermediate-risk patients ($CALR^{+}ASXL1^{+}$ or $CALR^{-}ASXL1$) and 3.2 years (HR, 8.7; 95% CI, 3.3–23.1) in high-risk patients ($CALR^{-}ASXL1^{+}$). The *CALR/ASXL1* mutations-based prognostic model was also effective in identifying short- (median survival 6 years; HR, 18.7; 95% CI, 2.3–153.9) and long-term (median survival not reached) survivors with IPSS low-/intermediate-1-risk disease (Figure 5) and short-term survivors with high-/intermediate-2-risk disease (median survival 3.1 years for molecular high risk and 3.7 years for molecular intermediate risk; HR 3.9, 95% CI 1.1–13.4 for molecular high risk and 3.0, 95% CI 0.9–9.8 for molecular intermediate risk) (Figure 6). In the Florence cohort, both $CALR^{-}ASXL1^{+}$ (HR, 8.0; 95% CI, 1.7–38.2) and $CALR^{+}ASXL1^{+}$ or $CALR^{-}ASXL1^{-}$ (HR, 4.5; 95% CI, 1.04–19.1) patients had inferior leukemia-free survival, compared with $CALR^{+}ASXL1^{-}$ patients.

DISCUSSION

Our observations from the current study of 570 patients with PMF confirm the prognostic advantage of harboring a *CALR* mutation, especially in the absence of a concomitantly

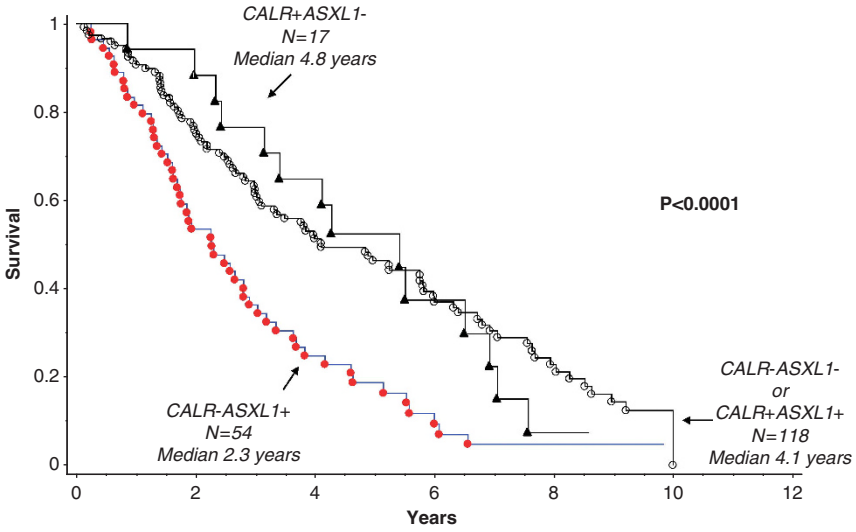

Figure 3. Kaplan–Meier estimates of overall survival in 189 Mayo Clinic patients with high- or intermediate-2-risk PMF, according to the DIPSS-plus,[8] stratified by the presence or absence of *CALR* and *ASXL1* mutations.

