

# Targeted deep sequencing in polycythemia vera and essential thrombocythemia

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## Key Points

- More than half of patients with PV or ET harbor DNA mutations/variants other than *JAK2/CALR/MPL*.
- The presence of some of these mutations adversely affects overall, leukemia-free, or myelofibrosis-free survival.

Polycythemia vera (PV) is characterized by *JAK2* and essential thrombocythemia (ET) by *JAK2*, calreticulin (*CALR*), and myeloproliferative leukemia virus oncogene (*MPL*) mutations; we describe the occurrence and prognostic relevance of DNA sequence variants/mutations other than *JAK2/CALR/MPL*. A myeloid neoplasm–relevant 27-gene panel was used for next-generation sequencing of bone marrow or whole blood DNA and conventional tools were used for analysis. “Adverse variants/mutations” were identified by age-adjusted multivariable analysis of impact on overall, leukemia-free, or myelofibrosis-free survival. Fifty-three percent of 133 Mayo Clinic patients with PV and 53% of 183 with ET harbored 1 or more sequence variants/mutations other than *JAK2/CALR/MPL*; the most frequent were *TET2* and *ASXL1*. “Adverse variants/mutations” in PV included *ASXL1*, *SRSF2*, and *IDH2* and in ET *SH2B3*, *SF3B1*, *U2AF1*, *TP53*, *IDH2*, and *EZH2*; combined prevalence was 15% and 15%, respectively. Adverse variants/mutations were associated with inferior survival in both PV (median, 7.7 vs 16.9 years) and ET (median, 9 vs 22 years) and the effect was independent of conventional prognostic models with respective hazard ratio (95% confidence interval) of 2.8 (1.5–5.1) and 2.6 (1.4–4.8); these observations were validated in 215 Italian patients with PV and 174 with ET. In both Mayo Clinic and Italian cohorts, leukemic or fibrotic progression was also predicted by adverse variants/mutations. Number of mutations did not provide additional prognostic information. We conclude that targeted deep sequencing in PV and ET allows for genetic risk stratification that is independent of clinically derived prognostic models.

## Introduction

Despite seminal descriptions of novel<sup>1–6</sup> and other less specific<sup>7,8</sup> mutations in the last 10 years, the molecular pathogenesis of myeloproliferative neoplasms (MPNs) remains incompletely understood; however, there has been increasing use of these mutations in diagnostics and disease prognostication.<sup>9</sup> Mutations in MPNs are operationally classified into those that involve *JAK2*, calreticulin (*CALR*), and myeloproliferative leukemia virus oncogene (*MPL*) and those that involve other genes, such as additional sex combs like transcriptional regulator 1 (*ASXL1*).<sup>9</sup> The former are believed to be “driver” mutations and are now formally integrated into the World Health Organization (WHO) diagnostic criteria for polycythemia vera (PV; 98% *JAK2* mutational frequency), essential thrombocythemia (ET; 60% *JAK2*, 22% *CALR*, and 3% *MPL* mutational frequency) and primary myelofibrosis (PMF).<sup>10</sup> In terms of disease

prognostication, type 1/type 1–like *CALR* mutations have been associated with superior survival in PMF<sup>11,12</sup> and *JAK2* mutations with thrombosis in ET.<sup>13</sup>

The prognostic contribution of mutations other than *JAK2*, *CALR*, or *MPL* has been demonstrated in PMF, where *ASXL1*, serine/arginine-rich splicing factor 2 (*SRSF2*), isocitrate dehydrogenase 1 or 2 (*IDH1/2*), and enhancer of zeste 2 polycomb repressive complex 2 subunit (*EZH2*) were identified as being unfavorable to survival.<sup>7</sup> More recent studies have suggested the prognostic relevance of not only the specific type but also the number of mutations.<sup>8,14</sup> These observations are noteworthy considering the increasing application of next-generation sequencing (NGS) technology in clinical practice. In the current study, we used custom-designed targeted sequencing of 27 myeloid neoplasm–relevant genes that are also often included in most commercially available NGS gene panels used in myeloid cancer. Our main objective was to determine the prognostic and phenotypic relevance of sequence variants/mutations, other than *JAK2*, *CALR* and *MPL*, in PV and ET.

## Methods

The current study was approved by the institutional review boards of the Mayo Clinic and the University of Florence, Florence, Italy. Diagnoses and treatment approaches were in accordance with what was considered standard of care at the time of initial diagnosis.<sup>15</sup> Targeted capture assays for Mayo Clinic patients (the main study cohort) were carried out on bone marrow or whole-blood DNA for the following genes: *TET2*, *DNMT3A*, *IDH1*, *IDH2*, *ASXL1*, *EZH2*, *SUZ12*, *SRSF2*, *SF3B1*, *ZRSR2*, *U2AF1*, *PTPN11*, *TP53*, *SH2B3*, *RUNX1*, *CBL*, *NRAS*, *JAK2*, *CSF3R*, *FLT3*, *KIT*, *CALR*, *MPL*, *NPM1*, *CEBPA*, *IKZF1*, and *SETBP1*. Additional details regarding NGS analysis are outlined in the supplemental Methods, which also includes the methods used in the validation cohort from the University of Florence. Prognostic evaluation of sequence variants/mutations considered both the “number” of sequence variants/mutations and the specific genes affected. “Adverse sequence variants/mutations” were identified by age-adjusted multivariable analysis of their impact on overall survival or multivariable analysis of impact on leukemia-free or myelofibrosis-free survival.

