

Increased Mast Cell Numbers in Bone Marrow of Patients with Myeloproliferative Neoplasms and Pruritus

Miyeloproliferatif Neoplazmlı ve Pruritusu Olan Hastaların Kemik İliğinde Artmış Mast Hücreleri Sayısı

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Abstract

Pruritus may be a debilitating symptom in cases of myeloproliferative neoplasm (MPN). Since mast cells (MCs) play a pivotal role, we hypothesized that bone marrow (BM) MCs contribute to the occurrence of pruritus. In this exploratory study, untreated MPN patients were included. BM aspirate/biopsy/blood samples and responses to the MPN-Symptom Assessment Form were obtained. Thirty-two patients were included (essential thrombocythemia, n=19; polycythemia vera, n=7; primary myelofibrosis, n=6). Eleven had clinically significant pruritus (35%). The absolute MC count and proportion per total nucleated BM cells were significantly higher in patients with pruritus compared to those without (mean of 25.5 vs. 58.1 MCs/mm², p=0.032 and 0.59% vs. 1.66%, p=0.041, respectively). MC immunophenotypes and serum levels of tryptase, interleukin-6, tumor necrosis factor alpha, and soluble CD117 were not correlated. Thus, this study showed that absolute and relative MC numbers are increased in cases of MPN with pruritus. However, whether MCs play a pathophysiological role in causing pruritus or are bystanders remains to be determined.

Keywords: Myeloproliferative neoplasms, Mast cells, Pruritus

Öz

Pruritus, miyeloproliferatif neoplazm (MPN) olgularında güçten düşürücü bir semptom olabilir. Mast hücreleri (MH) önemli bir rol oynadığından, kemik iliği (Kİ) MH'lerinin pruritusun ortaya çıkmasına katkıda bulunduğunu varsaydık. Bu keşifsel çalışmaya tedavi edilmemiş MPN hastaları dahil edildi. Kİ aspiratı/biyopsisi/kan örnekleri ile MPN Semptom Değerlendirme Formu'na verilen yanıtlar elde edildi. Otuz iki hasta dahil edildi (esansiyel trombositemi, n=19; polisitemia vera, n=7; primer miyelofibrozis, n=6). On bir hastada klinik olarak anlamlı pruritus vardı (%35). Mutlak MH sayısı ve toplam çekirdekli Kİ hücreleri içindeki oranı, pruritusu olan hastalarda olmayanlara kıyasla anlamlı derecede daha yüksekti (sırasıyla ortalama 25,5'e karşı 58,1 MH/mm², p=0,032 ve %0,59'a karşı %1,66, p=0,041). MH immünofenotipleri ile serum triptaz, interlökin-6, tümör nekroz faktörü alfa ve çözünür CD117 düzeyleri arasında korelasyon saptanmadı. Dolayısıyla bu çalışma, prurituslu MPN olgularında mutlak ve göreceli MH sayılarının arttığını gösterdi. Bununla birlikte, MH'lerin pruritusu neden olmada patofizyolojik bir rol oynayıp oynamadığı veya yalnızca sürece eşlik eden hücreler olup olmadığı henüz belirlenmemiştir.

Anahtar Sözcükler: Miyeloproliferatif neoplazmlar, Mast hücreleri, Pruritus



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Introduction

Myeloproliferative neoplasms (MPNs) include essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF) and are characterized by abnormal myeloid proliferation, resulting in erythrocytosis, thrombocytosis, leukocytosis, and/or bone marrow (BM) fibrosis. Acquired activating mutations, mostly in the *JAK2*, *CALR*, or *MPL* genes [1], lead to elevated levels of many proinflammatory and profibrotic cytokines in the micro-milieu of the BM [2,3], which might contribute to constitutional symptoms and pruritus [4,5].

The role of mast cells (MCs) in the pathogenesis of pruritus is attributed to histamine production combined with non-histaminergic causes [6]. Earlier studies found that the mean total number of MCs in BM was increased in MPN patients [7,8]. In MPN, MCs carry the *JAK2* V617F mutation [9]. Since *JAK2* is important in MC activation pathways, mutated MCs could theoretically display abnormal degranulation and/or cytokine production leading to pruritus [10].

We hypothesized that MCs are involved in MPN-associated pruritus, either through an increase in absolute numbers or through altered activation. We designed an exploratory study to test this hypothesis.

