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

Philadelphia chromosome (Ph)-negative myeloproliferative neoplasms (MPN) [essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF)] vary in morphological features, disease presentation, and, most importantly, clinical outcome.[1] The World Health Organization (WHO) Classification of Myeloid Neoplasms was revised in 2016, and includes refined criteria for characterizing these diseases, particularly at early stages.[2] Part of the criteria includes the evaluation of 'phenotypic driver' mutations in MPN: *JAK2* V617F, *CALR* and *MPL* W515L/K. However, their common presence in PMF, ET, and PV indicates that molecular screening alone cannot reliably distinguish between these diseases. In particular, early ('prepolycythemic' or 'masked') PV and *JAK2*-mutant ET can share similar molecular and histomorphological characteristics, as can pre-fibrotic PMF and ET.[3] Therefore, histological evaluation of the bone marrow biopsy remains an essential major diagnostic criterion for diagnosing MPN.[2] In this study, we examined the correlation between bone marrow morphology, using both WHO-defined and additional parameters, and genetic mutations in a cohort of 115 Ph-negative MPN patients.

Median age of all patients was 64 years, with no significant difference in age and sex between patients with PMF, ET, PV, and MPN, Unclassifiable (MPN-U). Fifty-eight (50%) patients were newly diagnosed, whereas 57 (50%) had received therapy, including hydroxyurea, anagrelide, darbepoetin, peginterferon alfa-2a, and JAK2 small molecular inhibitors. Review of histological and clinical data according to the 2016 WHO Classification confirmed the original diagnoses in 92 cases (80%) and led to a revised diagnosis in 23 cases (20%) (*Online Supplementary Table S1*). The final analysis included 43 (37.4%) patients with PMF, 29 (25.2%) with ET, 23 (20.0%) with PV, and 20 (17.4%) with MPN-U (Tables 1-3 and *Online Supplementary Table S1*). Of these, 14 PMF cases were pre-fibrotic, whereas 29 showed overt fibrosis. In PV, 10 cases had progressed to post-PV myelofibrosis and 11 ET cases had progressed to post-ET myelofibrosis; this high number likely reflects institutional referral bias. Eight cases of MPN-U represented early stages of PV, PMF, or ET in which clinical, labora-

Table 1. Histological features of MPN patients according to phenotypic driver mutation.

		All MPN (n = 115)				
		JAK2 (n=72)	*CALR* (n=21)	*MPL* (n=6)	TN (n=16)	*P*
Cellularity		62.3%	55.2%	32.0%	53.3%	0.07
Reticulin fibrosis grade	0-1	31 (44%)	12 (57%)	1 (20%)	5 (42%)	0.46
	2-3	39 (56%)	9 (43%)	4 (80%)	7 (58%)	
Osteosclerosis grade	0-1	61 (85%)	16 (76%)	2 (40%)	11 (92%)	0.06
	2-3	11 (15%)	5 (24%)	3 (60%)	1 (8.3%)	
Vascularity	Decreased	12 (18%)	2 (11%)	2 (40%)	1 (9.1%)	0.02
	Normal	35 (54%)	15 (79%)	3 (60%)	3 (27%)	
	Increased	18 (28%)	2 (11%)	0	7 (64%)	
Megakaryocyte count	Decreased	4 (5.6%)	0	1 (20%)	2 (18%)	0.40
	Normal	6 (8.3%)	2 (9.5%)	1 (20%)	1 (8.3%)	
	Increased	62 (86%)	19 (90%)	3 (60%)	9 (82%)	
Megakaryocyte size	Small	25.1%	12.9%	22.0%	38.3%	1.0
	Medium	49.2%	61.0%	66.0%	46.7%	
	Large	25.6%	26.2%	12.0%	15.0%	
Predominant megakaryocytic nuclear morphology		Bulbous	Staghorn	None	Hyper-chromatic	
Megakaryocyte clusters	Present	65 (90%)	20 (95%)	4 (80%)	10 (83%)	0.62
Mean megakaryocyte cluster size		6.85	7.65	4.75	6.00	
Myeloid/erythroid ratio	Decreased	10 (14%)	4 (19%)	2 (40%)	1 (8.3%)	0.61
	Normal	21 (29%)	8 (38%)	1 (20%)	3 (25%)	
	Increased	41 (57%)	9 (43%)	2 (40%)	8 (67%)	
Myeloid left shift	Present	15 (21%)	4 (19%)	1 (20%)	5 (42%)	0.43
Erythroid islands	Present	34 (47%)	12 (57%)	3 (60%)	4 (33%)	0.56
Osteoblastic activity	Present	36 (50%)	17 (81%)	1 (20%)	6 (50%)	0.03
Osteoclastic activity	Present	17 (24%)	5 (24%)	0	1 (8.3%)	0.41

TN: triple negative.

tory, and morphological features were not readily distinguishable; the remaining 12 cases represented advanced stages of MPN in which fibrosis obscured the underlying diagnosis.

