JAK2, *CALR*, *MPL* and *ASXL1* mutational status correlates with distinct histological features in Philadelphia chromosome-negative myeloproliferative neoplasms

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ONLINE SUPPLEMENTARY MATERIALS

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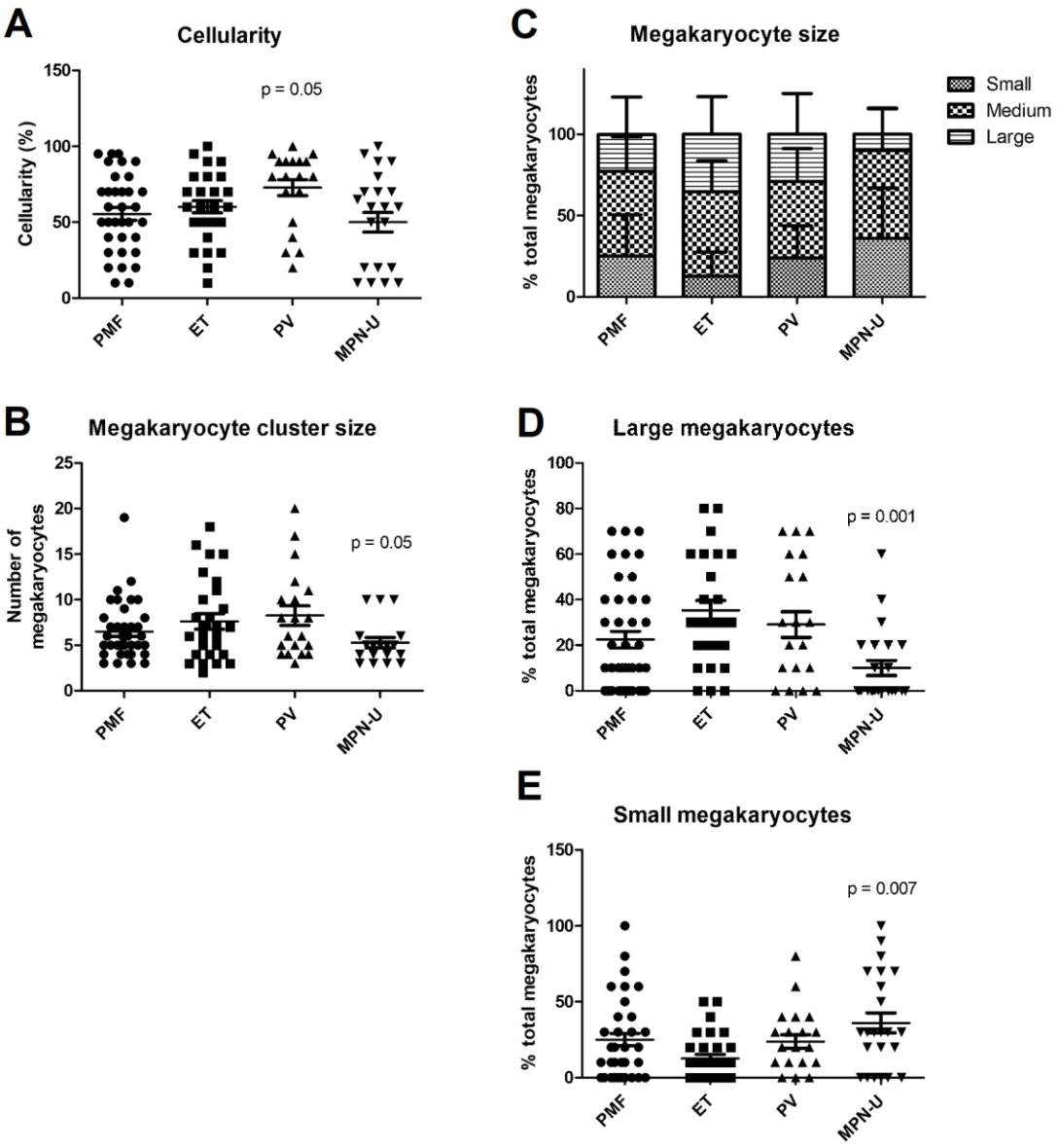

Supplementary Figure 1. MPN disease types exhibit distinct histologic features. (A) Increased bone marrow cellularity in PV (n = 23). (B) Smaller megakaryocyte clusters in MPN-U (n = 20). (C to E) Fewer large megakaryocytes and more small megakaryocytes in MPN-U. Statistical significance was calculated using one-way analysis of variance (ANOVA).

