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ORIGINAL REPORT

Leukocytosis and Risk Stratification Assessment in Essential Thrombocythemia

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ABSTRACT

Purpose
Established risk factors for thrombosis in essential thrombocythemia (ET) include age and previous vascular events. We aimed to refine this risk stratification by adding baseline leukocytosis.

Patients and Methods
We enrolled 657 patients with ET followed for a median of 4.5 years who developed 72 major thrombosis. Cox proportional hazard model was performed to analyze the thrombotic risk and to discriminate ET patients with or without thrombosis, multivariable C statistic index was used. We searched for leukocytes cutoff with the best sensitivity and specificity by a receiver operating characteristic curve.

Results
Results confirmed that age and prior events are independent risk factors for thrombosis and showed a gradient between baseline leukocytosis and thrombosis. On the contrary, no significant association was found either for $JAK2^{V617F}$ allele burden and for other laboratory parameters, including platelet number. In the model with conventional risk factors alone, C statistic ratio for total thrombosis was 0.63 and when leukocytosis was added, the change was small (C = 0.67). In contrast, in younger and asymptomatic patients (low-risk category), C statistic value indicated an high risk for thrombosis in patients with leukocytosis, similar to that calculated in conventionally defined high-risk group (C = 0.65). The best leukocyte cutoff values for predicting the events was found to be 9.4 ($\times 10^9$/L).

Conclusion
We suggest to include baseline leukocytosis in the risk stratification of ET patients enrolled in clinical trials.

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INTRODUCTION

Essential thrombocythemia (ET) is a clonal disease often associated with a JAK2 mutational (*JAK2 617V>F*) status. Its natural history encompasses vascular occlusive or hemorrhagic complications and a propensity to transform into polycythemia vera, myelofibrosis (MF), and ultimately to acute leukemia (AL).

Identification of new risk factors for venous and arterial thrombosis is an enduring theme and is the object of recent studies. Age and previous history of thrombosis are the two established main factors for assessing the vascular risk and have been utilized to stratify patients in clinical trials.[1,2]

Very recently, new biomarkers including leukocytosis and the status of *JAK2 617V>F*[3] have been recognized as being independently related to cardiovascular risk in patients with ET. Concerning leukocytosis, we found that the relative risk (RR) of this association was found significantly high, after adjusting for many parameters including conventional risk factors, especially in the subgroup of younger and asymptomatic patients.[4] These data are in keeping with results obtained by investigators at Mayo Clinic who made also the important observation of a significant correlation between leukocytosis and survival in patients with ET.[5]

Nevertheless, before leukocytosis can be operationally included in prognostic algorithms for stratifying patients with ET to different treatments, the strength of its value as a predictor for incident cardiovascular events should be confirmed in larger series of patients. In fact, in the Mayo Clinic cohort, leukocytosis was correlated with the episodes of thrombosis registered in the previous history or at presentation of the disease but not with the events seen in follow-up.[6]

Moreover, it is still unknown whether leukocytosis has an incremental role in addition to conventional risk factors to discriminate the group that will have thrombosis and the group that will not. Finally, the cutoff value of leukocytosis with highest sensitivity and specificity in relation to cardiovascular event occurrence remains to be assessed.

The aim of this article is to tackle these issues by using a novel statistical approach that has been recently proposed as being particularly suitable for assessing the prognostic value of risk factors.[7]

PATIENTS AND METHODS

Study Sample

A total of 657 patients with ET (Polycythemia Vera Study Group criteria) observed in two Italian academic institutions (Ospedali Riuniti di Bergamo, Italy and Policlinico Careggi, Florence, Italy) constituted the study sample. Permission was obtained from the institutional review boards to review the medical records.

Patients were classified as being at low or high risk for thrombosis according to standard risk factors (age $\geq$ 60 years and/or a previous major thrombotic event). Low-risk patients were followed with no cytoreductive therapy whereas high-risk patients were given in the great majority hydroxyurea and in a small group busulfan, according to age. The aim was to reduce platelet number to 600×10^9/L.

