Clinical utility of the absolute number of circulating CD34-positive cells in patients with chronic myeloproliferative disorders

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Background and Objectives. Flow cytometry enumeration of peripheral blood CD34-positive cells provides reliable measurements of circulating hematopoietic progenitors in humans. Since the absolute number of circulating CD34-positive cells has been previously found to be elevated in myelofibrosis with myeloid metaplasia (MMM), we prospectively studied the clinical utility of this parameter in the work-up of patients with chronic myeloproliferative disorders.

Design and Methods. Of the 248 consecutive patients enrolled in this study, 106 had polycythemia vera (PV), 90 essential thrombocythemia (ET), and 52 myelofibrosis with myeloid metaplasia (MMM). The study population included both newly diagnosed and established cases, and of these latter some patients were on cytoreductive treatment while others were chemotherapy naive. Both cross-sectional and longitudinal investigations were carried out. Flow cytometry enumeration of CD34-positive cells was performed using a single-platform assay.

Results. Median numbers and ranges of circulating CD34-positive cells were 2.3×10^6/L (0-5) in 20 control subjects, 2.2×10^6/L (0-14) in those with PV, 2.4×10^6/L (0-14) in those with ET, and 114×10^6/L (6-2,520) in MMM patients. Analysis of variance demonstrated that values were markedly higher in MMM patients than in the remaining groups, and counts did not appear to fluctuate over short periods of follow-up. In both cross-sectional and longitudinal investigations on patients at clinical onset and/or out of cytoreductive treatment, a CD34-positive count of $\geq 15\times10^6$/L was always associated with MMM, clearly indicating a disease-related specificity

Interpretation and Conclusions. The absolute number of circulating CD34-positive cells is normal or anyhow lower than 15×10^6/L in patients with uncomplicated PV or ET, whereas it is equal or above this cut-off in those with MMM, likely reflecting abnormal hematopoietic progenitor cell trafficking. Thus, enumeration of circulating CD34-positive cells may be useful in the work-up of patients with myeloproliferative disorders.

Key words: CD34-positive cell, myeloproliferative disorder, myelofibrosis with myeloid metaplasia, polycythemia vera, essential thrombocythemia.

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Myelofibrosis with myeloid metaplasia (MMM) is a chronic myeloproliferative disorder (CMD) characterized by clonal hematopoietic proliferation, reactive marrow fibrosis and extramedullary hematopoiesis.[1,2] Initial clues to the diagnosis include detection of splenomegaly on physical examination and recognition of teardrop-shaped red cells, immature granulocytes and nucleated red cells in the peripheral blood. Necessary diagnostic criteria are diffuse bone marrow fibrosis and absence of Philadelphia chromosome in bone marrow cells (or absence of *BCR-ABL* rearrangement in peripheral blood cells).[3]

At variance with chronic myeloid leukemia, Philadelphia-negative chronic myeloproliferative disorders [i.e., polycythemia vera (PV), essential thrombocythemia (ET) and MMM] lack a reliable molecular marker so far. Although the *PRV-1* gene is over-expressed in granulocytes from patients with PV,[4] this pattern does not appear to be specific.[5] Current diagnostic criteria for CMDs include combinations of clinical and laboratory parameters that were established decades ago,[6,7] and there is a need to define more stringent diagnostic criteria and identify reliable parameters[8-10] that can help clinicians in diagnostic and prognostic decision-making.[11,12]

Two years ago Barosi and co-workers[13] reported elevated numbers of circulating CD34-positive cells in patients with MMM, and suggested that this parameter may allow MMM to be distinguished from other Philadelphia-negative CMDs. However, they studied very few patients with PV or ET. Andreasson and co-workers,[14,15] found increased numbers of circulating CD34-positive cells in patients with PV or ET, although values were lower than those observed in patients with MMM.[16] We, therefore, conducted a prospective study aimed at defining the utility of the absolute number of circulating CD34-positive cells in the work-up of patients with myeloproliferative disorders.

Design and Methods

This prospective study enrolled 248 consecutive patients admitted to the Outpatient Department of the Division of Hematology, IRCCS Policlinico San Matteo, Pavia, Italy, from January 2001 to January 2003. The procedures followed were in accordance with the ethical standards of the institutional committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983.