All statistical analyses considered clinical and laboratory parameters obtained at time of diagnosis. Differences in the distribution of continuous variables between categories were analyzed by either the Mann-Whitney or the Kruskal-Wallis test. Patient groups with nominal variables were compared by  $\chi^2$  test. Survival analysis was considered from the date of diagnosis to date of death or last contact; because of the considerable effect of age on survival, univariate analysis for overall survival was adjusted for age. Leukemia-free and myelofibrosis-free survival calculations considered the transformation events as the uncensored variable. Survival curves were prepared by the Kaplan-Meier method and compared by the log-rank test. The Cox proportional hazard regression model was used for multivariable analysis. *P* values <.05 were considered significant. All analyses were conducted using the Stat View (SAS Institute, Cary, NC).

## Results

A total of 316 Mayo Clinic patients with PV (*n* = 133) or ET (*n* = 183) were evaluated; Tables 1 and 2 outline the presenting clinical and laboratory details. “Driver” mutation distribution was 98% *JAK2* for PV and 52% *JAK2*, 26% *CALR*, and 4% *MPL* for ET. According to

conventional prognostic models,<sup>16,17</sup> survival risk distribution at time of diagnosis was 45% high, 26% intermediate, and 29% low for PV, and 21% high, 39% intermediate, and 40% low for ET. Median follow-up was 10.4 years for PV and 10.1 years for ET. The respective numbers of documented deaths, leukemic transformations, and fibrotic progressions were, for PV, 62 (47%), 7 (5.3%), and 14 (11%) and for ET 61 (33%), 6 (3.3%), and 27 (15%).

## Prevalence and cosegregation of sequence variants/mutations

Sequence variants/mutations other than *JAK2*, *CALR*, or *MPL* were seen in 70 patients with PV (52.6%) and 96 with ET (52.5%) (Figure 1); supplemental Table 1 provides additional information on specific variants/mutations. The respective percentages of patients with 1, 2, or  $\geq 3$  sequence variants/mutations were 30%, 20%, and 3% for PV and 41%, 8%, and 4% for ET. The most frequent sequence variants/mutations in both disorders were *TET2* and *ASXL1* (Figure 1). There were no significant associations between *JAK2/CALR/MPL* mutational status and number or type of other sequence variants/mutations. Among the most frequent sequence variants/mutations, *ASXL1* in ET cosegregated with *EZH2* (*P* < .001), *IDH2* (*P* = .004), and *RUNX1* (*P* = .002) and in PV with *IDH2* (*P* = .003) and *KIT* (*P* = .02); *TET2* in PV cosegregated with *SH2B3* (*P* = .01).

## Phenotypic correlations of sequence variants/mutations

Phenotypic correlations were examined for sequence variants/mutations with a recurrence rate of at least 5% (Figure 1). Parameters examined for possible association included age, sex, risk category,<sup>16,17</sup> karyotype, thrombosis history, leukocyte count, hemoglobin level, platelet count, palpable splenomegaly, pruritus, and microvascular symptoms. The most noteworthy association was between *TET2* variants/mutations and thrombosis in ET (*P* = .01), which was independent of both age and *JAK2/CALR/MPL* mutational status; relative risk on age-adjusted multivariable analysis was 3.4 (95% confidence interval [CI], 1.4-8.4), compared with 4.8 (95% CI, 1.6-14.2) for *JAK2* vs *CALR*. However, such association was not evident in either PV or an external cohort of 174 Italian patients with ET seen at the University of Florence (*P* = .58). In ET, *TET2* and *SF3B1* variants/mutations were associated with older age (*P* = .04 and 0.04, respectively), *SF3B1* with higher platelet count (*P* = .02), and *ASXL1* with palpable splenomegaly (*P* = .009). In PV, the only association noted was between *SH2B3* variants/mutations and palpable splenomegaly (*P* = .045).