Supplementary Table 1. Demographic, clinical, and histological features of MPN patients according to disease type

		PMF (n=43)	ET (n=29)	PV (n=23)	MPN-U (n=20)	Total (n=115)	*P* Value
Mean age		65.9	60.0	62.0	63.4	63.1	0.30
Female		18 (42%)	18 (62%)	14 (61%)	9 (45%)	59 (50%)	0.26
New diagnosis		19 (44%)	14 (48%)	12 (52%)	13 (65%)	58 (50%)	0.48
JAK2 inhibitor treatment	Prior	5 (12%)	1 (3.4%)	2 (8.7%)	3 (15%)	11 (9.6%)	0.21
	Current	7 (16%)	1 (3.4%)	2 (8.7%)	0	10 (8.7%)	
Diagnoses revised		6 (14%)	5 (17%)	3 (10%)	11 (55%)	25 (22%)	**0.001**
Phenotypic driver mutation	*JAK2*	28 (65%)	12 (41%)	21 (91%)	11 (55%)	72 (63%)	**0.004**
	CALR	5 (12%)	12 (41%)	0	4 (20%)	21 (18%)	
	Type 1	3	6	0	3	12	
	Type 2	2	4	0	1	7	
	Other	0	2	0	0	2	
	MPL	4 (9.3%)	1 (3.4%)	0	1 (5%)	6 (5.2%)	
	TN	6 (14%)	4 (14%)	2 (8.7%)	4 (20%)	16 (14%)	
Mean cellularity		55.4	59.0	71.7	47.5	58.4	**0.022**
Reticulin fibrosis grade	0-1	14 (33%)	17 (61%)	12 (55%)	8 (40%)	51	0.40
	2-3	28 (67%)	11 (29%)	10 (45%)	12 (60%)	61	
Osteosclerosis grade	0-1	34 (81%)	25 (86%)	20 (87%)	15 (75%)	94	0.49
	2-3	8 (19%)	4 (14%)	3 (13%)	5 (25%)	20	
Vascularity	Decreased	6 (15%)	2 (7.7%)	4 (20%)	6 (32%)	18	0.12
	Normal	23 (59%)	20 (77%)	8 (40%)	8 (42%)	59	
	Increased	10 (26%)	4 (15%)	8 (40%)	5 (26%)	27	
Megakaryocyte count	Decreased	3 (7.1%)	0	1 (4.3%)	3 (15%)	7	0.24
	Normal	2 (4.8%)	2 (6.9%)	4 (17%)	2 (10%)	10	
	Increased	37 (88%)	27 (93%)	18 (78%)	15 (75%)	97	
Megakaryocyte size	Small	24.5	13.1	23.5	39.5		**<0.01**
	Medium	52.9	53.1	50.0	50.0		
	Large	22.4	33.8	26.5	10.5		
Predominant megakaryocytic nuclear morphology		Bulbous	Staghorn	Bulbous	Hyperchromatic		**0.0009**
Megakaryocyte clusters	Present	39 (93%)	27 (93%)	22 (96%)	15 (75%)	103	**0.08**
Mean megakaryocyte cluster size		5.78	6.93	7.78	3.75	0.008	
Myeloid/erythroid ratio	Decreased	6 (14%)	5 (17%)	1 (4.3%)	5 (25%)	17	**0.005**
	Normal	7 (17%)	14 (48%)	11 (48%)	2 (10%)	34	
	Increased	29 (69%)	10 (34%)	11 (48%)	13 (65%)	63	
Myeloid left shift	Present	11 (26%)	4 (14%)	4 (17%)	7 (35%)	26	0.30
Erythroid islands	Present	21 (50%)	17 (59%)	12 (52%)	5 (25%)	55	0.12
Osteoblastic activity	Present	17 (40%)	20 (69%)	13 (57%)	12 (60%)	62	0.11
Osteoclastic activity	Present	10 (24%)	6 (21%)	5 (22%)	2 (10%)	23	0.64

Supplementary Table 2

Peripheral blood parameters in MPN patients according to phenotypic driver mutation