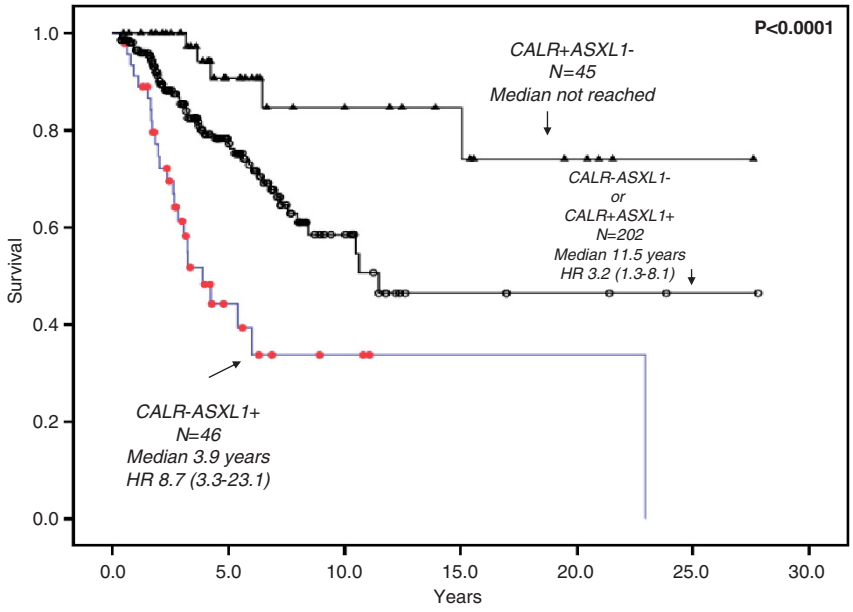

Figure 4. Kaplan–Meier estimates of overall survival in patients of the Italian series ($n = 293$) stratified according to the mutational status of *CALR* and *ASXL1*. The three tiers included $CALR^+/ASXL1^-$ ($n = 45$), $CALR^-/ASXL1^+$ ($n = 46$) and $CALR^-/ASXL1^-$ or $CALR^+/ASXL1^+$ ($n = 202$). The different survival curves were statistically significant, $P < 0.0001$. The HR is presented together with the 95% CI, using $CALR^+/ASXL1^-$ as the reference population (HR = 1.0).

occurring *ASXL1* mutation. Survival was best in the presence of *CALR* and absence of *ASXL1* mutation (that is, $CALR^+ASXL1^-$) and worst otherwise (that is, $CALR^-ASXL1^+$). This is somewhat similar to the scenario in AML with $NPM1^+FLT3\text{-ITD}^-$ vs $NPM1^-FLT3\text{-ITD}^+$ mutational status, respectively.[1] Furthermore, the presence of *CALR* mutations appears to attenuate, but not fully overcome, the unfavorable prognosis in *ASXL1*-mutated patients (that is, $CALR^+ASXL1^+$). Conversely, the absence of *ASXL1* mutations is associated with better survival, even in *CALR*-unmutated cases (that is, $CALR^-ASXL1^-$). DIPSS-plus had limited added value in molecularly defined high-risk patients (that is, $CALR^-ASXL1^+$) but was effective in identifying short-lived patients among the molecularly low- (that is, $CALR^+ASXL1^-$) and intermediate-risk (that is, $CALR^-ASXL1^-$ or $CALR^+ASXL1^+$) groups. On the other hand, although the value of molecular risk stratification was most evident in DIPSS-plus low-/intermediate-1-risk patients, it was also apparent in high-/intermediate-2-risk patients. These results were validated in an independent patient cohort and performed similarly in the context of IPSS.

How does one translate this new information into clinical practice? First, our observations provide strong evidence for the prognostic relevance of performing *CALR* and *ASXL1* mutation determination in all patients with PMF. Second, survival in PMF is significantly compromised (median <2.5 years) in the presence of either $CALR^-ASXL1^+$ mutation status or DIPSS-plus high-risk score. In other words, a high-risk molecular signature identifies DIPSS-plus low- or intermediate-1-risk patients whose survival might not be better than those with DIPSS-plus high-risk disease.