## Prognostic relevance of sequence variants/mutations in PV

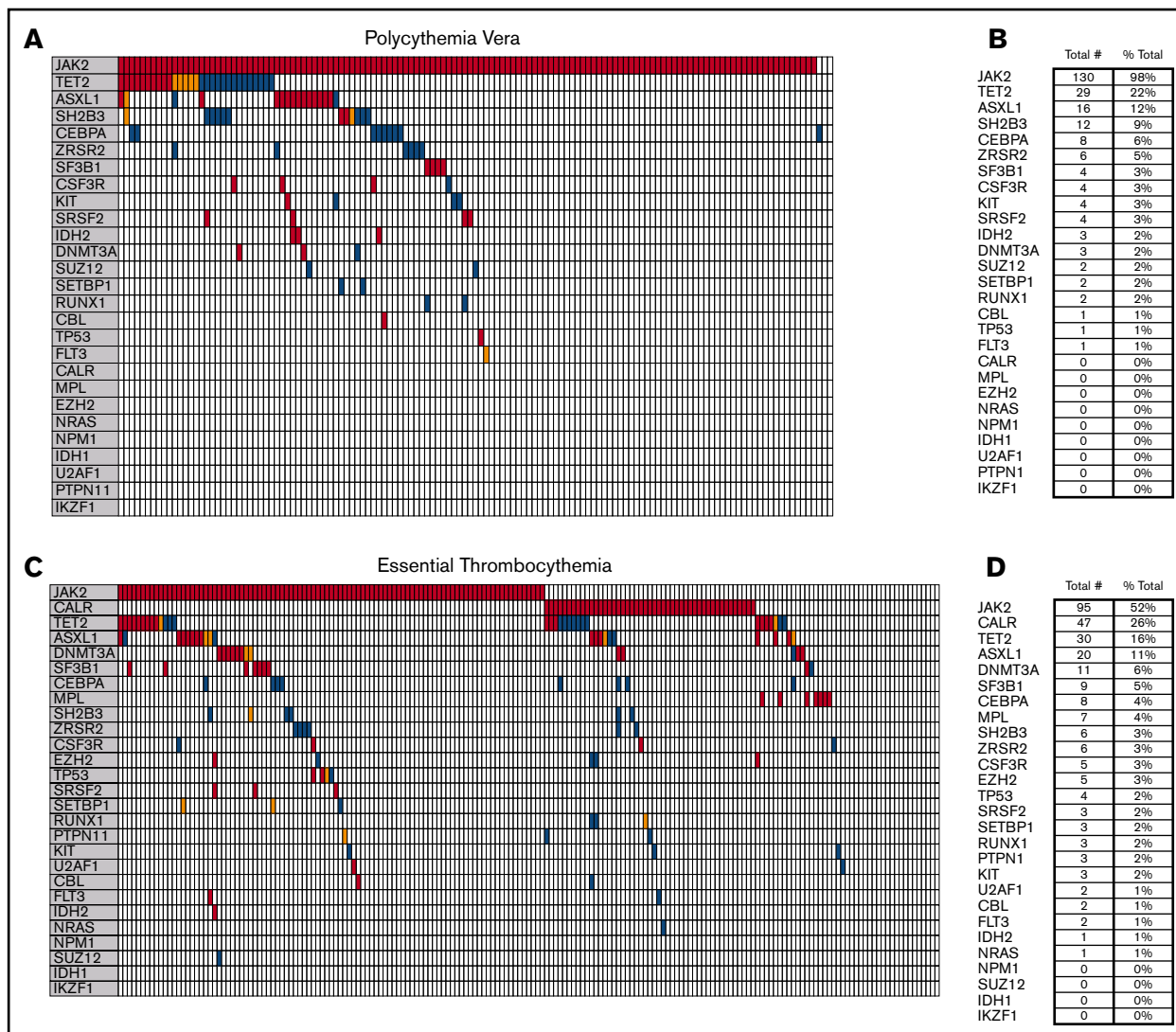
Median survival of the 133 Mayo Clinic patients with PV was 14.2 years. Age-adjusted univariate analysis identified *SRSF2*, *ASXL1*, and *IDH2* variants/mutations as being significantly associated with inferior survival; *ASXL1* (hazard ratio [HR], 2.2; 95% CI, 1.1-4.3) and *SRSF2* (HR, 6.1; 95% CI, 2.0-19.2) remained significant on multivariable analysis. For leukemia-free and myelofibrosis-free survival, univariate analysis showed significant associations with *SRSF2*, *IDH2*, and *RUNX1* for the former and with *SRSF2* and *RUNX1* for the latter; on multivariable analysis, *SRSF2* (HR, 74.5, 95% CI, 4.4-1261.7) and *IDH2* (HR, 55.5; 95% CI, 3.5-887.4) remained significant for leukemia-free survival and *SRSF2* (HR, 27.2; 95% CI, 2.7-274.3) for myelofibrosis-free survival.



**Table 2. Clinical and laboratory details: patients with ET**

ET	Mayo Clinic patients			University of Florence, Italy patients		
	All patients, n = 183	Patients with adverse variants, n = 27 (15%)	Patients without adverse variants, n = 156 (85%)	All patients, n = 174	Patients with adverse variants, n = 31 (18%)	Patients without adverse variants, n = 143 (82%)
Age, median (range), y	58 (15-90)	69.6 (24-88)	57 (15-90)	54.8 (17.8-85.0)	61.2 (33.5-81.1)	53.4 (17.8-85.0)
Female (%)	107 (59)	12 (44)	95 (61)	103 (59)	14 (45)	89 (62)
Hemoglobin, median (range), g/dL	13.6 (6.9-17.4)	13.3 (9-16.5)	13.8 (6.9-17.4)	14.2 (11.1-17.0)	13.7 (11.2-16.2)	14.2 (11.1-17.0)
Leukocytes, median (range), $\times 10^9/L$	8.8 (1.9-53.4)	10.1 (4-21.3)	8.6 (1.9-53.4)	8.9 (3.8-26.0)	9.7 (4.8-15.6)	8.5 (3.8-26.0)
Platelets, median (range), $\times 10^9/L$	900 (206-3036)	1014 (354-2466)	863 (206-3036)	786 (480-2000)	848 (654-1526)	778 (480-2000)
Thrombosis history at diagnosis	27 (15)	5 (19)	22 (14)	40 (23)	6 (19)	34 (24)
Cardiovascular risk factors, n (%)	89 (49)	18 (67)	71 (46)	73 (42)	14 (45)	59 (41)
Microcirculatory symptoms, n (%)	36 (20)	1 (4)	35 (22)	78 (45)	19 (61)	59 (41)
Palpable splenomegaly, n (%)	28 (15)	4 (15)	24 (15)	44 (25)	11 (36)	33 (23)
<b>IWG risk category, n (%)</b>						
Low	73 (40)	5 (19)	68 (44)	59 (34)	5 (16)	54 (38)
Intermediate	71 (39)	10 (37)	61 (39)	80 (46)	17 (55)	63 (44)
High	39 (21)	12 (44)	27 (17)	35 (20)	9 (29)	26 (18)
Abnormal karyotype, n (%)	23 (15), N evaluable = 150	2 (10)	21 (16)	3 (5), N evaluable = 65	0 (0)	3 (5)
<b>"Driver" mutation distribution, n (%)</b>						
JAK2	95 (52)	19 (70)	76 (49)	101 (58)	20 (65)	81 (57)
CALR	44 (24)	3 (11)	41 (26)	48 (28)	4 (13)	44 (31)
MPL	7 (4)	1 (4)	6 (4)	12 (7)	4 (13)	8 (6)
Triple-negative	37 (20)	4 (15)	33 (21)	13 (7)	3 (10)	10 (7)
<b>P</b>						
Age, median (range), y				<b>.002</b>		<b>.02</b>
Female (%)				.1		.06
Hemoglobin, median (range), g/dL				.08		.7
Leukocytes, median (range), $\times 10^9/L$				<b>.03</b>		<b>.04</b>
Platelets, median (range), $\times 10^9/L$				<b>.02</b>		.5
Thrombosis history at diagnosis				.6		.4
Cardiovascular risk factors, n (%)				<b>.04</b>		.4
Microcirculatory symptoms, n (%)				<b>.02</b>		.06
Palpable splenomegaly, n (%)				.9		.1
<b>IWG risk category, n (%)</b>				<b>.003</b>		<b>.05</b>
Low						
Intermediate						
High						
Abnormal karyotype, n (%)				.4		.6
<b>"Driver" mutation distribution, n (%)</b>				.2		.09
JAK2						
CALR						
MPL						
Triple-negative						