Histological review of the entire cohort confirmed the presence of morphological differences between PMF, ET, and PV, which were consistent with the 2016 WHO Classification (*Online Supplementary Table S1*). PV showed an elevated mean marrow cellularity (71.7%) compared to PMF, ET, and MPN-U (range 47.5-59.0%; *P*=0.022) (*Online Supplementary Figure S1A*). PMF, ET and MPN-U were characterized by predominantly bulbous, staghorn, and hyperchromatic megakaryocytic nuclei, respectively (*P*=0.0009). Although megakaryocyte clusters were present in all entities, MPN-U showed fewer and smaller megakaryocyte clusters (3.8 cells per cluster *vs.* 5.8-7.8 cells per cluster; *P*=0.008) (*Online Supplementary Figure S1B*). In addition, MPN-U was associated with smaller megakaryocyte size

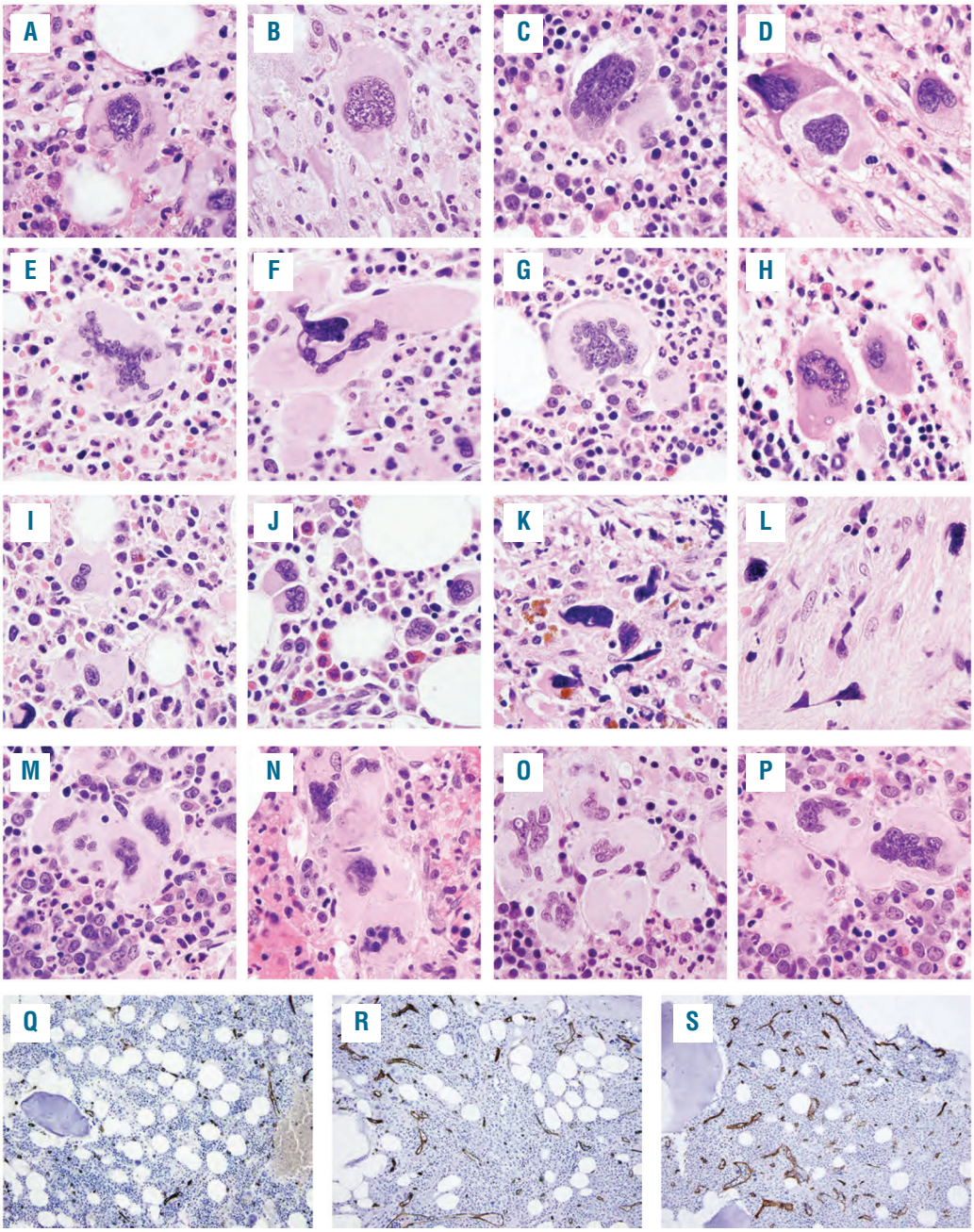

Figure 1. Representative morphological features of different myeloproliferative neoplasm (MPN) disease types. Bulbous or 'cloud-like' megakaryocytic nuclei (A-D) with rounded contours and vesicular chromatin are most characteristic of pre-fibrotic primary myelofibrosis (PMF). 'Staghorn' nuclei (E-H) with hyperlobation, elongated contours, and enlarged cell size, are commonly seen in essential thrombocythemia (ET). Hypolobated nuclei (I and J) are typically found in small megakaryocytes and sometimes show widely separated nuclear lobes. Hyperchromatic nuclei (K and L) are commonly found in overtly fibrotic PMF and myeloproliferative neoplasm, Unclassifiable (MPN-U). Note the highly fibrotic stroma in (L). Tight megakaryocyte clusters (M and N) show a syncytial quality in clustered cells. In contrast, loose megakaryocyte clusters (O and P) are characterized by intervening cells or stroma (P). CD34 immunostains demonstrate decreased (Q), normal (R), and increased (S) marrow vascularity (see *Online Supplementary Methods*).

(P<0.01) (*Online Supplementary Figure S1C-E*). Both MPN-U and PMF were characterized by increased myeloid to erythroid ratio (P=0.005) compared to PV and ET. MPN-U displayed a trend toward fewer erythroid islands (P=0.12), while PV displayed a trend towards increased marrow vascularity (P=0.12). Osteoblastic activity was more frequently observed in ET (P=0.11). Therefore, we not only confirmed the reproducibility of WHO-defined criteria in diagnosing PMF, ET, and PV, as we and others had previously demonstrated,[4,5] but further identified additional morphological criteria that are helpful in distinguishing between MPN entities.