		All MPN					PMF					ET		
		JAK2 **(n=67)**	***CALR*** **(n=21)**	***MPL*** **(n=7)**	**TN (n=16)**	***P*** **Value**	***JAK2*** **(n=28)**	***CALR*** **(n=5)**	***MPL*** **(n=4)**	**TN (n=6)**	***P*** **Value**	***JAK2*** **(n=12)**	***CALR*** **(n=12)**	***P*** **Value**
Disease	PMF	28	5	4	6	0.004								
	ET	12	12	1	4									
	PV	21	0	0	2									
	MPN-U	6	4	2	4									
WBC		19.5	11.9	13.3	10.7	0.58	14.7	6.7	14.2	6.6	0.30	22.5	8.76	0.29
	% neutrophil	67.1	55.7	64.2	58.8	**0.01**	69.0	60.2	68.8	56.1	0.17	61.0	57.8	0.60
	% lymphocyte	14.1	22.0	14.7	27.1	**0.0004**	14.1	20.4	11.1	31.7	**0.003**	16.8	20.1	0.41
	% monocyte	5.77	7.97	7.83	6.67	0.29	4.54	9.50	7.40	5.85	0.06	7.98	7.45	0.83
	% eosinophil	1.82	1.43	1.23	1.33	0.58	1.66	1.60	0.98	0.67	0.46	2.46	1.50	0.19
	% basophil	1.77	0.72	0.47	0.49	**0.003**	1.73	0.60	0.03	0.20	0.08	1.56	0.77	0.11
	% blasts	0.91	0.38	1.67	2.00	0.26	1.11	0.60	2.00	2.00	0.71	0.17	0.08	0.56
Hemoglobin		11.1	11.8	9.95	10.1	0.23	10.3	9.94	9.33	8.78	0.50	11.0	12.5	0.16
Hematocrit		35.0	35.6	29.6	31.0	0.20	31.9	30.7	27.8	26.8	0.45	36.0	37.2	0.68
Platelet		319	675	413	440	**0.001**	294	465	317	264	0.68	506	834	**0.04**

Supplementary Table 3

Peripheral blood parameters in PMF patients according to reticulin fibrosis grade

		Pre-fibrotic PMF (n=14)	Overtly fibrotic PMF (n=29)	*P* Value
WBC		15.9	11.0	0.20
	% neutrophil	71.9	63.4	0.07
	% lymphocyte	15.2	17.9	0.50
	% monocyte	7.19	4.61	**0.04**
	% eosinophil	2.34	1.02	**0.005**
	% basophil	0.72	1.47	0.20
	% blasts	0.29	1.72	0.07
Hemoglobin		12.2	8.89	**0.0001**
Hematocrit		38.0	27.1	**0.0001**
Platelet		608	168	**0.0001**

Supplementary Table 4

Non-phenotypic driver mutations detected by next-generation sequencing in PMF patients

		JAK2 **(n=28)**	***CALR*** **(n=5)**	***MPL*** **(n=4)**	**TN (n=6)**	***P*** **Value**
Average mutational burden		2.4	2.0	2.3	1.2	0.23
Mutations in	*ASXL1*	8 (29%)	1 (20%)	1 (25%)	2 (33%)	0.97
	TET2	3 (11%)	2 (40%)	0	0	0.15
	DNMT3A	4 (14%)	0	1 (25%)	0	0.51
	SF3B1	3 (11%)	0	0	2 (33%)	0.27
	SRSF2	3 (11%)	1 (20%)	0	0	0.62
	U2AF1	4 (14%)	0	0	0	0.50
	NRAS	3 (11%)	0	1 (25%)	0	0.50
	KRAS	0	0	1 (25%)	0	n/a
	CBL	2 (7.1%)	0	0	0	0.77
	IDH2	1 (3.6%)	0	0	0	n/a
	IDH1	1 (3.6%)	1 (20%)	0	0	0.36
	EZH2	0	1 (20%)	0	0	n/a