Presenting clinical and laboratory details of 183 Mayo Clinic patients with ET and 174 University of Florence, Italy patients with ET, stratified by the presence or absence of at least 1 "adverse sequence variant/mutation"; the letter in ET SH2B3, SF3B1, U2AF1, TP53, IDH2, or EZH2. Bold values represent statistically significant values.



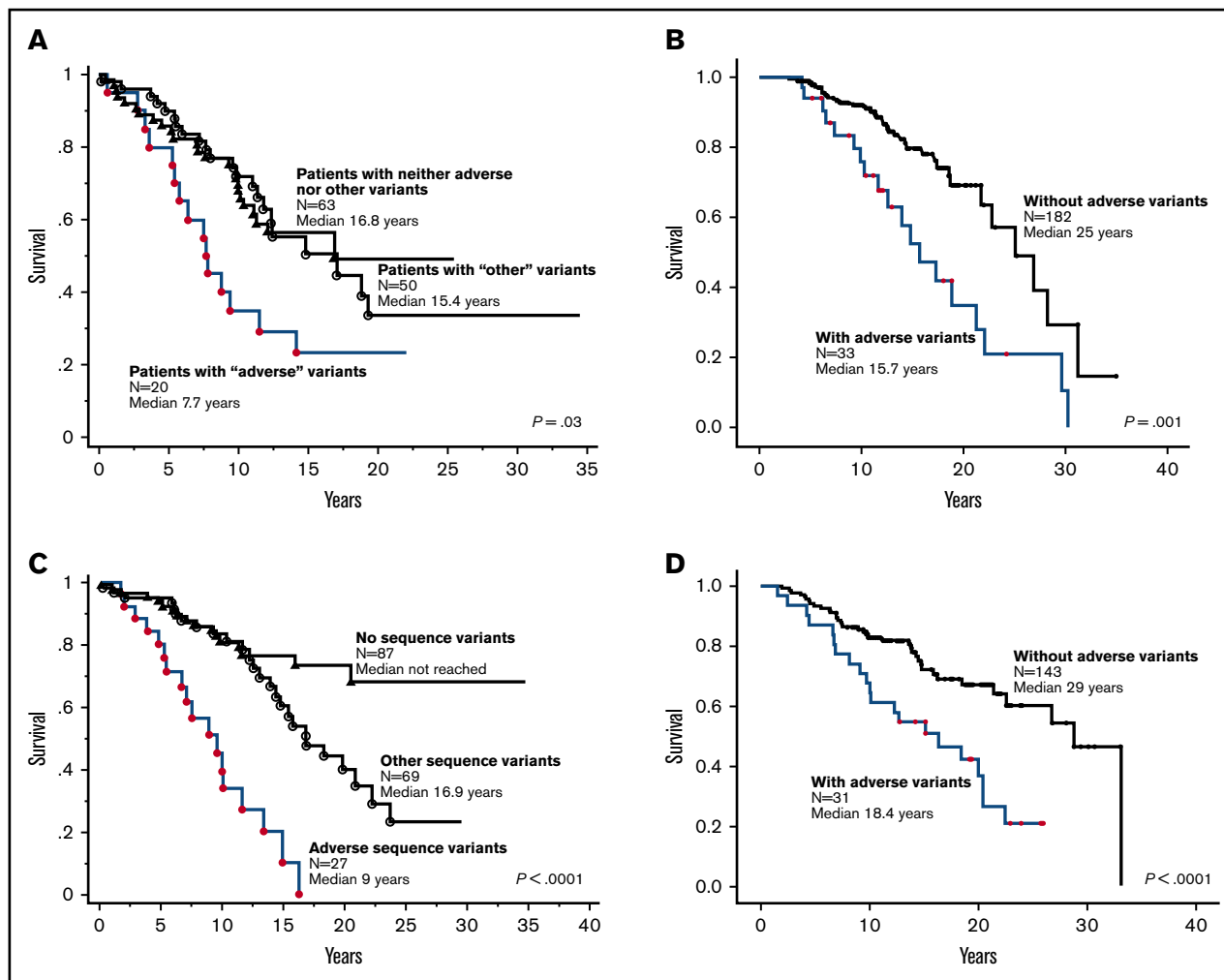
**Figure 1. Twenty-seven-gene panel DNA sequence variants in Mayo Clinic patients with PV (n = 133) and ET (n = 183).** (A,C) Individual variant/mutational cosegregation plot for both PV and ET. Each column represents 1 of the sequenced subjects. Variant/mutations are depicted by representative colored bars. Red, Variants previously associated with a hematologic malignancy, identified as being somatic, and present with  $\leq 1\%$  minor allele frequency (MAF). Pink, Variants previously associated with a hematologic malignancy and present with  $\leq 1\%$  MAF. Blue, Variants not previously associated with a hematologic malignancy and present with  $\leq 1\%$  MAF. (B,D) Variant/mutation totals in PV and ET ranked by gene and corresponding overall frequency percentage.