Materials and Methods

Previously untreated MPN patients were prospectively included at the moment of diagnostic BM examination. Clinical

characteristics were analyzed based on responses to the MPN-Symptom Assessment Form (MPN-SAF) [4] and samples obtained from BM aspirate, BM biopsy, and peripheral blood. For a more detailed description of the methods, see Appendix 1.

Results

Pruritus

Thirty-two patients were included (Table 1). The MPN-SAF subitem of pruritus was considered to be positive for clinically significant pruritus at a score of ≥ 4 for this subitem [11].

Eleven patients (35%) reported significant pruritus. Patients with PV had significantly higher pruritus scores than those with ET and PMF (Figure 1A). The mean MPN-SAF pruritus subscore was significantly higher in *JAK2*-mutated cases (score of 3 vs. 0.3, respectively, $p=0.041$; Figure 1B). There were no significant differences between patients with or without pruritus regarding age, sex, or degree of myelofibrosis (Table 2).

Mast Cells

The mean absolute MC count was not significantly different between MPN subtypes (Figures 2A and 2B). Values of MCs/mm² were associated with myelofibrosis grade (Figures 2C and 2D). A positive association was found between MPN-SAF pruritus subscores and MCs/mm² as well as the percentage of MCs/total nucleated cells in BM (Figure 3). There were no significant differences between patients with or without pruritus regarding serum cytokine levels or blood counts (Table 2).

	ET (n=19)	PV (n=7)	PMF (n=6)
Age, years, mean (SEM)	61 (2)	66 (3)	75 (4)
Male, n (%)	10 (53%)	3 (43%)	3 (50%)
Thromboembolism, n (%)	3 (16%)	1 (14%)	0
Hemoglobin, mmol/L, mean (SEM)	9.4 (0.3)	10.2 (0.9)	7.0 (1.0)
Leukocytes, 10 ⁹ /L, mean (SEM)	9.5 (0.9)	10.5 (1.7)	24.1 (5.3)
Eosinophils, 10 ⁹ /L, mean (SEM)	0.29 (0.04)	0.3 (0.13)	0.78 (0.31)
Platelets, 10 ⁹ /L, mean (SEM)	705 (56)	513 (91)	841 (229)
<i>JAK2</i> mutation*, n (%)	15 (79%)	7 (100%)	3 (50%)
<i>CALR</i> mutation, n (%)	3 (16%)	0	2 (33%)
MPN-SAF total score, mean (SEM)	66 (14)	90 (8)	67 (16)
MPN-SAF pruritus, mean (SEM)	2 (1)	5 (1)	3 (1)
MCs, % of total nucleated cells, mean (SEM)	0.83 (0.20)	0.50 (0.11)	1.80 (1.15)
MCs per mm ² , mean (SEM)	39.97 (6.83)	25.11 (6.22)	74.10 (30.60)
Tryptase, µg/mL, mean (SEM)	7.3 (0.8)	7.6 (1.2)	13.8 (6.5)
IL-6, pg/mL, mean (SEM)	17.5 (8.9)	14.2 (14.1)	4.4 (4.4)
TNF-α, pg/mL, mean (SEM)	67.4 (52.5)	145.7 (142.4)	32.3 (11.7)
sCD117, ng/mL, mean (SEM)	68.5 (4.3)	61.9 (4.4)	50.3 (3.6)

*: 21 patients had V617F and 3 had exon 12 mutation in *JAK2*. None had *MPL* mutation. Mutation status was missing for 2 patients.
 ET: Essential thrombocythemia; PV: polycythemia vera; PMF: primary myelofibrosis; SEM: standard error of the mean; MPN-SAF: Myeloproliferative Neoplasm-Symptom Assessment Form; MC: mast cell; IL-6: interleukin-6; TNF-α: tumor necrosis factor alpha; sCD117: soluble CD117.

There were nonsignificant correlations between serum tryptase levels and absolute MC count/mm² and MC percentage. Furthermore, serum tryptase levels correlated with the interleukin (IL)-6 levels ($R=0.424$, $p=0.011$), but not tumor necrosis factor alpha (TNF- α) or soluble CD117 (sCD117).