Patients and diagnostic criteria

The diagnosis of PV was established according to the modified criteria proposed by Pearson *et al.*,[17] and that of ET according to Murphy *et al.*[18] The Italian Consensus Conference criteria[3] were utilized for the diagnosis of MMM. Twenty normal subjects were studied to define a normal reference group. One hundred and six patients had PV, 90 had ET, and 52 had MMM. Of these last, 32 patients had primary MMM, 14 had post-PV MMM, and 6 had post-ET MMM. Of the 106 PV patients, 30 (28%) were studied at clinical onset and 76 (72%) during follow-up (59 on cytoreductive treatment and 17 on phlebotomy). Of the 90 ET patients, 19 (21%) were studied at clinical onset and 71 (79%) during follow-up (47 on cytoreductive treatment and 24 on low-dose aspirin). Of the MMM patients, 10 (19%) were studied at clinical onset and 42 (81%) during follow-up: 32 had primary MMM, 14 post-PV MMM, and 6 post-ET MMM.

Sequential studies were performed in 91 patients, of whom 53 had PV, 25 had ET and 13 had MMM. These subjects were systematically re-evaluated one year after the first study.

Flow cytometry enumeration of circulating CD34+ cells

Flow cytometry enumeration of circulating CD34-positive cells was carried out by one of us (LV) in a blind manner. Peripheral blood samples were drawn into K3-EDTA-anticoagulated tubes for complete blood counts and enumeration of the percentage of CD34-positive cells by flow cytometry. Cells (50 µL of blood) were incubated for 15 minutes at room temperature with fluorescein isothiocyanate (FITC)-conjugated CD45 monoclonal antibody (Becton-Dickinson, San José, CA, USA) and phycoerythrin (PE)-conjugated CD34 monoclonal antibody (HPCA 2, Becton-Dickinson, San José, CA, USA) for progenitor cells. Laser dye styryl (LDS751) for nuclear identification, and ammonium chloride for lysing red blood cells were added to micro-bead tubes (TruCount, Becton-Dickinson, San José, CA, USA). Analyses were performed using a FACSCalibur flow cytometer (Becton-Dickinson, San José, CA, USA) and a single-platform assay following the cell-gating guidelines recommended by the International Society of Hematotherapy and Graft Engineering (ISHAGE)[19] and the subsequent modifications of the European Working Group of Clinical Cell Analysis.[20] The Cell Quest 3.1 program (Becton-Dickinson, San José, CA, USA) was utilized to acquire CD34-positive cells according to logical gates analysis of the ISHAGE protocol.

Statistical analyses

Numeric variables are summarized by their median and range while categorical variables are described by counts and relative frequencies. Analysis of variance (ANOVA) techniques were employed to test for differences among circulating CD34-positive cell count in different conditions. CD34-positive cell counts were log-transformed in order to reduce departures from normality of their distribution. The Kolmogorov-Smirnov test was employed to check for normality of the log-CD34-positive cell counts. The Scheffé post-hoc test was used to carry out comparisons between pairs of disease categories. In MMM patients, univariate associations between log-CD34-positive cell count and numeric parameters such as hemoglobin, white cell count, platelet count, splenomegaly (cm), hepatomegaly (cm), disease duration, age at diagnosis, and age at CD34-positive cell evaluation were investigated using Pearson's correlation coefficient. Multiple regression analysis was also performed in order to test for multivariate associations. In the longitudinal investigation, the non-parametric Wilcoxon rank sign test for paired data was employed to test for differences between subsequent circulating CD34-positive cell counts. ROC analysis was employed for defining the best cut-offs.

Table 1. Main clinical characteristics and hematologic data of the patients studied.

	PV	*ET*	*MMM*
	(n=106)	*(n=90)*	*(n=52)*
Age at the time of study, years	64 (18-87)	63 (19-91)	63 (31-88)
Male/female ratio	77/29	23/67	30/22
Patients at clinical onset	30/106	19/90	10/52
Patients on cytoreductive treatment	59/106	47/90	30/52
Hb, g/dL	16.7 (12.3-23.1)	13.8 (11.0-15.5)	11.5 (7.6-14.2)
White blood cells, 10^9/L	7.3 (3.1-17.6)	7.8 (3.7-24.0)	10.0 (2.0-96.0)
Platelet count, 10^9/L	249 (102-1170)	726 (250-1300)	300 (3-1100)
Splenomegaly, no.	16/106	24/90	50/52*
Hepatomegaly, no.	25/106	13/90	31/52

*Abbreviations: PV: polycythemia vera, ET: essential thrombocythemia; MMM: myelofibrosis with myeloid metaplasia. *Two patients had been splenectomized.*

Table 2. Absolute number of circulating CD34-positive cells in patients with chronic myeloproliferative disorders. Median values and ranges are shown.