Based on their above-outlined independent impact on overall, leukemia-free, or myelofibrosis-free survival, *ASXL1*, *SRSF2*, and *IDH2* variants/mutations were identified as being “adverse” in PV and at least 1 of the 3 was present in 20 of the 133 patients (15%). Patients with adverse variants/mutations displayed significantly worse survival when compared with both patients with other sequence variants/mutations (HR, 2.1; 95% CI, 1.1-4.1) and those without any sequence variant/mutation (HR, 2.2; 95% CI, 1.2-4.2; Figure 2A); there was no difference in survival between patients with other sequence variants/mutations and those without any sequence variant ( $P = .92$ ).

The difference in survival between patients with and without adverse variants/mutations was independent of age, the IWG prognostic model for PV,<sup>17</sup> and karyotype, with respective HRs (95% CIs) of 2.7 (1.5-4.9), 2.8 (1.5-5.1), and 2.4 (1.2-4.6). Additional multivariable analysis that included the individual variables used in the IWG

prognostic model<sup>17</sup> confirmed the independent prognostic contribution of adverse variants/mutations (HR, 3.0; 95% CI, 1.6-5.5), along with age (HR, 5.6; 95% CI, 3.1-10.0 for age  $\geq 65$  years) and leukocytosis (HR, 2.0; 95% CI, 1.2-3.5 for leukocyte count  $\geq 15 \times 10^9/L$ ); venous thrombosis was no longer significant in the particular multivariable model ( $P = .32$ ). The presence of adverse variants/mutations was also associated with shorter leukemia-free (Figure 3A;  $P = .02$ ; HR, 5.6; 95% CI, 1.3-25.4) and myelofibrosis-free (Figure 4A;  $P = .11$ ; HR, 2.5; 95% CI, 0.8-8.1) survivals, although the latter did not reach significance.

Patients with 3 sequence variants/mutations (n = 4) had significantly worse survival, compared with those with 2 (n = 26;  $P = .04$ ), 1 (n = 40;  $P = .003$ ), or no (n = 63;  $P = .003$ ) sequence variant/mutation, whereas there was no difference in survival among the latter 3 groups ( $P = .29$ ), especially after adjusting for age ( $P = .72$ ).



**Figure 2. Overall survival curves.** (A) Survival in 133 Mayo Clinic patients with PV stratified by the presence or absence of “adverse” (*ASXL1*, *SRSF2*, *IDH2*) or “other” (*TET2*, *SH2B3*, *SF3B1*, *SETBP1*, *DNMT3A*, *CSF3R*, *CEBPA*, *SUZ12*, *ZRSR2*, *KIT*, *RUNX1*, *FLT3*, *CBL*, and *TP53*) DNA sequence variants/mutations. (B) Survival in 215 Italian patients with PV stratified by the presence or absence of adverse (*ASXL1*, *SRSF2*, *IDH2*) variants/mutations. (C) Survival in 183 Mayo Clinic patients with ET stratified by the presence or absence of “adverse” (*SH2B3*, *IDH2*, *SF3B1*, *U2AF1*, *EZH2*, *TP53*) or “other” (*TET2*, *ASXL1*, *PTP11*, *SUZ12*, *ZRSR2*, *CBL*, *CEBPA*, *CSF3R*, *DNMT3A*, *SRSF2*, *FLT3*, *KIT*, *NRAS*, *RUNX1*, *SETBP1*) sequence variants/mutations. (D) Survival in 174 Italian patients with ET stratified by the presence or absence of adverse (*SH2B3*, *IDH2*, *SF3B1*, *U2AF1*, *EZH2*, *TP53*) variants/mutations.

Furthermore, the shorter survival seen in the 4 patients with 3 sequence variants/mutations was fully accounted for by the presence of adverse variants/mutations in all of them.

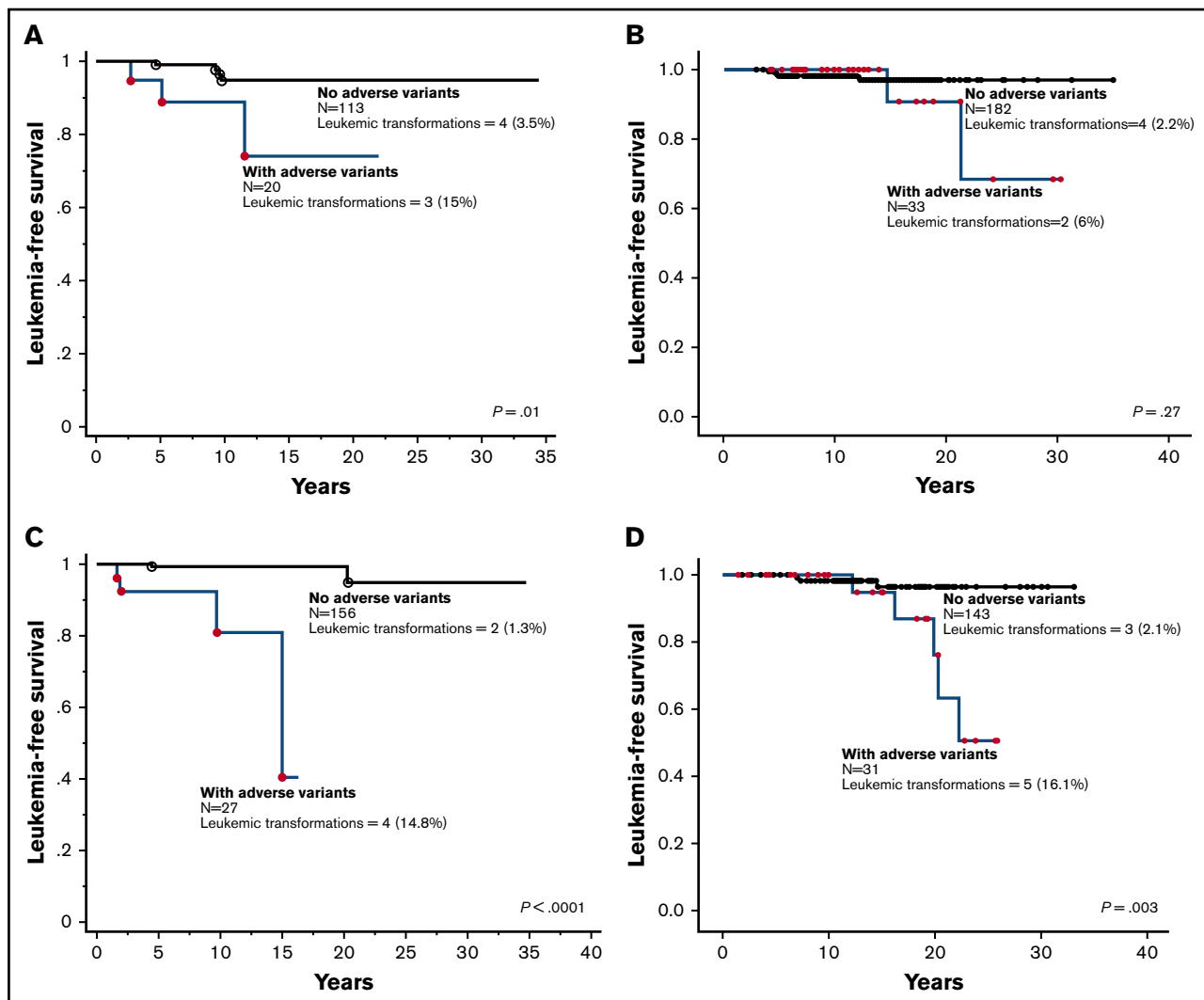
### Validation of prognostic relevance of adverse sequence variants/mutations in PV