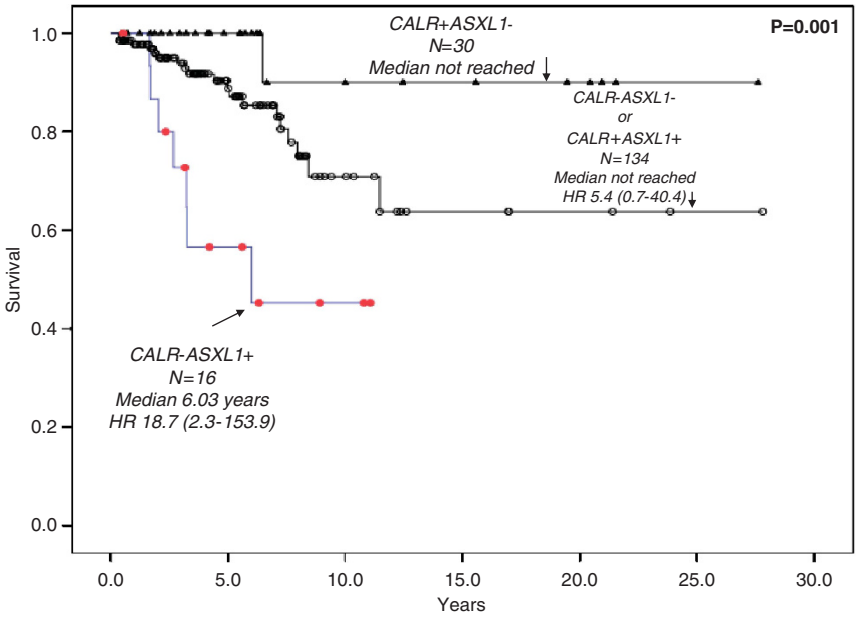

Figure 5. Kaplan–Meier estimates of overall survival in patients of the Italian series belonging to IPSS[11] low- and intermediate-1-risk category ($n=180$) stratified according to the mutational status of *CALR* and *ASXL1*. The three tiers included $CALR^+/ASXL1^-$ ($n=30$), $CALR^-/ASXL1^+$ ($n=16$) and $CALR^-/ASXL1^-$ or $CALR^+/ASXL1^+$ ($n=134$). The different survival curves were statistically significant, $P=0.001$. The HR is presented together with the 95% CI, using $CALR^+/ASXL1^-$ as the reference population (HR = 1.0).

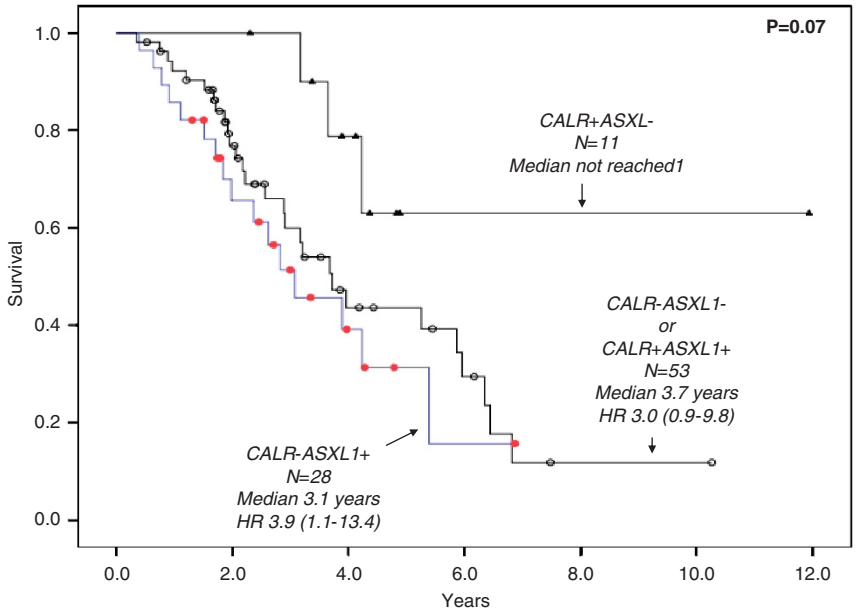

Figure 6. Kaplan–Meier estimates of overall survival in patients of the Italian series belonging to the IPSS[11] intermediate-2- and high-risk category ($n=92$) stratified according to the mutational status of *CALR* and *ASXL1*. The three tiers included $CALR^+/ASXL1^-$ ($n=11$), $CALR^-/ASXL1^+$ ($n=28$) and $CALR^-/ASXL1^-$ or $CALR^+/ASXL1^+$ ($n=53$). The different survival curves were trend significant, $P=0.07$ The HR is presented together with the 95% CI, using $CALR^+/ASXL1^-$ as the reference population (HR = 1.0).