	Polycythemia vera	*Essential thrombocythemia*	*Myelofibrosis with myeloid metaplasia*
		Circulating CD34-positive cells ($\times 10^6$/L)	
Cross sectional analysis			
All patients	2.2 (0-14) (n=106)	2.4 (0-14) (n=90)	114(6-2520) (n=52)
Patients without cytoreductive treatment	3.0 (0-12) (n=47)	3.4 (0-14) (n=43)	70 (16-743) (n=22)
Patients under cytoreductive treatment	1.8 (0-14) (n=59)	1. (0-10) (n=47)	124 (6-2520) (n=30)
Longitudinal analysis			
First evaluation	2.1 (0-14) (n=53)	1.9 (0-8) (n=25)	96 (16-260) (n=13)
Second evaluation after 12 months	1.3 (0-8) (n=53)	2.0 (1-7) (n=24)*	152 (12-535) (n=13)
Normal controls		2.3 (0-5) (n=20)	

** One of the 25 ET patients enrolled in the longitudinal study developed post-ET myelofibrosis between the first and the second evaluation, and was therefore excluded from the second analysis. Evolution into MMM was associated with a marked increase in the number of circulating CD34-positive cells (from 8 to 120 $\times$ 10^6/L).*

Results

The main clinical features and blood cell counts of the patients studied are summarized in Table 1. Circulating CD34-positive cell counts are shown in Table 2 and Figure 1.

Analysis of variance excluded any significant difference in the absolute number of circulating CD34-positive cells between PV, ET and controls, although upper limits were higher in patients with PV or ET. In contrast, the absolute number of CD34-positive cells was significantly higher in MMM patients than in the remaining groups (p<0.00001). Considering patients at clinical onset and/or out of cytoreductive treatment, ROC analysis demonstrated that a cut-off of $\geq 15\times10^6$/L accurately distinguished MMM patients from the other patients.

Analysis of variance showed that being on cytoreductive treatment involved significantly lower numbers of circulating CD34-positive cells in patients with PV (p=0.001) and ET (p<0.0001), but not in those with MMM (p>0.05). Accordingly, patients with newly diagnosed PV or ET had significantly higher numbers of circulating CD34-positive cells than those in follow-up (p=0.04).

The non-parametric Wilcoxon rank sign test for paired data was employed to analyze sequential investigations (longitudinal analysis in Table 2). There was a significant decrease in the number of CD34-positive cells between sequential evaluations in PV patients ($p < 0.0001$), reflecting a higher proportion of individuals under cytoreductive treatment after 12 months. In contrast, no significant difference was found in patients with ET and MMM. Again, counts $\geq 15\times10^6$/L were always associated with MMM.

Of particular interest was the case of a 62-year old woman, who was diagnosed with ET twelve years before entering this study. At baseline, she had a nearly normal CD34-positive cell count (8×10^6/L). One year later, the circulating CD34-positive cell count had markedly increased (120×10^6/L): a bone marrow biopsy showed diffuse fibrosis consistent with the diagnosis of post-ET MMM. In contrast, three patients (one with PV and 2 with ET) showed progressive splenomegaly but steadily low CD34-positive cell counts ($< 15\times10^6$/L) during the follow-up. Bone marrow biopsy excluded evolution into MMM in all of them.