Next-generation sequencing[6] was performed on all biopsies and showed that 72 (62.6%) patients carried a *JAK2* V617F mutation, 21 (18.3%) a *CALR* mutation, and 6 (5.2%) an *MPL* W515 mutation, while 16 (13.9%) had non-mutated *JAK2*, *CALR*, and *MPL* ('triple-negative', TN) (Table 1). *CALR* mutations were more common in ET patients (P=0.004) and were associated with a higher platelet count in all MPN patients (P=0.001) (*Online Supplementary Table S2*), consistent with previous studies.[7] Patients with *JAK2* mutation showed higher peripheral blood neutrophil and basophil percentages (P=0.0004 and P=0.003, respectively). In comparison, TN status (including 2 PV cases which lacked *JAK2* exon 12 mutations) was associated with a higher percentage of blood lymphocytes (P=0.0004) and blasts, although the latter was not statistically significant (*Online Supplementary Table S2*).

Comparison of molecular and biopsy findings demonstrated that certain histological features of MPN disease correlate with *JAK2, CALR*, and *MPL* mutational status (Table 1). *MPL* mutation was borderline associated with lower cellularity and greater osteosclerosis (P=0.07 and P=0.06, respectively). Sixty-four percent of TN cases showed increased vascularity, which was significantly higher than cases with *JAK2/MPL/CALR* mutations (P=0.02). *CALR* mutations were associated with increased osteoblastic activity (P=0.03). *CALR* mutations are subdivided into type 1 and type 2 mutations, with the former associated with lower DIPSS-plus score and possibly longer survival in PMF patients.[8,9] We found that *CALR* type 1 and type 2 mutations occurred at similar frequency in pre-fibrotic (MF 0-1, 46%) and overtly fibrotic (MF 2-3, 34%) patients. Furthermore, no significant differences were detected in megakaryocyte morphology, reticulin and collagen fibrosis, myelopoiesis, or erythropoiesis between *JAK2*, *CALR* type 1 and type 2, *MPL* mutant, or TN cases (Table 1).