In this analysis, vascular events included ischemic stroke, cerebral transient ischemic attack (TIA), acute myocardial infarction, peripheral arterial thrombosis, and venous thromboembolism. Diagnostic procedures for stroke included cerebral computed tomography or magnetic resonance imaging; characteristic neurological symptoms for TIA; for acute myocardial infarction electrocardiography and/or increased cardiac enzymes, angiography for peripheral arterial thrombosis and ultrasonography of the arms or the legs, or pulmonary ventilation–perfusion scan or computed tomography scan for venous thromboembolism.

JAK2 617V>F genotyping

JAK2 617V>F allele burden was measured by a quantitative real-time polymerase chain reaction assay, using 20 ng genomic DNA purified from peripheral blood granulocytes. Polymerase chain reaction amplification and detection were performed on ABI Prism 7300 analyzer (Applied Biosystems, Foster City, CA) according to the following cycling conditions: 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Primers flanking the mutant region (forward primer 5′-AAGCTTTCTCA CAAGCATTTGGTTT-3′; reverse primer 5′-AGAAAGGCATTAGAAAG CCTGTAGTT-3′) were employed together with TaqMan probes (Applied Biosystems, Milano, Italy) which were specific for either wild type (VIC-5′-TCTCCACAGACACATAC-3′MGB) or mutant *JAK2* allele (FAM-5′-TCCACAGAAACATAC-3′-MGB). All samples were analyzed in triplicate and the amount of *JAK2617V>F* allele was calculated by comparison with serial dilutions of mutant DNA, obtained from a polycythemia vera patient harboring 100% mutant allele, and wild-type DNA from healthy subjects. The mean of triplicate ΔC_T determinations ($C_T^{JAK2617V>F} - C_T^{JAK2WT}$) was used to calculate the percentage of mutant allele. Positive and negative controls were included in each assay; inter- and intra-assay variation was 3% and 5%, respectively.

Statistical Methods

Leukocytosis as a risk factor for thrombosis was tested using a Cox proportional hazards model. Quartile distribution was used in the model fitted for the prediction of total thrombosis while, focusing on different type of thrombosis, the second and third quartile were unified because of the rarity of the events. For this latter analysis, competing risks were taken into account in each model. All multivariable models have been fitted after adjusting for center, sex, standard risk factors (age $\geq$ 60 years and/or previous thrombotic event), and quartile distributions of laboratory parameters measured at diagnosis (hemoglobin, hematocrit, platelet count). *JAK2 617V>F* allele status was classified in four categories as follows: wild-type, 1% to 25%, 26% to 50%, and more than 50% of allele expression.

The ability to classify risk in individual patients was assessed by the C discrimination index introduced by Harrell et al[8] as a natural extension of the receiver operating characteristic (ROC) curve area to censored survival data and recently employed by Wang and colleagues[9] to explore the prediction of new biomarkers in cardiovascular diseases.

The C index measures the model ability to classify individual patients into risk groups at different prognosis by the probability of concordance between the rank of the model prognosis and the rank of the observed outcomes over pairs of population subjects. Specifically, C is the concordance probability that for any given pair of subjects *i* and *j*, labeled such as *i* fails while l is still event free, *i* is assigned a poorer prognosis than l. In the presence of right random censoring, the rank between the underlying failure times of a pair of subjects can be determined if, and only if, the shortest time between the two is not censored (comparable pair). As a consequence, the concordance between the rank of predictions and rank of outcome can be assessed only for comparable pairs, and in practice C is estimated by the ratio of concordant number of pairs over the total number of comparable pairs.[10] C values range from 0.5 (absence of discrimination) to 1 (perfect discrimination).