Multivariate regression analysis of data from patients with MMM showed that the absolute number of circulating CD34-positive cells was related to platelet count (inverse relationship, p=0.004) and hemoglobin (inverse relationship, p=0.02). These data might indicate that the greater the degree of bone marrow failure, the higher the number of circulating hematopoietic cells. While the longitudinal study did not show any significant effect of oral cytoreductive treatment on CD34-positive cell counts in MMM

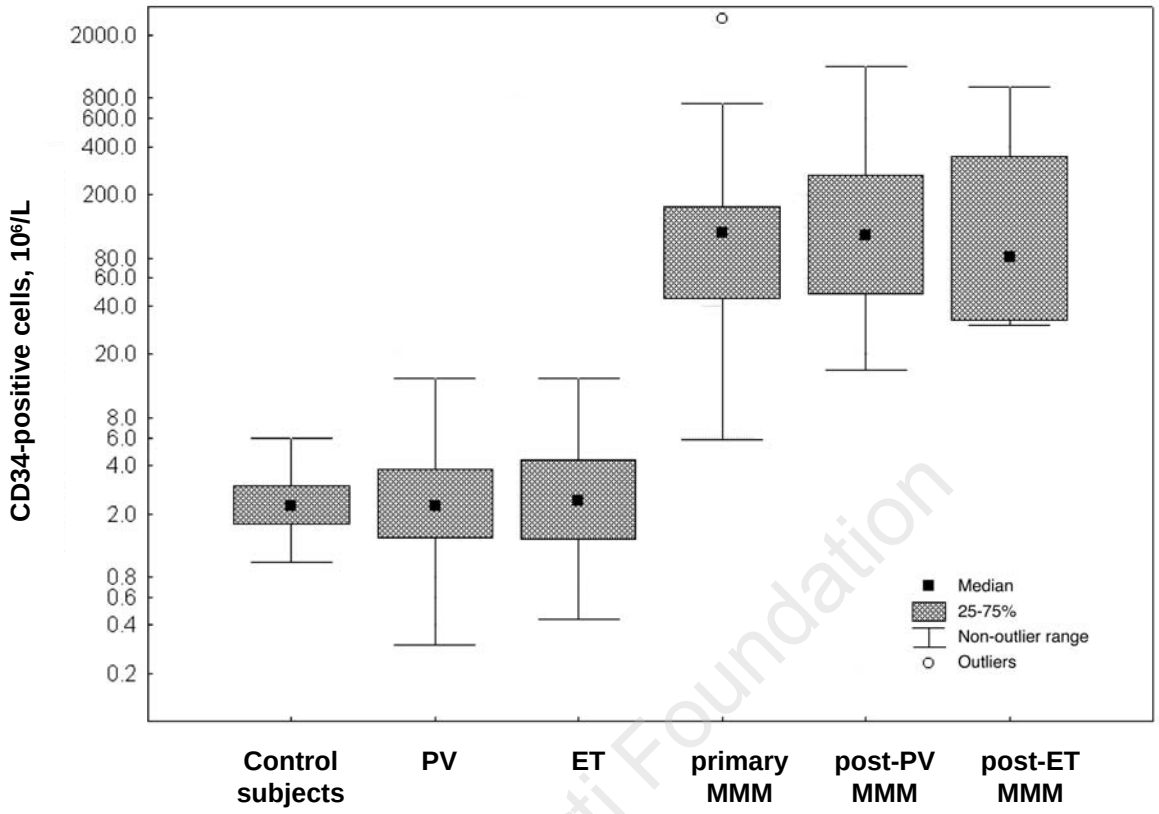

Figure 1. Box-plot of circulating CD34-positive cell counts in patients with chronic myeloproliferative disorders and in control subjects. ***PV: polycythemia vera; ET: essential thrombocytemia; MMM: myelofibrosis with myeloid metaplasia.***

patients, more aggressive treatments had a significant impact. Within MMM patients enrolled in the cross sectional study, one received aggressive chemotherapy and another one was treated with splenic irradiation. In both cases, there was a prolonged post-treatment pancytopenia, associated with nearly undetectable circulating CD34-positive cells.

Discussion

CD34 is a transmembrane glycophosphoprotein expressed on early hematopoietic cells.[21] Expression levels decline with hematopoietic differentiation, so that the earliest clonogenic cells (long-term culture-initiating cells, LTC-IC) express the highest levels, while the most differentiated ones (e.g., erythroid colony-forming units or CFU-E) show low expression of CD34.