Table 2. Histological features of pre-fibrotic primary myelofibrosis (PMF) patients according to phenotypic driver mutation.

		PMF (n = 43)				
		JAK2 (n=28)	*CALR* (n=5)	*MPL* (n=4)	TN (n=6)	*P*
Cellularity		59.1%	60.0%	26.7%	48.3%	0.20
Reticulin fibrosis grade	0-1	10 (36%)	2 (40%)	1 (33%)	1 (17%)	0.82
	2-3	18 (64%)	3 (60%)	2 (67%)	5 (83%)	
Osteosclerosis grade	0-1	23 (82%)	4 (80%)	1 (33%)	6 (100%)	0.12
	2-3	5 (18%)	1 (20%)	2 (67%)	0	
Vascularity	Decreased	4 (14%)	1 (25%)	1 (33%)	0	0.10
	Normal	16 (57%)	3 (75%)	2 (67%)	1 (20%)	
	Increased	6 (21%)	0	0	4 (80%)	
Megakaryocyte count	Decreased	1 (3.6%)	0	1 (33%)	1 (20%)	>0.05
	Normal	0	0	1 (33%)	1 (20%)	
	Increased	27 (96%)	5 (100%)	1 (33%)	3 (60%)	
Megakaryocyte size	Small	21.1%	14%	33.3%	45%	>0.05
	Medium	52.9%	54%	66.7%	45%	
	Large	25.7%	32%	0	10%	
Predominant megakaryocytic nuclear morphology		Bulbous	Bulbous	Bulbous	Bulbous	0.75
Megakaryocyte clusters	Present	26 (93%)	5 (100%)	2 (67%)	5 (100%)	0.29
Mean megakaryocyte cluster size		5.79	7.20	2.67	6.17	0.32
Myeloid/erythroid ratio	Decreased	2 (7.1%)	1 (20%)	2 (67%)	1 (20%)	0.08
	Normal	5 (18%)	2 (40%)	0	0	
	Increased	21 (75%)	2 (40%)	1 (33%)	4 (80%)	
Myeloid left shift	Present	5 (18%)	2 (40%)	0	4 (80%)	0.02
Erythroid islands	Present	13 (46%)	5 (100%)	2 (67%)	1 (20%)	0.06
Osteoblastic activity	Present	10 (36%)	4 (80%)	1 (33%)	2 (40%)	0.32
Osteoclastic activity	Present	6 (21%)	3 (60%)	0	1 (20%)	0.20

TN: triple negative.

Having shown that MPN entities exhibit distinct molecular and histological profiles, we went on to examine PMF cases specifically. Of 43 patients with PMF, 28 (65.1%) carried a *JAK2* mutation, 5 (11.6%) a *CALR* mutation, 4 (9.3%) an *MPL* mutation, and 6 (14.0%) were TN (Table 2 and *Online Supplementary Table S1*). Review of bone marrow histology showed that TN cases were associated with left-shifted myelopoiesis (P=0.02) (Table 2), and showed a trend towards increased myeloid to erythroid ratio (P=0.08), reduced erythroid islands (P=0.06), and increased vascularity (P=0.10). In contrast, *MPL*-mutant cases exhibited a trend towards decreased vascularity (P=0.10) and increased osteosclerosis (P=0.12). No significant differences were detected in megakaryocyte morphology, cellularity, reticulin and collagen fibrosis between mutational subgroups (Table 2). Compared to pre-fibrotic PMF, overtly fibrotic PMF was associated with reduced hemoglobin concentration (P=0.0001) and platelet count (P=0.0001), as well as reduced peripheral monocytes and eosinophils (*Online Supplementary Table S3*), likely reflecting overall compromised hematopoiesis. Overtly fibrotic PMF also showed decreased cellularity (P=0.01), increased marrow vascularity (P=0.01), erythroid left shift (P=0.001), and increased osteosclerosis (P=0.04) (Table 4). While PMF biopsies as a whole showed predominantly bulbous megakaryocyte nuclei (Tables 1-3), this finding was most prominent in pre-fibrotic cases; cases with overt fibrosis displayed mostly hyperchromatic nuclei (Table 4). These findings support the hypothesis that pre-fibrotic and overtly fibrotic PMF are histologically and clinically distinct.[10]