Twenty patients had MC counts high enough (>100) to be included in the analysis of flow cytometry data. A heatmap was

created to gain insight into the patterns of marker expression (Figure 4). No relevant correlations were observed. There were also no differences in MC immunophenotypes between MPN subgroups. Statistical analysis was not performed to avoid multiple testing bias given the small patient sample and the high number of markers tested.

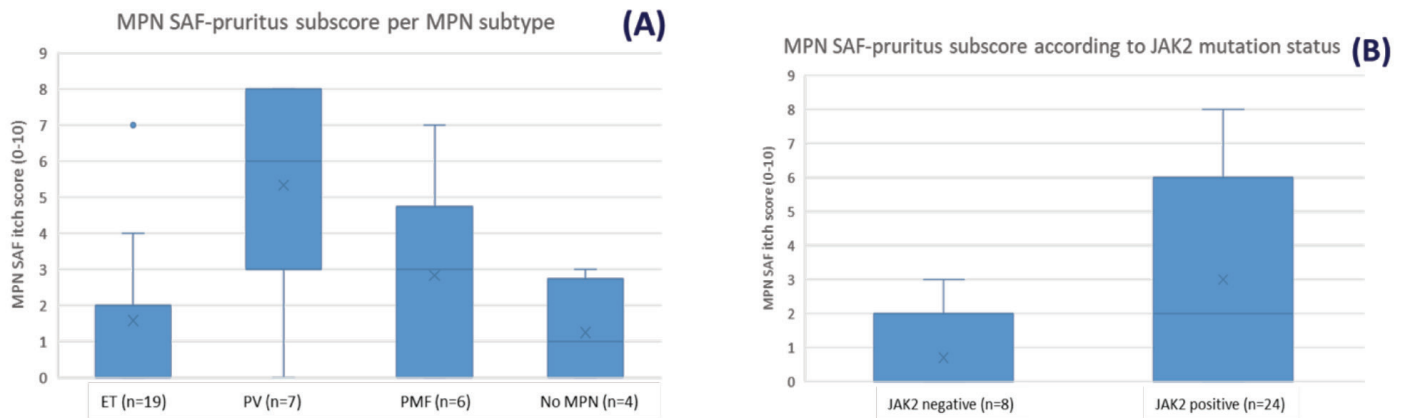


Figure 1. Myeloproliferative neoplasm-Symptom Assessment Form (MPN-SAF) pruritus subscores. A) Mean MPN-SAF pruritus subscores per MPN subtype. The mean score was significantly higher for polycythemia vera (PV) (mean: 5, standard error of the mean: 1) compared to essential thrombocythemia (ET) (mean: 2, standard error of the mean: 1) and primary myelofibrosis (PMF) (mean: 3, standard error of the mean: 1) ($p=0.037$, Kruskal-Wallis test). B) Mean MPN-SAF pruritus subscores in *JAK2*-negative versus *JAK2*-positive MPN. The mean score was significantly higher for *JAK2*-positive patients (mean: 3, standard error of the mean: 1) compared to *JAK2*-negative patients (mean: 0.3, standard error of the mean: 0.1) ($p=0.041$, Mann-Whitney U test).

Table 2. Patients with pruritus compared to those without pruritus.

	No pruritus (n=20)	Pruritus (n=11)	p
Male, n (%)	11 (55%)	4 (36%)	NS
Thromboembolism, n (%)	2 (10%)	2 (18.2%)	NS
MPN-SAF total score, mean (SEM)	57 (13)	91 (7)	0.009
Subtype			0.02
ET, n (%)	15 (75%)	(36%)	
PV, n (%)	1 (5%)	5* (45.5%)	
PMF, n (%)	4 (20%)	2 (18.2%)	
<i>JAK2</i> V617F, n (%)	14 (70%)	9 (82%)	0.042
<i>CALR</i> , n (%)	5 (25%)	0	NA
<i>MPL</i> , n (%)	0	0	NA
Age, years, mean (SEM)	64 (3)	69 (3)	NS
MCs, % of total nucleated cells, mean (SEM)	0.59% (0.12)	1.66% (0.68)	0.041
MCs per mm ² , mean (SEM)	25.52 (6.36)	58.08 (17.97)	0.032
Tryptase, μ g/L, mean (SEM)	7.3 (0.9)	10.3 (3.6)	NS
IL-6, pg/mL, mean (SEM)	16.7 (8.9)	12.8 (9.1)	NS
TNF- α , pg/mL, mean (SEM)	69.9 (52.3)	106 (89.8)	NS
sCD117, ng/mL, mean (SEM)	63.9 (4.3)	60.5 (2.7)	NS

*: Initially, 7 PV patients were included in this study. In 1 case, no pruritus data were available.