The observations from the Mayo Clinic patients were validated in an external cohort of 215 Italian patients with PV (Tables 1 and 2), followed for a median of 11.9 years and with 84 deaths (39%), 55 fibrotic progressions (26%), and 6 leukemic transformations (3%); the particular cohort was enriched for patients who experienced fibrotic progression, in order to increase the power for molecular prediction for fibrotic transformation. In multivariable analysis, the unfavorable survival effect of adverse variants/mutations on survival (Figure 2B; HR, 2.6; 95% CI, 1.5-4.6) was independent of the IWG prognostic model for PV<sup>17</sup>; HRs (95% CIs) were 2.3 (1.3-4.1) for adverse variants/mutations and 3.6 (1.7-7.8) for high- or

intermediate-risk category. Adverse variants/mutations also affected myelofibrosis-free survival (Figure 4B), but the effect on leukemia-free survival was not significant (Figure 3B). Multivariable analysis confirmed the individual prognostic contribution of *ASXL1* (HR, 2.41; 95% CI, 1.3-4.5) and *SRSF2* (HR, 3.84; 95% CI, 1.2-12.6) variants/mutations to overall survival and *ASXL1* to myelofibrosis-free survival (HR, 1.9; 95% CI, 1.2-3.6).

### Prognostic relevance of sequence variants/mutations in ET

Median survival of the 183 Mayo Clinic patients with ET was 19.9 years. Age-adjusted univariate analysis identified *IDH2*, *EZH2*, and *SH2B3* variants/mutations as significant risk factors for survival; *SH2B3* (HR, 3.0; 95% CI, 1.03-8.2) and *IDH2* (HR, 22.1; 95% CI, 2.8-176.9) remained significant on multivariable analysis. For leukemia-free and myelofibrosis-free survival, univariate analysis showed significant associations with *TP53*, *EZH2*, *SRSF2*, and