Therefore, the risk of aggressive therapy, including ASCT, might be justified not only in DIPSS-plus high or intermediate-2-risk disease but also in low or intermediate-1-risk patients who harbor a high-risk molecular signature (that is, $CALR^-ASXL1^+$). On the other hand, aggressive therapy might be less urgent in DIPSS-plus intermediate-2-risk patients who do not express high-risk mutational profile (that is, $CALR^-ASXL1^+$). We were particularly gratified with the highly indolent nature of the disease (median survival ~20 years) in DIPSS-plus low/intermediate-1-risk patients with low-risk molecular profile (that is, $CALR^+ASXL1^-$) and the risk of ASCT or investigational drug therapy may not be justified in such patients. A similar scenario was confirmed in an independent cohort of patients, in the context of IPSS.

Drug therapy in PMF remains inadequate and there is no firm evidence yet that, beyond their value in reducing splenomegaly and improving symptoms, JAK inhibitors provide survival advantage.[19] In other words, there is a dire need for new drugs with disease-modifying activity and continuing value for ASCT. To that effect, accurate disease prognostication for the selection of appropriate patients for specific treatment strategies is of

paramount importance. The International Working Group for MPN research and treatment (IWG-MRT) has led the effort in this regard with the development of the IPSS in 2009.[11] IPSS was first revised to dynamic IPSS (DIPSS)[20] and subsequently to DIPSS-plus.[10] A key element of DIPSS-plus is karyotype, whose prognostic value was further highlighted by the more recent identification of 'very high risk' cytogenetic abnormalities, which included monosomal karyotype and inv(3)/i(17q) abnormalities.[21] The observations from the current study signify the additional importance of molecular information in disease prognostication in PMF and provide the basis for revising DIPSS-plus accordingly.

It is difficult and somewhat premature to speculate on the biological explanation for our observation because of our limited insight on the pathogenetic contribution of either *CALR* or *ASXL1* mutations. The adverse prognostic effect of *ASXL1* mutations is not specific to PMF and has been realized across different types of myeloid malignancies. Mutant *ASXL1* or loss of *ASXL1* induces myelodysplastic syndrome-like disease in mice, putatively through loss of PRC2-mediated histone methylation, which in turn leads to increased expression of HOXA9 and microRNA-125a.[22,23] Mutant *CALR* displays a significantly modified C terminal that is less acidic and lacks the endoplasmic reticulum retention motif. Currently, little is known about the consequences of *CALR* mutations on the function of wild-type protein, which includes regulation of intracellular calcium homeostasis and the control of protein folding/misfolding.[24] Transfection of mutant *CALR* into a Ba/F3 murine cell line was shown to induce IL-3-independent growth that was associated with increased STAT5 phosphorylation.[6] However, the particular observation needs to be validated and further elaborated upon. Regardless, it is possible that a *CALR*, compared with a *JAK2*-mutated clone is genetically more stable and biologically more capable of handling the epigenetic alteration associated with mutant *ASXL1*.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This study was supported by the Mayo Clinic Harvey-Yulman Charitable Foundation for Myelofibrosis Tissue Bank and Clinical Database of Molecular and Biological Abnormalities and by a special grant from Associazione Italiana per la Ricerca sul Cancro-'AIRC 5 per Mille'- to AGIMM, 'AIRC-Gruppo Italiano Malattie Mieloproliferative' (no. 1005) to AMV; for a description of the AGIMM project, see at http://www.progettoagimm.it). Partially supported by Ministero della Università e Ricerca (MIUR; FIRB project #RBAP11CZLK and PRIN 2010NYKNS7 to AMV).

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