MPN-SAF: Myeloproliferative Neoplasm-Symptom Assessment Form; SEM: standard error of the mean; ET: essential thrombocythemia; PV: polycythemia vera; PMF: primary myelofibrosis; MC: mast cell; IL-6: interleukin-6; TNF- α : tumor necrosis factor alpha; sCD117: soluble CD117; NS: not significant ($p>0.05$); NA: statistical analysis not applicable. Pruritus: MPN-SAF pruritus score of >3 .

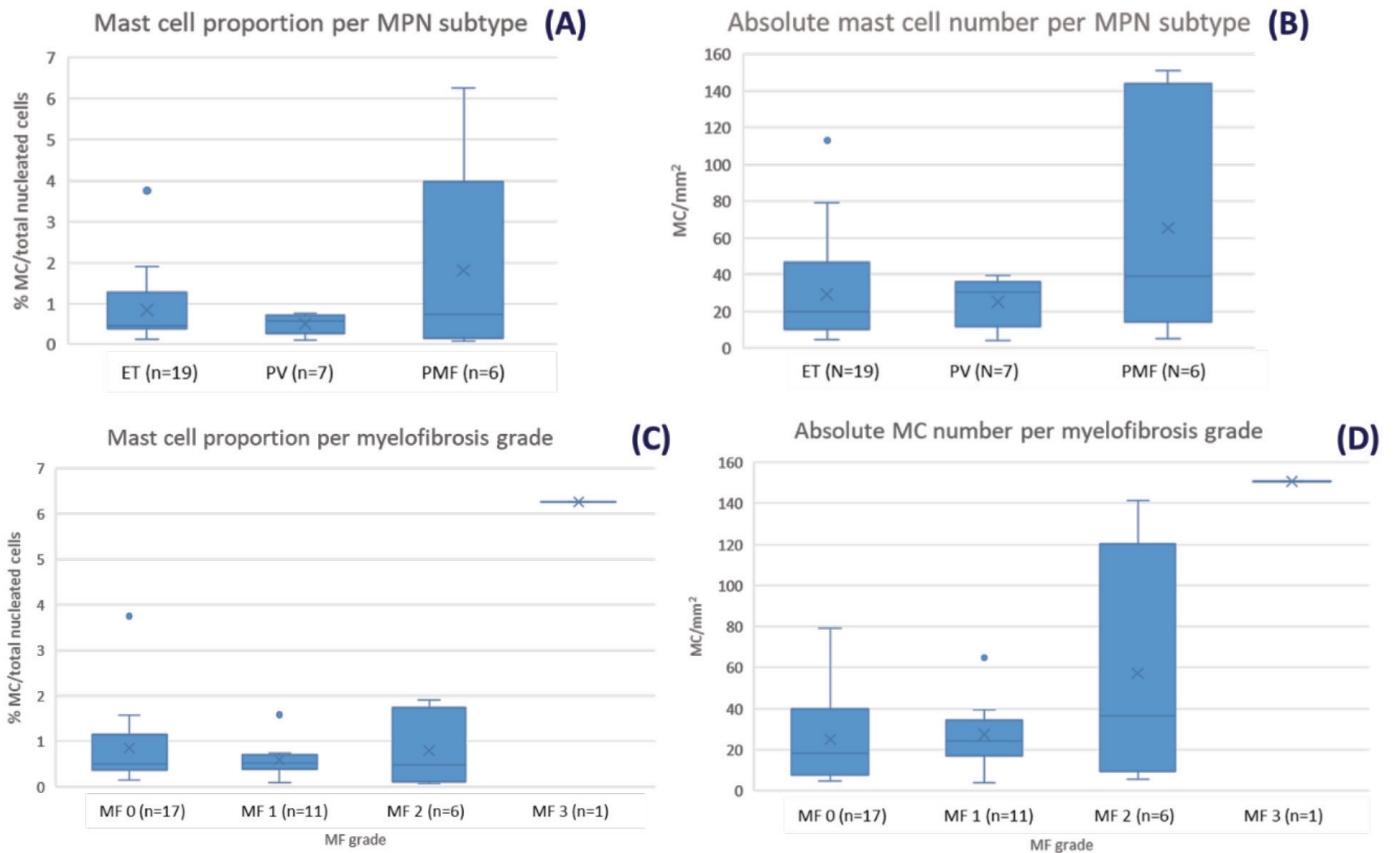


Figure 2. Bone marrow mast cell (MC) counts according to myeloproliferative neoplasm (MPN) subtype. A) The mean percentage of MCs per total nucleated cells was higher in primary myelofibrosis (PMF) compared to essential thrombocythemia (ET) and polycythemia vera (PV) without reaching statistical significance ($p=0.859$, Kruskal-Wallis test). B) The mean absolute count of MCs/mm² was higher in PMF compared to ET and PV without reaching statistical significance ($p=0.463$, Kruskal-Wallis test). See Table 1 for absolute numbers. C) The percentage of MCs per total nucleated cells