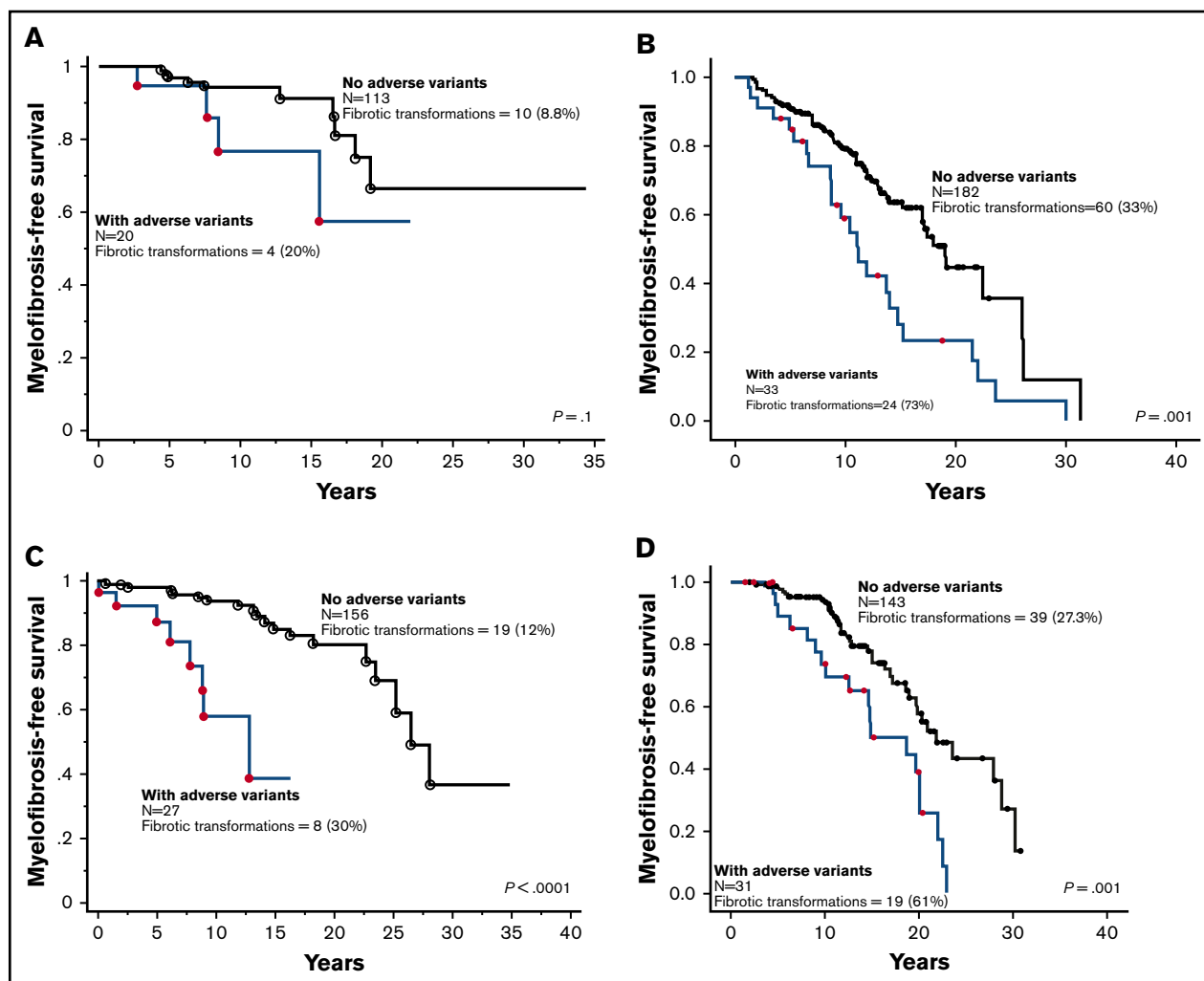
**Figure 3. Leukemia-free survival curves.** (A) Leukemia-free survival in 133 Mayo Clinic patients with PV stratified by the presence or absence of “adverse” (*ASXL1*, *SRSF2*, *IDH2*) DNA sequence variants/mutations. (B) Leukemia-free survival in 215 Italian patients with PV stratified by the presence or absence of adverse variants/mutations. (C) Leukemia-free survival in 183 Mayo Clinic patients with ET stratified by the presence or absence of “adverse” (*SH2B3*, *IDH2*, *SF3B1*, *U2AF1*, *EZH2*, *TP53*) sequence variants/mutations. (D) Leukemia-free survival in 174 Italian patients with ET stratified by the presence or absence of adverse sequence variants/mutations.

*IDH2* variants/mutations for the former and with *SF3B1* and *U2AF1* for the latter; on multivariable analysis, *TP53* (HR, 82.8; 95% CI, 7.5-916) and *EZH2* (HR, 146.8; 95% CI, 11.1-1935.6) remained significant for leukemia-free survival and *SF3B1* (HR, 8.1; 95% CI, 2.5-25.8) and *U2AF1* (HR, 30.3; 95% CI, 3.4-271.0) for myelofibrosis-free survival.

Based on their above-outlined independent impact on overall, leukemia-free, or myelofibrosis-free survival, *SH2B3*, *IDH2*, *U2AF1*, *SF3B1*, *EZH2*, and *TP53* variants/mutations were identified as being “adverse” and at least 1 of them was present in 27 (14.8%) of the 183 patients with ET. Patients with adverse variants/mutations displayed significantly worse overall survival, when compared with both patients with other sequence variants/mutations (HR, 4.3; 95% CI, 2.2-8.5) and those without any sequence variant/mutation (HR, 7.0; 95% CI, 3.5-14.1; Figure 2C). In univariate analysis, patients with other sequence variants/mutations also showed significantly worse survival, when compared with patients without any

sequence variant/mutation (HR, 2.0; 95% CI, 1.1-3.7); however, in multivariable analysis that included age, the difference in survival remained significant for patients with adverse variants/mutations (HR, 3.4; 95% CI, 1.6-7.0) but not for those with other sequence variants/mutations (HR, 1.5; 95% CI, 0.8-2.8). The presence of adverse variants/mutations was also associated with significantly shorter leukemia-free (Figure 3C; HR, 36.2; 95% CI, 3.8-347.9) and myelofibrosis-free (Figure 4C; HR, 6.6; 95% CI, 2.6-16.4) survivals.

Patients without any sequence variant/mutation ( $n = 87$ ) had significantly longer survival when compared with those with 3 or 4 ( $n = 7$ ;  $P < .0001$ ) or 1 ( $n = 75$ ;  $P = .0009$ ) sequence variant but not to those with 2 sequence variants ( $n = 14$ ;  $P = .28$ ). However, when analysis was repeated after adjusting for age and excluding patients with adverse variants/mutations ( $n = 27$ ), the survival impact of the number of mutations was no longer evident ( $P = .21$ ); 6 of the 7 patients with 3 or more sequence variants also had adverse variants. In other words, in both ET and PV, patients with



**Figure 4. Myelofibrosis-free survival curves.** (A) Myelofibrosis-free survival in 133 Mayo Clinic patients with PV stratified by the presence or absence of “adverse” (*ASXL1*, *SRSF2*, *IDH2*) DNA sequence variants/mutations. (B) Myelofibrosis-free survival in 215 Italian patients with PV stratified by the presence or absence of adverse variants/mutations. (C) Myelofibrosis-free survival in 183 Mayo Clinic patients with ET stratified by the presence or absence of “adverse” (*SH2B3*, *IDH2*, *SF3B1*, *U2AF1*, *EZH2*, *TP53*) sequence variants/mutations. (D) Myelofibrosis-free survival in 174 Italian patients with ET stratified by the presence or absence of adverse sequence variants/mutations.

multiple sequence variants were older and more likely to harbor adverse variants, thus giving the appearance of unfavorable prognostic impact during unadjusted survival analysis.