A ROC analysis, widely employed in diagnostic tests but also used for prognostic evaluation,[11] was performed to determine the cutoff of leukocytes. Because standard methods do not exist for deriving ROC curves for time-to-event data, we used occurrence as compared with nonoccurrence of events within 20 years from ET diagnosis as the outcome for the analysis.

RESULTS

Presenting Characteristics

Patients' characteristics are presented in Table 1. Median age was 52 years, (range, 8 to 93); 246 were older than 60 years (37%) and 519 (79%) were asymptomatic at presentation. In 56% of patients, the *JAK2 617V>F* was present and in 17 (3%) the allele burden was superior to 51%. A total of 151 of patients (23%) had a prior history of thrombosis which was made up of an arterial event in 104 (16% of total patient population). By age and previous events, patients were divided in low risk (n = 341; 52%) and high risk (n = 316; 48%). During up to 38 years of follow-up (median, 4.48 years; range 0 to 38), 59% of patients received cytotoxic therapy and total major vascular complications were 50 (14.6%) in low risk (which had been left untreated after diagnosis in 90% of patients) and 48 (15.2%) in high risk treated patients. Seventy-two were the first events in the follow-up object of this analysis. Stroke, TIA, and acute coronary syndromes constituted the majority (48%) of events.

Leukocytosis As a Risk Factor for Thrombosis

Baseline WBC counts ranged between 3.28 to 35×10^9/L (median, 8.73×10^9/L); 10th and 90th percentiles were 6.0 and 12.9×10^9/L, respectively.

Multivariable analysis of risk factors for thrombosis (Table 2) confirmed the value of age and previous events and, interestingly, showed that by dividing patients in quartile distribution of leukocytes, the higher the baseline leukocyte number, the higher the hazard ratio (HR) leading to an increment of thrombosis probability up to three times as great as persons with normal leukocyte counts. Different quartiles of baseline hemoglobin concentration and platelet number did not influence the rate of thrombosis. *JAK2 617V>F* allele burden showed a linear correlation with the level of leukocytes ($P < .0001$) but it was not found to be an independent risk factor when total thrombotic events were considered. However, relative risk for arterial thrombosis was significantly increased in the

Table 1. Characteristics of Patients With ET (n = 657)

Characteristic	No.	%
No. of patients	657	
Center		
Bergamo	395	60
Florence	262	40
Sex		
Male	212	32
Female	445	68
Median age at diagnosis, years	52	
Range	8-93	
Median WBC × 10^9/L	8.73	
Range	3.28-35	
Median hemoglobin, g/dL	14	
Range	8.9-17.6	
Median HCT, %	42	
Range	30-54	
Median PLT, × 10^9/L	772	
Range	450-2,269	
JAK2 617V>F	366	56
Quantitative *JAK2 617V>F**		
0	289	46
1-25	170	27
26-50	155	24
51-75	12	2
76-100	5	1
History of vascular events	151	23
AMI	42	6
Stroke/TIA	37	6
PAT	25	4
VTE	47	7
Treated patients		
Never treated	144	22
Chemotherapy	388	59
Aspirin	431	66
Median follow-up, years	4.48	
Range	0-38	
Vascular events in the follow-up	98	15
AMI	20	3
Stroke/TIA	27	4
PAT	16	2
VTE	35	5

Abbreviations: ET, essential thrombocythemia; HCT, hematocrit; PLT, platelet; AMI, acute myocardial infarction; TIA, transient ischemic attack; PAT, peripheral arterial thrombosis; VTE, venous thromboembolism.
*Percentages calculated for 631 patients for whom quantitative *JAK2 617V>F* determination was available.