was higher in cases with myelofibrosis (MF) grade of 3 compared to MF 0-2, but because only one patient with MF 3 was included, statistical analysis was not applicable. D) The mean absolute count of MCs/mm² seemed to be increased in cases of MF 2 and MF 3 compared to lower MF grades, but because only one patient with MF 3 was included, statistical analysis was not applicable.

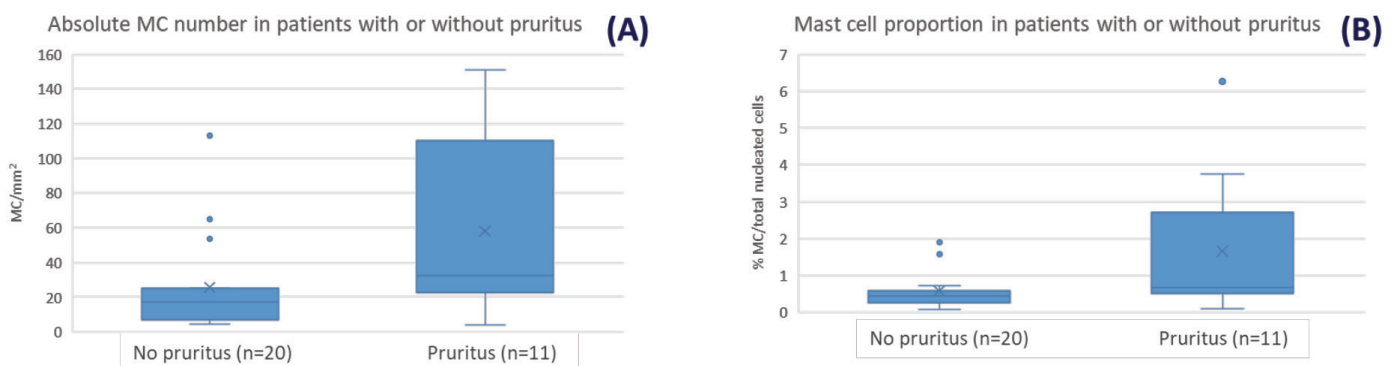


Figure 3. Bone marrow mast cell (MC) counts associated with pruritus. A) The mean percentage of MCs per total nucleated cells was significantly higher in myeloproliferative neoplasm (MPN) patients with pruritus as defined by a Myeloproliferative Neoplasm-Symptom Assessment Form (MPN-SAF) pruritus subscore of >3 compared to those without pruritus ($p=0.041$, Mann-Whitney U test). B) The mean absolute count of MCs/mm² was significantly higher in MPN patients with pruritus as defined by MPN-SAF pruritus subscore of >3 compared to those without pruritus ($p=0.032$, Mann-Whitney U test).