Under normal conditions, CD34-positive cells represent a small proportion (less than 1%) of bone marrow nucleated cells. There is little exchange of hematopoietic stem cells from the marrow through the blood and into the marrow under basal conditions[22] and, in steady state, CD34-positive cells form less than 0.1% of peripheral blood nucleated cells in humans. This percentage can, however, increase to 1-5% with administration of mobilizing chemotherapy and/or colony-stimulating factor.[23]

Using different approaches, increased numbers of circulating pluripotent and committed colony-forming cells have been found in patients with myeloproliferative disorders.[24,25] Not surprisingly, elevated numbers of circulating CD34-positive cells have also been previously reported in these conditions,[13-16] suggesting abnormalities in bone marrow stem cell homeostasis. The findings of this study clearly indicate that the absolute number of circulating CD34-positive cells is within the reference range in most patients with PV or ET, while it is markedly elevated in those with MMM.

Previous studies[14-16] have reported increased CD34-positive cell counts in patients with PV or ET. Although we did not find any significant difference between these patients and control subjects, the box-plot reported in Figure 2 indicates that a few patients with PV or ET tend to have higher counts. Only long-term follow-up studies will allow it to be established whether these individuals are indeed

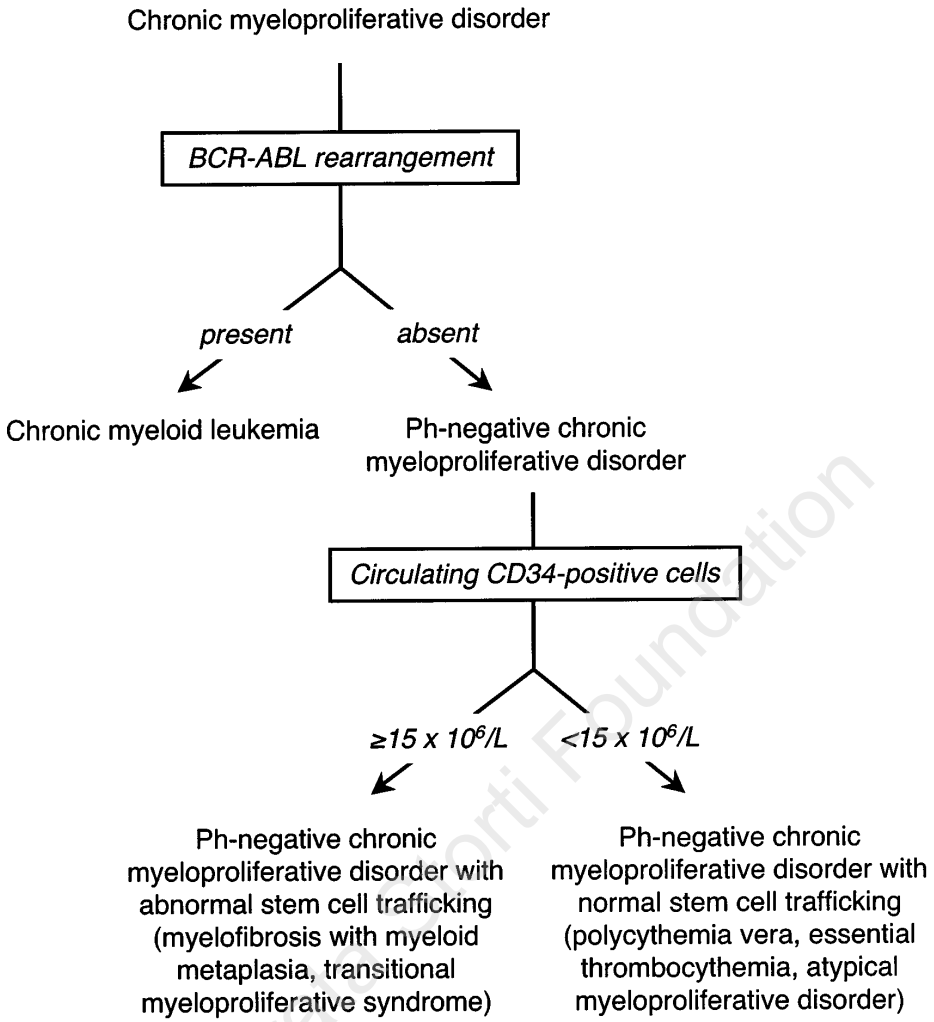

Figure 2. Separation of chronic myeloproliferative disorders based on the evaluation of *BCR-ABL* rearrangement in peripheral blood cells and on the enumeration of circulating CD34-positive cells. This diagram is a schematic representation of our current knowledge in this field, and might be employed as a working diagnostic pathway in clinical studies.