Table 2. Risk Factors for Thrombosis in the Follow-Up (multivariable analysis)

Factor	HR	95% CI	*P*
Center (Florence *v* Bergamo)	1.1	0.6 to 2.0	.77
Sex (male *v* female)	1.0	0.6 to 1.9	.91
High-risk patients*	**2.1**	**1.2** to **3.4**	**.008**
Hemoglobin, g/dL (< 13)	1 (ref)		
13-14	1.7	1.0 to 6.2	.06
14-15	0.9	0.3 to 2.8	.87
≥ 15	0.9	0.2 to 3.4	.87
HCT, % (< 39.5)	1 (ref)		
39.5-42	0.8	0.4 to 1.8	.60
42-45	1.5	0.5 to 4.1	.46
≥ 45	1.4	0.4 to 5.3	.61
PLT, × 10^9/L (< 650)	1 (ref)		
650-772	0.6	0.3 to 1.2	.14
772-977	0.7	0.3 to 1.3	.24
≥ 977	0.6	0.3 to 1.2	.16
WBC, × 10^9/L (< 7.2)	1 (ref)		
7.2-8.7	**2.4**	**1.1** to **5.6**	**.04**
8.7-10.4	**2.7**	**1.2** to **6.2**	**.02**
≥ 10.4	**3.0**	**1.3** to **6.9**	**.008**
Quantitative *JAK2 617V>F*, %			
0	1 (ref)		
1-25	1.2	0.6 to 2.5	.69
26-50	1.5	0.8 to 2.8	.25
> 50	1.8	0.5 to 5.7	.34

NOTE. Bold font indicates statistical significance.
Abbreviations: HR, hazard ratio; ref, referent; HCT, hematocrit; PLT, platelet.
*Patients age ≥ 60 years and/or with prior thrombosis.

presence of *JAK2 617V<F* allele burden values greater than 50% (HR, 4.9; 95% CI, 1.4 to 16.5; $P = .01$; data not shown).

Table 3 reports types of thrombosis in relation to the entity of leukocytosis, adjusted for variables known at diagnosis and considering for each outcome of interest the other thrombosis as competing events. Myocardial infarction was the major complication which occurred in the presence of leukocyte counts greater than 10 × 10^9/L (HR, 8.08; 95% CI, 1.00 to 65.5; $P = .05$).

As shown in Figure 1, cumulative probability of incident thrombosis was significantly higher in low-risk patients with leukocyte number over the median value (8.7 × 10^9/L), as compared with the group with lower leukocyte count (55% *v* 20% at 20 years from diagnosis; $P = .003$). This probability was comparable to high-risk patients. In this latter group, leukocytosis was also associated with an increased incidence of thrombosis, even though the difference with patients with normal leukocyte number was not statistically significant (39% *v* 69%; $P = .33$). It is likely that this is due to the small number patients at risk.

Leukocytosis As Prognostic Test for Thrombosis

C statistic estimates are calculated in all patients and in those with high and low risk by using the quartile distribution of leukocytes (Table 4). When considering the discrimination ability of conventional risk factors, independently of the leukocyte number, in total patient population, C statistic gave a probability of concordance of 0.63 for predicting major thrombosis and 0.64 for arterial thrombosis. When the analysis included leukocyte quartiles, the C statistic estimates ranged from 0.66 to 0.67 for major and arterial thrombosis respectively, indicating a marginal role of leukocytosis in total population. Conversely, if patients were divided according to conventional risk criteria, the C statistical value found in the subgroup of young and asymptomatic patients was greater than the one calculated in elderly patients and/or with a history of thrombosis (0.65 and 0.60, respectively). This indicated a better discriminatory ability of the variable leukocytosis in predicting major vascular events in the subgroup of ET patients defined at low risk by conventional criteria. In this latter group, the value of leukocytes exhibiting the highest sensitivity (64%) and specificity (67%) by ROC analysis was 9.4 × 10^9/L.