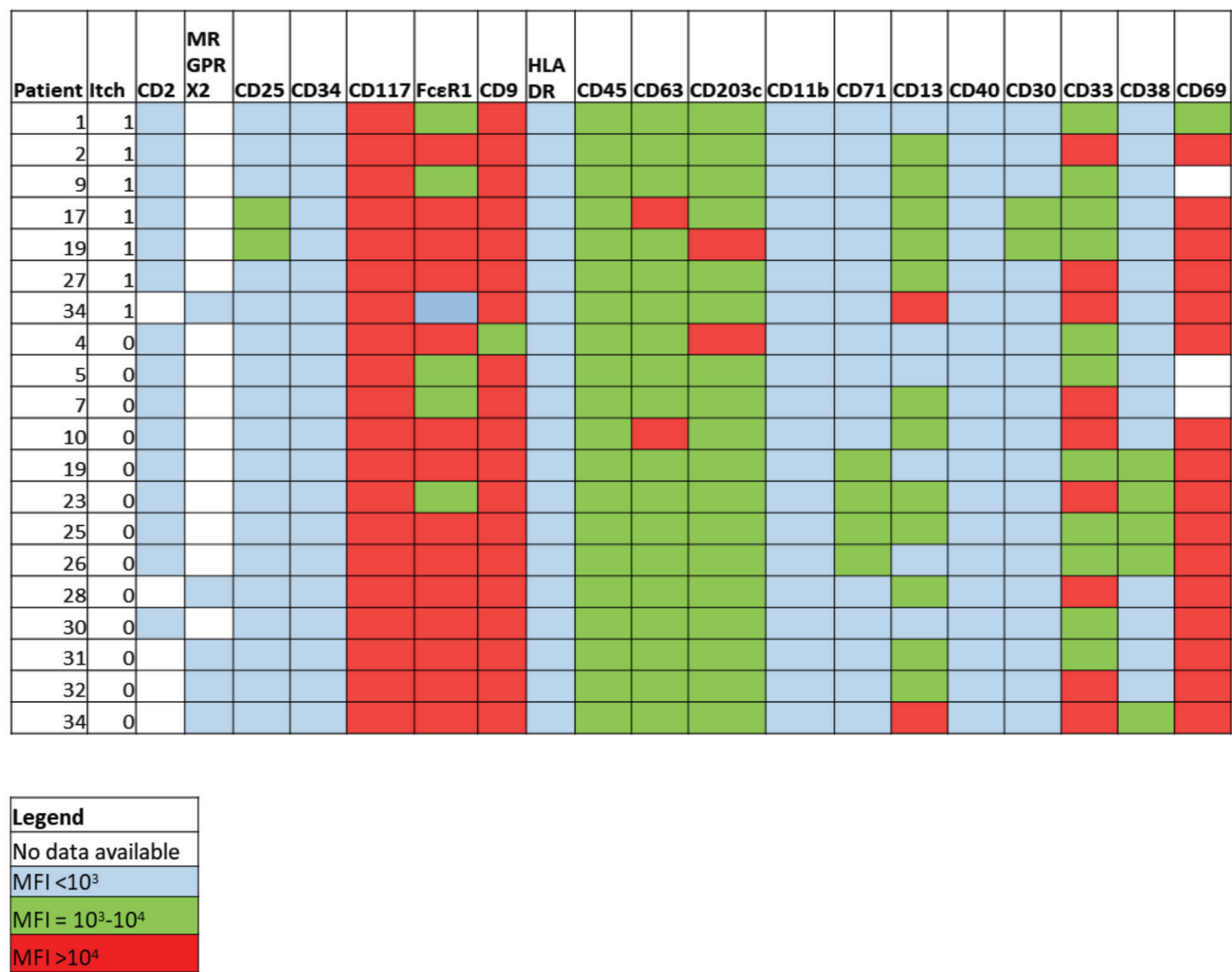


Figure 4. Heatmap of the immunophenotype of bone marrow mast cells (MCs) measured by flow cytometry. The expression of MC-specific surface markers, particularly the markers related to MC activation, were not significantly altered in patients with pruritus compared to those without pruritus.

MFI: Mean fluorescent intensity.

Systemic Mastocytosis

Spindle-shaped MCs were observed in eight patients, with only one at a degree of >25%, constituting a minor World Health Organization (WHO) criterion for systemic mastocytosis (sM). Furthermore, two patients had spindle-shaped MCs at rates of 10%-25% and five others at rates of <10%. Five of 8 patients (63%) with any degree of spindle-shaped MCs had MPN-SAF pruritus subscores of ≥4 versus 6/24 (25%) without pruritus (p=0.079). The mean serum tryptase levels were also higher in this subgroup (12.3 µg/L (standard error of the mean: 3.9) vs. 6.7 µg/L [standard error: 0.6], p=0.058), but the levels of IL-6, TNF-α, and sCD117 did not differ. In two patients, aberrant CD25 expression was found by flow cytometry; both of these cases lacked other WHO criteria for sM, although both reported

significant pruritus. Moreover, the MCs of both of these patients displayed moderate but aberrant CD30 expression. The presence of the D816V *KIT* mutation was not routinely investigated in this trial. No patient fulfilled the diagnostic criteria for sM based on the available information.