The difference in survival between patients with and without adverse variants/mutations was independent of age (HR, 2.6; 95% CI, 1.4-4.8), IWG prognostic model for ET<sup>16</sup> (HR, 2.6; 95% CI, 1.4-4.8), and karyotype (HR, 4.7; 95% CI, 2.4-9.2). Additional multivariable analysis that included the individual variables used in the IWG prognostic model<sup>16</sup> confirmed the independent prognostic contribution of adverse variants/mutations (HR, 2.4; 95% CI, 1.2-4.7), along with age (HR, 3.2; 95% CI, 1.6-6.2 for age  $\geq 60$  years) and leukocytosis (HR, 2.1; 95% CI, 1.2-3.7 for leukocyte count  $\geq 11 \times 10^9/L$ ); thrombosis was no longer significant in the particular multivariable model ( $P = .68$ ).

### Validation of prognostic relevance of adverse sequence variants/mutations in ET

The observations from the Mayo Clinic patients were validated in an external cohort of 174 Italian patients with ET (Tables 1 and 2)

followed for a median of 13 years and with 62 deaths (36%), 58 fibrotic progressions (33%), and 8 leukemic transformations (5%); the particular cohort was enriched for patients who experienced fibrotic progression, in order to increase the power for molecular prediction for myelofibrosis-free survival. In multivariable analysis, the unfavorable survival effect of adverse variants/mutations (Figure 2D; HR, 2.5; 95% CI, 1.5-4.4) was independent of the IWG prognostic model for ET<sup>16</sup>: HRs (95% CIs) were 2.0 (1.1-3.6) for adverse variants, 3.5 (1.6-7.7) for the intermediate-risk category, and 8.7 (3.6-21.0) for the high-risk category. Multivariable analysis confirmed the individual prognostic contribution of *U2AF1* to overall (HR, 2.9; 95% CI, 1.1-8.7) and myelofibrosis-free (HR, 3.2; 95% CI, 1.1-9.0) survival and that of *TP53* to leukemia-free survival (HR, 7.3; 95% CI, 1.5-35.9). Adverse variants/mutations also affected leukemia-free (Figure 3D; HR, 6.7; 95% CI, 1.6-28.1) and myelofibrosis-free survival (Figure 4D; HR, 2.6; 95% CI, 1.4-4.5).

### Discussion

The revelation, in the current study, of DNA sequence variants/mutations, other than *JAK2/CALR/MPL*, in the majority of patients



with PV or ET is consistent with the results from a smaller previous study that showed the presence of proven mutations in 44% and 29% of the respective diseases.<sup>8</sup> The current study identifies prognostically important specific sequence variants/mutations and demonstrates the subsidiary role, in this regard, of the “number” of mutations.

The detrimental nature of some of the adverse variants identified in the current study has also been recognized in other chronic myeloid malignancies, including PMF (*ASXL1*, *SRSF2*, *EZH2*, *IDH1/2*, *U2AF1*),<sup>7,18</sup> chronic myelomonocytic leukemia (*ASXL1*),<sup>19</sup> and myelodysplastic syndromes (*ASXL1*, *EZH2*, *TP53*, *SRSF2*).<sup>20,21</sup> Similarly, *TP53*, *IDH2*, *SRSF2*, and *SH2B3* mutations were previously found to be overrepresented in blast-phase MPNs.<sup>8,22,23</sup> In this regard, the possible leukemogenic cooperation of mutant *JAK2* with *IDH1/2* mutations has previously been clinically suggested<sup>24</sup> and that of *TP53* loss with mutant *JAK2* demonstrated in vivo.<sup>23</sup> Consistent with the latter observation, all 5 *TP53*-mutated patients with ET, in the current study, were also *JAK2*-mutated, and leukemic transformation in another study<sup>8</sup> was temporally linked to the consequent loss of the wild-type *TP53* allele in *TP53*-mutated patients.

The observations from the current study are practically relevant and timely considering the increasing use of targeted NGS in routine myeloid cancer practice. We show that the occurrence of sequence variants/mutations other than *JAK2/CALR/MPL*, in patients with PV or ET, is not infrequent and neither the type nor number of genes involved is necessarily detrimental to disease outcome, unless it involves specific genes with prognostic relevance. On the other hand, patients with adverse variants/mutations, regardless of their conventional risk category, might require closer monitoring. In this regard, the current study suggests the possibility of refining clinically derived prognostic models by the incorporation of molecular information.

Additional studies are warranted in order to refine and possibly expand the pool of clinically relevant DNA sequence variants/mutations that could be targeted by NGS. Such a strategy would ideally also include sequence variants/mutations that identify patients with increased risk of thrombosis. In this regard, the discrepant results between the Mayo Clinic and Florence patient cohorts, in regards to the possible association between *TET* variants/mutations

and thrombosis in ET, remains unexplained and deserves further investigation.

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## Authorship

Contribution: A.T. designed and sponsored the study, contributed patients, collected clinical data and patient samples, reviewed the molecular data, performed statistical analysis, and wrote the paper; T.L.L. performed and analyzed the molecular analysis; P.G. contributed patients, collected clinical data and patient samples, performed and analyzed the molecular data, performed statistical analysis, and helped with manuscript writing; C.M.F. performed the molecular analysis; G.R., A. Pacilli, A. Pancrazzi, C.M., and T.F. performed sequencing and analysis of data; Y.E. and D.B. collected clinical data; C.A.H. performed pathology review; R.P.K. performed cytogenetics review; A. Pardanani contributed patients and reviewed the molecular data; N.G. contributed patients and collected clinical data; A.M.V. designed the study, contributed patients, collected clinical data and patient samples, reviewed the molecular data, and helped with data analysis and manuscript writing; and all authors reviewed and approved the paper.

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