DISCUSSION

The strength of the association between leukocytosis and the incidence of thrombosis was investigated in a large cohort of patients with ET,

Table 3. Sites of Thrombosis According to Leukocytosis at Diagnosis

Site	White Blood Cells*					
	7.1 to 10 × 10^9/L			More Than 10 × 10^9/L		
	HR	95% CI	*P*	HR	95% CI	*P*
Major thrombosis	**2.21**	**1.05 to 4.65**	**.036**	**3.27**	**1.54 to 6.95**	**.002**
Arterial thrombosis	2.07	0.83 to 5.20	.121	**3.12**	**1.20 to 8.08**	**.019**
Acute myocardial infarction	5.82	0.64 to 53.2	.118	**8.08**	**1.00 to 65.5**	**.050**
Stroke/TIA	0.88	0.29 to 2.67	.824	1.32	0.42 to 4.11	.631
Venous thrombosis	1.40	0.49 to 4.04	.534	2.51	0.86 to 7.29	.092

NOTE. Multivariable models adjusted for information collected at diagnosis, including center, sex, standard risk factors, hemoglobin, hematocrit, platelet count. Bold font indicates statistical significance.
Abbreviations: HR, hazard ratio; TIA, transient ischemic attack.
*Reference category: WBC ≤ 7 × 10^9/L.

uniformly treated in two centers and observed for a median of 4.48 years. Multivariable analysis confirmed age and previous thrombosis as risk factors, and gave further details on the association between leukocytosis and the vascular risk. The impact of leukocytosis was mostly observed on myocardial infarction risk, thus suggesting a stronger association of leukocytes with this event (HR, 8.08; $P = .05$). Interestingly, the same observation was made in patients with PV enrolled in the European Collaboration in Low-Dose Aspirin in Polycythemia Vera study, in which patients with WBC count above 15 × 10^9/L had a significant increase of myocardial infarction risk (HR, 2.84; 95% CI, 1.25 to 6.46).[12] Even though not statistically significant ($P = .09$), a trend toward the link of leukocytosis with the incidence of arterial thrombosis was also reported by Mayo Clinic investigators[6] in their large ET cohort. To further confirm this association, we observed a gradient between the degree of leukocytosis and vascular outcomes in our patients. Noteworthy was the finding that the vascular risk attributable to leukocytosis was significantly evident in younger and asymptomatic patients, in whom cumulative probability of thrombosis at 20 years from diagnosis rose from 20% to 55%. In contrast, the presence of high leukocyte count did not significantly increase the probability in the high-risk category. Whether leukocytosis should be considered a marker of the whole myeloproliferative process or rather to have a defined, causative role in cardiovascular events remains to be established. However, many data are now available supporting the likelihood of thrombogenicity played by leukocytes in myeloproliferative disorders,[13] and the hypothesis of a causative role of leukocytes in the processes of thrombogenesis has led to suggest short-term use of hydroxyurea in subjects with non-myeloproliferative disorders with very high vascular risk.[14]

Even though the leukocyte counts were correlated with *JAK2 617V>F* allele burden ($P < .0001$), we fail to show a significant correlation between the percentage of mutant alleles and thrombosis in the entire sample of 631 examined patients. However, if multivariable analysis was limited to arterial thrombosis, a *617V>F* allele burden greater than 50% resulted to be independently associated with this outcome (HR, 4.9; $P = .01$). This confirms previous results reporting that wild type and heterozygous V617 F patients had a similar incidence of major thrombosis while a significant increase of vascular complications occurred in the homozygous patients with ET.[3]

Thus, results from this study confirm and extend the observation that leukocytosis at diagnosis is an independent and strong risk factor predicting major vascular events in ET, particularly in the category of younger and asymptomatic patients. In the high-risk group, we found a higher incidence of thrombosis in patients with leukocytosis but the