Discussion

In this small study, we explored the hypothesis that MCs are related to MPN-associated pruritus. We found that one-third of MPN patients had significant pruritus, which was associated with increased absolute and relative MC numbers in the BM but not with alterations in MC immunophenotype or associated serum markers.

Previous studies have provided evidence that MCs are important players in the BM microenvironment. In a study using a BM organoid model of MPN, MCs proved to be a signaling hub reinforcing TNF- α and IL-4 signaling, thereby creating a proinflammatory loop [12]. Other studies showed increased MC numbers in BM and upregulated activation markers on the MCs of MPN patients [7,8,9]. The number of MCs correlated positively with the grade of reticulin fibrosis, splenomegaly, and the presence of the *JAK2* V617F mutation in one study (n=34) [13].

The MCs of MPN patients produce more transforming growth factor beta and IL-13 in vitro [14], and increased levels of IL-4 and IL-13 in BM are associated with the grade of fibrosis [15]. Skin MCs of MPN patients are more easily activated compared to those of healthy individuals [16]. This could be explained by the fact that MCs from MPN patients also carry the *JAK2* V617F mutation [9]. A small uncontrolled cohort study described a positive effect of omalizumab, a monoclonal against immunoglobulin E, in MPN-associated pruritus, again suggesting a role for MCs [17].

Our study was designed as a first exploration of MC numbers and phenotypes in relationship to pruritus, not to corroborate pathophysiological mechanisms. The question thus remains whether the increased MCs that we found in patients with MPNs play a pathophysiological role in causing pruritus or are merely bystanders attracted secondarily to the increased cytokine levels associated with MPNs. MCs are known to produce many other signaling substances besides histamine and tryptase [18], potentially causing a non-histaminergic form of pruritus [19]. Interestingly, the JAK-STAT pathway is also involved in non-histaminergic pruritus through modulation of peripheral nerve activity and epidermal barrier integrity [20,21]. Alternatively, this might explain our observation that pruritus was present despite low numbers of MCs in the PV cohort. Based on these data, linking the increase in MCs that we and others have consistently found in both the BM and skin of MPN patients to the activating *JAK2* mutation and (non-histaminergic) pruritus seems a promising route for further translational research in MPN. In addition, since pruritus was more prominent among patients with PV, the possibility remains that disease subtype may influence the observed association between MC burden and pruritus.

In our cohort, several patients had signs of sM that would not have been noticed in routine diagnostic studies for MPNs. A higher presence of spindle-shaped MCs or the aberrant expression of CD25 and/or CD30 was observed in patients with significant pruritus, although the patient numbers were too small for statistical analysis. In one patient, both >25% spindle-shaped MCs and the presence of two MC aggregates in the BM biopsy were noted, suggesting concurrent sM. Two other patients had aberrant CD25 expression on MCs. Unfortunately, no data on D816V *KIT* mutations were available, precluding firm conclusions

on the cooccurrence of sM in our cohort. Coexisting mastocytosis might be possible, limiting the interpretation of our results. We suggest that additional diagnostics for sM be performed for every patient with (suspected) MPN, especially those with clinically significant pruritus. This might have prognostic and therapeutic implications.

Conclusion

In addition to the impact of incomplete data on mastocytosis, the interpretation of our data might also be limited by the imbalance of MPN subtypes and the limited sample size, causing the study to be underpowered for extensive statistical testing. The absence of significant differences in MC phenotype and serum markers may reflect limited statistical power rather than true biological equivalence. However, the consistent association of MC numbers with pruritus in this exploratory, prospective MPN cohort offers a basis for more extensive research on this topic.

Ethics

Ethics Committee Approval: Ethical approval for this study was obtained from the Medical Ethical Committee of Erasmus MC, Rotterdam, the Netherlands (approval no.: MEC-2018-1504/NL64166.078.17; date: January 28, 2019).

Informed Consent: All patients provided written informed consent.