Supplementary Materials and Methods

Study population

An institutional review board-approved search of the pathology archives at Brigham & Women's Hospital (BWH) and Massachusetts General Hospital (MGH) identified a total of 115 patients diagnosed with PMF, ET, or PV on bone marrow biopsy with concurrent hematologic and molecular sequencing data. Forty-three (37.4%) patients were diagnosed with PMF, 29 (25.2%) with ET, 23 (20.0%) with PV, and 20 (17.4%) with MPN-U. All original diagnoses were rendered according to the 2008 WHO Classification; histological review (see below) was based on the 2016 WHO Classification. Additional patient information including age, gender, laboratory values, date of original diagnosis of MPN, and treatment history were obtained from the electronic medical record. Exclusion criteria included patients diagnosed with MPN/myelodysplastic syndrome (MDS) overlap disease, those that had progressed to acute leukemia, chemotherapeutic treatment for prior cancer diagnoses, or stem cell transplant. Patients receiving JAK2 inhibitor therapy were included in the analysis. The study was conducted in accordance with the principles set forth by the Declaration of Helsinki.

Mutational analysis

Targeted sequencing of 95 commonly mutated genes in myeloid neoplasms was performed on DNA isolated from peripheral blood or bone marrow aspirates using amplicon library generation (TruSeq Custom Amplicon, Illumina, San Diego, CA) and next generation sequencing[1] (MiSeq, Illumina, San Diego, CA) as part of each patient's clinical evaluation. Data processing and analysis were performed using MuTect for single-nucleotide variants with subsequent manual review and annotation (including evaluation of allele frequencies). Likely pathogenic variants were defined as frameshift, nonsense, splice-site mutations, insertions-deletions, or known pathogenic missense alterations.

Histological analysis

Bone marrow trephine biopsies (hematoxylin and eosin, reticulin, trichrome, CD34) and aspirate smears (Wright-Giemsa) were evaluated by two hematopathologists (RH, OP) and a trainee

hematopathologist (WW) and graded on the following 24 histomorphological characteristics: cellularity, reticulin fibrosis grade, osteosclerosis grade, megakaryocyte abundance, megakaryocyte size, megakaryocyte size distribution, megakaryocyte nuclear morphology, megakaryocyte clustering, number of megakaryocytes per cluster, density of megakaryocyte clusters (tight or loose), location of megakaryocyte clusters (paratrabecular or non-paratrabecular), myeloid to erythroid ratio (M:E), myeloid left shift, myeloid dysplasia, erythroid left shift, erythroid dysplasia, erythroid islands, intrasinusoidal hematopoiesis, lymphoid aggregates, increased plasma cells, increased eosinophils, presence of osteoblasts, presence of osteoclasts. For each biopsy, a silver impregnation reticulin stain was evaluated for reticulin fibrosis grade and a trichrome stain was evaluated for collagen fibrosis grade.

Megakaryocyte abundance was scored as decreased (fewer than 2 megakaryocytes per HPF), normal (2-4 megakaryocytes per HPF), or increased (more than 4 megakaryocytes per HPF). Clustering of megakaryocytes was classified as tight or loose, according to previously described features[2,3]. Megakaryocyte nuclear morphology was scored as normal, bulbous, staghorn, hypolobated, and hyperchromatic according previously described features[4]. The predominant megakaryocyte morphology was the morphologic subtype comprising the highest fraction in each case. Erythroid islands were scored as present or absent as previously defined[5]. Erythroid left shift was defined morphologically as increased pronormoblasts relative to normoblasts. Osteosclerosis grade was assessed using recently defined criteria[6].

For comparison of PMF stage, pre-fibrotic PMF was defined as cases showing reticulin fibrosis grade 0-1; overtly fibrotic PMF showed reticulin fibrosis grade 2-3. For each biopsy, an immunohistochemical stain for CD34 (clone QBEnd/10) was used to assess bone marrow vascularity, which was scored as decreased (fewer than 10 capillaries per 20X objective), normal (10-25 capillaries), or increased (more than 25 capillaries) using a 20x objective. Based on the above observations and other diagnostic features, a 2016 WHO Classification diagnosis was assigned to all cases.

Statistical analysis

Numerical and categorical values were represented by the mean and frequency count, respectively. Statistical significance between qualitative variables was assessed using 2-way ANOVA, 1-way ANOVA, or Student's t test, with Bonferroni post hoc correction, as appropriate. Correlation between categorical variables was evaluated by Chi-square test or Fisher's exact test, as appropriate. All statistical analyses were performed using PRISM software (Irvine, CA). *P* values <0.05 were considered as significant.

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