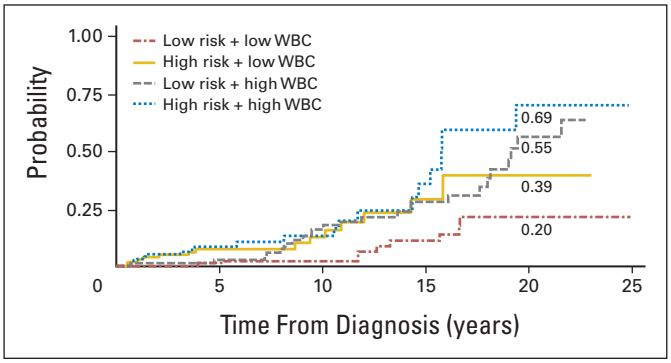

Fig 1. Cumulative probability of thrombosis in the follow-up according to standard risk factors and categories of WBC count at diagnosis. Rates reported are calculated at 20 years from diagnosis. Low-risk category: patients younger than 60 years and with no prior thrombosis. High-risk category: patients ≥ 60 years and/or with prior thrombosis. Low WBC category: patients with leukocyte count lower than 8.7 × 10^9/L at diagnosis. High WBC category: patients with leukocyte count ≥ 8.7 × 10^9/L at diagnosis.

Table 4. Discriminatory Ability of Cox Models for Predicting Major Thrombosis and Arterial Thrombosis in the Follow-Up

Event and Model	C Statistic	95% CI
Major thrombosis		
1	0.63	0.56 to 0.71
2	0.67	0.59 to 0.75
3	0.65	0.54 to 0.76
4	0.60	0.49 to 0.72
Arterial thrombosis		
1	0.64	0.57 to 0.72
2	0.68	0.60 to 0.76
3	0.64	0.51 to 0.76
4	0.63	0.52 to 0.74

NOTE. Model 1: center, sex, and standard risk factor (previous thrombosis and/or age ≥ 60 years); model 2: model 1 + quartile distribution of WBC count (× 10^9/L); model 3: quartile distribution of WBC count (× 10^9/L) in low-risk patients; and model 4: quartile distribution of WBC count (× 10^9/L) in high-risk patients.

difference with patients with normal leukocyte number did not reach statistical significance. This could be due to the small number patients at risk in the last part of the observation. However, we were well aware that before leukocytosis is considered as a prognostic test to be fruitfully employed for stratifying patients to different treatments in clinical trials, it would have been worthy determining its contribution in addition to the established prognostic factors. Moreover, its discriminatory performance in recognizing those patients who will have the events in the future and those who will not, needed to be carefully assessed.[15] This information is not provided by the relative risk analysis, that limits the comparison of the effect of leukocytosis on the events in patients located at each end of the distribution of these cells, thus ignoring most people in the middle of their distribution.[16] To solve this issue, C statistic has been proposed, and recently it has been employed to evaluate the contribution of new risk factors in cardiovascular diseases.[9]

In our patients, the probability of concordance between leukocytosis and events, measured by C statistic multivariable analysis,[7] led to interesting findings. The ratio between the sum of concordant pairs of patients was searched in different models. In the model with conventional risk factors alone, C statistic ratio for total thrombosis was 0.63 and when leukocytosis was added, the change was small (C = 0.67). Thus, the ability of leukocytosis to add to existing risk factors did not seem to be relevant in the whole population of patients with ET. On the contrary, in younger and asymptomatic patients (low-risk category), which accounted for 52% of our patients, C statistic was slightly higher (C = 0.65) than that calculated in conventional high-risk group (C = 0.60), indicating that leukocytosis per se can discriminate two subgroups of patients within the low-risk category who are at different risk of future vascular complications. Thus, results of this statistical approach suggest the opportunity to re-evaluate risk stratification in patients with ET conventionally considered to be at low risk for cardiovascular events by utilizing baseline leukocytosis. Overall, this attitude might have major implications in clinical decision making, specifically in this ET subgroup, in which treatment is highly controversial.[17] In our ROC analysis, the best sensitivity and specificity was selected for leukocyte value of 9.4×10^9/L.

In conclusion, our work provides further evidence to strengthen the concept of leukocytosis as risk factor for thrombosis, and suggests that leukocyte counts may be of prognostic value mostly in low-risk patients with ET. These findings provide the evidence to include leukocytosis in the stratification of patients with ET in future clinical trials.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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