Footnotes

Authorship Contributions

Surgical and Medical Practices: P.W.; Concept: M.H., P.t.B.; Design: M.H., W.D., V.v.d.V., P.t.B.; Data Collection or Processing: M.H., P.W., T.v.d.B., W.D., K.L., S.v.d.M., F.N., V.v.d.V., P.t.B.; Analysis or Interpretation: M.H., P.W., T.v.d.B., W.D., K.L., S.v.d.M., F.N., V.v.d.V., P.t.B.; Literature Search: M.H., V.v.d.V., P.t.B.; Writing: M.H., P.W., T.v.d.B., W.D., K.L., F.N., V.v.d.V., P.t.B.

Conflict of Interest: No conflict of interest was declared by the authors.

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Appendix 1. Methods.

Histopathology

Sequential formalin-fixed paraffin-embedded sections of 4 µm in thickness were stained for tryptase using ultraView (Roche Diagnostics, Basel, Switzerland). In brief, following deparaffinization and heat-induced antigen retrieval with CC1 (#950-500, Ventana, Roche Diagnostics) for 64 min, the tissue samples were incubated with tryptase for 16 min at 37 °C. Incubation was followed by hematoxylin II counterstaining for 8 min and then a blue coloring reagent for 8 min according to the manufacturer's instructions (Ventana).

The number of mast cells (MCs) was quantified using open-source QuPath software for digital pathology image analysis. First, an object classifier was trained to recognize tryptase-positive cells as MCs by deep learning method. Subsequently, the surface area of each sample was calculated, after which the total amount of nucleated cells and the amount of tryptase-positive MCs were analyzed by the trained software. From these data, the number of MCs/mm² and the percentage of MCs/total nucleated cells could be calculated. Finally, reticulin staining was performed to determine the degree of myelofibrosis (MF), with scores ranging from MF 0 to MF 3 as is customary.

Flow Cytometry

Flow cytometric immunophenotyping was performed using three eight-color tubes with CD45, CD34, and CD117 as the backbone. Bone marrow (BM) samples were first bulk-lysed according to EuroFlow (Zutphen, the Netherlands) protocols, and 50 µL of a cell suspension of 100x10⁶ cells/mL was used per tube. Samples were stained according to EuroFlow protocols and 3x10⁶ cells were acquired on a FACSCanto or FACSLytic flowcytometer (BD Biosciences, Franklin Lakes, NJ, USA) set up according to EuroFlow. A multiplex panel of 18 surface markers related to MC activation of monoclonality was used, detecting CD2, CD9, CD11b, CD13, CD25, CD30, CD33, CD34, CD38, CD40, CD45, CD63, CD71, CD69, CD117, CD203c, FcεR1, HLA-DR, and MRGPRX2. Data were analyzed using Infinicyt software (BD Biosciences); MCs were gated based on strong CD117 positivity. Only patients

with >100 counted MC events were included for analysis. The expression of surface markers was graded according to mean fluorescent intensity (MFI) as follows: MFI of <103 = 0, MFI of 103-104 = 1, and MFI of >104 = 2.

Serum Markers

Serum was obtained at the same visit at which the questionnaire was administered and BM samples were collected. All sera were stored at -20 °C until analysis, at which time the levels of serum tryptase, interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and soluble CD117 (sCD117) were measured. Serum tryptase levels were measured by fluorescence enzyme immunoassay on a Phadia 250 system using the ImmunoCAP tryptase assay according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). IL-6, TNF-α, and sCD117 levels were measured by enzyme-linked immunosorbent assay (IL-6 and TNF-α: R&D Systems, Abingdon, UK; sCD117: Thermo Fisher Scientific).

Ethical Considerations

Ethical approval for this study was obtained from the Medical Ethical Committee of Erasmus MC, Rotterdam, the Netherlands (approval no.: MEC-2018-1504/NL64166.078.17; date: January 28, 2019). All patients provided written informed consent.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 28.0 for Windows (IBM Corp., Armonk, NY, USA) and two-sided values of p<0.05 were considered to indicate statistical significance. Categorical variables were summarized as numbers with percentages. Continuous variables were summarized as mean values with standard error of the mean unless specifically stated otherwise. To compare categorical data, the Fisher exact test was used, while Mann-Whitney U and Kruskal-Wallis tests were used to compare continuous variables. Spearman rho values were calculated to determine correlations between two continuous variables. Statistical analysis was not performed if >1 of the subgroups contained only one patient.