Overtly fibrotic PMF also demonstrated frequent mutations in the chromatin modifier gene *ASXL1* (Table 4). The most common non-phenotypic driver mutations in PMF included *ASXL1* (28%), *TET2* (12%), *DNMT3A* (12%), and *SF3B1* (12%) (*Online Supplementary Table S4*). There was no significant difference in the co-occurrence of *ASXL1* mutations among *JAK2, CALR, MPL,* and TN disease (P=0.97). *ASXL1* mutations occurred twice as frequently in *CALR*-mutant patients with type 1 *versus* type 2 mutations (31% *vs.* 17%, respectively); however, this finding was not statistically significant (data not shown). Histological examination showed that *ASXL1* mutations correlated with increased reticulin fibrosis (P=0.01), increased osteosclerosis (P=0.02), and reduced myeloid to erythroid ratio (P=0.03), all features of advanced stage PMF (Table 4). *ASXL1*-mutant cases showed trends towards increased marrow vascularity, hyperchromatic megakaryocyte nuclei, and osteoclastic activity; however, these findings did not reach statistical significance (Table 4). Mutations in *ASXL1* are known to confer poor prognosis in PMF patients;[11] we found that this clinical correlation is reflected in the histological findings in the biopsy.

In conclusion, we demonstrated that the morphological diagnostic parameters laid out in the 2016 WHO

Table 3. Histological features of essential thrombocythemia (ET) patients according to phenotypic driver mutation.

		ET (n = 24)		
		JAK2 (n=12)	*CALR* (n=12)	*P*
Cellularity		60.0%	62.5%	0.79
Reticulin fibrosis grade	0-1	6 (55%)	9 (75%)	0.30
	2-3	5 (45%)	3 (25%)	
Osteosclerosis grade	0-1	10 (83%)	10 (83%)	1.00
	2-3	2 (17%)	2 (17%)	
Vascularity	Decreased	1 (10%)	1 (9.1%)	0.10
	Normal	7 (70%)	8 (73%)	
	Increased	2 (20%)	2 (18%)	
Megakaryocyte count	Decreased	0	0	>0.05
	Normal	1 (8.3%)	1 (8.3%)	
	Increased	11 (92%)	11 (92%)	
Megakaryocyte size	Small	14.2%	11.7%	
	Medium	52.5%	59.2%	1.00
	Large	33.3%	29.2%	
Predominant megakaryocytic nuclear morphology		Bulbous	Staghorn	0.62
Megakaryocyte clusters	Present	11 (92%)	12 (100%)	0.31
Mean megakaryocyte cluster size		7.27	8.25	0.64
Myeloid/erythroid ratio	Decreased	3 (25%)	2 (17%)	0.86
	Normal	5 (42%)	6 (50%)	
	Increased	4 (33%)	4 (33%)	
Myeloid left shift	Present	10 (83%)	11 (92%)	0.54
Erythroid islands	Present	7 (58%)	6 (50%)	0.68
Osteoblastic activity	Present	8 (67%)	10 (83%)	0.35
Osteoclastic activity	Present	5 (42%)	1 (8.3%)	0.06

Classification accurately distinguish PMF, ET, and PV, but the application of these criteria may be further enhanced by additional detailed morphological analysis. In particular, we identified increased marrow vascularity, increased osteosclerosis, and erythroid left shift as characteristic features of overtly fibrotic PMF, whereas MPN-U exhibits small megakaryocytes with hyperchromatic nuclei and fewer clusters. Because there are currently no standard criteria for the assessment of vascularity and several other morphological parameters included in our study, our findings suggest that a more uniform approach towards the evaluation of MPN biopsies may improve the diagnostic accuracy of these diseases. Our analysis was limited by the number of examined cases and we hope that systematic analyses with larger cohort sizes will establish the significance of many of these associations in future studies.

From a molecular genetics perspective, *CALR* mutations were significantly associated with the presence of osteoblast activity, while *MPL* mutation was associated with reduced cellularity, increased osteosclerosis, and absence of vascular proliferation. In contrast, increased vascularity was observed in triple-negative cases. We also

Table 4. Histologic features of primary myelofibrosis patients according to fibrosis grade and *ASXL1* mutational status.