evolving into MMM. From a practical point of view, examining data on patients out of cytoreductive treatment, reported in Table 2, it is apparent that there is no substantial overlap in CD34-positive cell counts between MMM and the remaining myeloproliferative disorders, and that a cut-off of $\geq 15\times10^6$/L can accurately distinguish MMM patients from those with other Philadelphia-negative CMDs. Increased numbers of circulating CD34-positive cells may be found in high-risk myelodysplastic syndromes.[26] However, in these latter conditions as well as in acute myeloid leukemia,[27] the elevated counts essentially reflect leukemic blasts expressing CD34 (*Mario Cazzola, unpublished observations, 2003*).

An evidence-based initial separation of myeloproliferative disorders, based on evaluation of *BCR-ABL* rearrangement and enumeration of circulating CD34-positive cells, is reported in Figure 2. CD34 counts might be lower than 15×10^6/L in occasional cases of MMM (e.g., variants of this condition),[28] but these patients should be considered as atypical cases. It is also possible that occasional patients with PV or ET show CD34-positive cell counts $\geq 15\times10^6$/L. These individuals with elevated numbers of circulating hematopoietic progenitors should likely be considered as having transitional myeloproliferative con-

ditions, possibly representing evolution into myelofibrosis with myeloid metaplasia.

Although MMM can be diagnosed by the presence of immature cells in the peripheral blood, the absolute number of circulating CD34-positive cells likely has a different biological significance, possibly representing a quantitative measurement of hematopoietic progenitor cell trafficking. Obviously, flow cytometry enumeration of circulating CD34-positive cells should not supplant morphologic examination of peripheral blood smears, which will always represent the starting point of the diagnostic process, but rather integrate the information obtained from the morphologic examination. The findings of this study also suggest that enumeration of CD34-positive cells might be used to follow patients with PV or ET in order to predict or promptly diagnose evolution into MMM. For all the above reasons, we believe that flow cytometry enumeration of circulating CD34-positive cells should be included in the work-up of patients with myeloproliferative disorders.

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Pre-publication Report & Outcomes of Peer Review

Contributions

All authors gave substantial contributions to the conception and design of the study (FP, EM, ML, MC), or acquisition of data (LV, LM, ER, EP), or analysis (LM, CP) and interpretation of data (FP, LM). The final manuscript was written by FP and MC and critically revised and approved by all the remaining authors. The authors wish to thank Erica Consensi and Annamaria Tenore for their invaluable help in flow cytometry immunophenotyping.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This paper was processed by Professor Edoardo Ascari, who manages papers submitted by authors associated with Haematologica's core editorial functions. The manuscript was peer-reviewed by two external reviewers and by Dr. Jerry Spivak, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Spivak and Professor Ascari. Manuscript received on July 14, 2003; accepted on September 10, 2003.

In the following paragraphs, Dr. Spivak summarizes the peer-review process and its outcomes.

What is already known on this topic

Extramedullary hematopoiesis is a feature common to all the chronic myeloproliferative disorders but is most marked at the time of diagnosis in idiopathic myelofibrosis (IMF). In keeping with this observation, the number of circulating CD34+ hematopoietic progenitor cells is higher in IMF than its companion myeloproliferative disorders, polycythemia vera (PV) and essential thrombocytosis (ET).

What this study adds

The present study is the largest prospective study of circulating $CD34^+$ cell number in patients with PV, ET or IMF, and clearly substantiates the seminal observation of Barosi *et al.* that an increase in the number of circulating $CD34^+$ cells is an integral feature of IMF.

Caveats

As also previously observed, the number of circulating $CD34^+$ cells was increased in PV and ET patients who developed myelofibrosis. Thus, the number of circulating $CD34^+$ cells did not distinguish the underlying myeloproliferative disorder when myelofibrosis was present, nor was the initial circulating $CD34^+$ cell count predictive of which PV or ET patients would develop marrow fibrosis. Finally, whether the number of circulating $CD34^+$ cells will prove useful in distinguishing IMF from myelodysplasia with marrow fibrosis remains to be determined.