		Fibrosis grade			*ASXL1* status		
		0-1 (n=14)	2-3 (n=29)	*P*	Wild type (n=31)	Mutant (n=12)	*P*
Mean age		65.4	66.1	0.88	66.8	63.6	0.48
Female		9 (64%)	9 (31%)	0.04	15 (48%)	3 (25%)	0.16
New diagnosis		10 (71%)	9 (31%)	0.02	17 (55%)	2 (17%)	0.08
JAK2 inhibitor treatment	Prior	0	5 (17%)		3 (9.7%)	3 (25%)	0.01
	Current	0	8 (28%)		3 (9.7%)	4 (33%)	
Phenotypic driver mutation	*JAK2*	10 (71%)	18 (62%)	0.79	20 (65%)	8 (67%)	0.97
	CALR	2 (14%)	3 (10%)		4 (13%)	1 (8.3%)	
	MPL	1 (7.1%)	3 (10%)		3 (9.7%)	1 (8.3%)	
	TN	1 (7.1%)	5 (17%)		4 (13%)	2 (17%)	
Mean cellularity		69.3%	48.4%	0.01	60.2%	43.3%	0.79
Reticulin fibrosis	0-1				16 (52%)	0	0.001
	2-3				14 (48%)	12 (100%)	
Osteosclerosis	0-1	14 (100%)	20 (71%)	0.04	28 (93%)	6 (50%)	0.001
	2-3	0	8 (29%)		2 (6.7%)	6 (50%)	
Vascularity	Decreased	5 (38%)	1 (3.8%)	0.01	6 (22%)	0	0.10
	Normal	7 (54%)	16 (62%)		15 (56%)	8 (67%)	
	Increased	1 (7.1%)	9 (35%)		6 (22%)	4 (33%)	
Megakaryocyte count	Decreased	0	3 (11%)	0.41	2 (6.7%)	1 (8.3%)	>0.05
	Normal	1 (7.1%)	1 (3.6%)		2 (6.7%)	0	
	Increased	13 (93%)	24 (86%)		26 (84%)	11 (92%)	
Megakaryocyte size	Small	18.6%	27.5%	0.23	22.7%	29.2%	1.00
	Medium	59.3%	49.6%		52.7%	53.3%	
	Large	21.4%	22.9%		24.3%	17.5%	
Predominant megakaryocytic nuclear morphology		Bulbous	Hyper-chromatic/ hypolobated	0.02	Bulbous	Hyperchromatic	0.08
Megakaryocyte clusters	Present	13 (93%)	26 (93%)	1.00	28 (93%)	11 (92%)	0.85
Mean megakaryocyte cluster size		7.27	4.71	0.14	7.3	8.3	0.64
Myeloid/erythroid ratio	Decreased	1 (7.1%)	5 (18%)	0.27	2 (6.7%)	4 (33%)	0.03
	Normal	4 (29%)	3 (11%)		7 (23%)	0	
	Increased	9 (64%)	20 (71%)		21 (70%)	8 (67%)	
Myeloid left shift	Present	2 (14%)	9 (32%)	0.21	6 (20%)	5 (42%)	0.15
Erythroid left shift	Present	0	14 (50%)	0.001			
Erythroid islands	Present	8 (57%)	13 (46%)	0.51	14 (47%)	7 (58%)	0.49
Osteoblastic activity	Present	4 (29%)	13 (46%)	0.27	11 (37%)	6 (50%)	0.43
Osteoclastic activity	Present	1 (7.1%)	9 (32%)	0.07	5 (17%)	5 (42%)	0.09

found that secondary *ASXL1* mutations are associated with advanced stage disease in PMF. One limitation is that we did not assess disease mutational burden, which in the case of *JAK2* V617F has been associated with a proportional risk of evolution to myelofibrosis in both PV and ET.[11] We also could not determine the sequence of mutagenic events involving phenotypic driver genes and other mutations, a process that has been shown to direct phenotypic differentiation of MPN.[12,13]

While it remains unclear how the MPN mutational landscape translates into distinct morphological characteristics, we hypothesize that different morphological phenotypes in MPN are partly a result of differences in transcriptional output, which reflects both the phenotypic driver mutations and other recurrent gene mutations.[14,15] Therefore, integrated analysis of molecular and multiple histological features will allow for more accurate diagnosis and monitoring of different MPN disease types.

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