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**Impact of leukocytosis on thrombotic risk and survival in 532 patients with Essential Thrombocythemia: a retrospective study.**

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**ABSTRACT**

Established risk factors for thrombosis in Essential Thrombocythemia (ET) include age (≥60 years) and previous vascular events. Recently, also leukocytosis has been proposed in risk stratification of ET patients. We report a retrospective study on 532 ET patients followed for a median of 7.6 years. Sixty-four patients (12%) developed 95 thrombotic events during follow-up. Together with the high risk condition, a WBC value above $11x10^9$/L, corresponding to the fourth percentile value, significantly correlated with a higher thrombotic risk (p=0.033) by Cox proportional hazards. Moreover, the cumulative risk of thrombosis was significantly higher in high-risk patients with WBC>$11x10^9$/L. JAK2 V617F mutation did not correlate with thrombosis. Overall, 123 (23%) patients died. Three independent parameters were noted as prognostic factors for survival in multivariate analysis: age>60 years, leukocytosis>$11x10^9$/L and haemoglobin level below normal values. Based on these parameters, three groups of risk were defined, with significantly different survivals. Baseline leukocytosis correlated with a higher thrombotic risk in high risk patients and identified a cohort of patients with worse survival.

## INTRODUCTION

Essential Thrombocythemia (ET) is a Philadelphia-negative chronic myeloproliferative neoplasm (CMPN) characterized by the abnormal proliferation of a malignant megakaryocytic clone, responsible for persistent thrombocytosis [1,2].

The clinical course is burdened by an increased incidence of vascular complications, whose pathogenesis is multifactorial. In the last years, many retrospective studies have failed to show a clear correlation between platelet count and thrombotic events [3-5] and the role of thrombocytosis in the assessment of vascular risk has been progressively minimized. Conversely, age (≥60 years) and previous vascular events remain the main established risk factors for thrombosis and on this basis patients are currently stratified in low and high risk.

Recently, also the presence of JAK2V617F mutation [6-8] and baseline leukocyte (WBC) count have been considered as disease-related cardiovascular risk factors of ET patients. This latter has been found to play a biological role in determining thrombosis, mainly acute coronary syndromes, in CMPN and particularly in ET, although it is presently not completely defined whether elevated WBC levels actually contribute directly to causing thrombosis [9].

Consequently, several clinical studies have investigated the association between leukocytosis at diagnosis and risk of thrombosis, with not univocal results. Indeed, some Authors, including our group, have reported that leukocyte count at presentation did not predict subsequent vascular events [3,10-12], although none of these analyses investigated possible differences among low and high-risk patients. On the contrary, some other studies concluded that leukocytosis was associated with thrombosis, particularly in low-risk patients [13-15], but no consensus was found on the numerical cut-off which should be utilized for the definition of leukocytosis. Furthermore, the implications of baseline WBC count on long-term survival remain to be determined.

The aim of this article is to contribute to these issues by reporting a long-term retrospective study on 532 ET patients followed for a median of 7.6 years, with particular focus on the evaluation of the additional risk factors for thrombosis and survival.

## METHODS

### *Definitions*

The diagnosis of ET was made according to the criteria proposed by the Polycythemia Vera Study Group (PVSG) [16] and/or, since 2002, the WHO criteria [17]. Additional risk factors for thrombosis (blood pressure >140/90 mmHg, overweight, smoking habit, mellitus diabetes, and hypercholesterolemia, defined as fasting serum total cholesterol concentration >200mg/dL) and renal and liver function were evaluated at diagnosis and at 6-months interval thereafter.

The definition of acute leukemia and myelodisplastic syndrome were made according to the French-American-British criteria [18]; diagnosis of myelofibrosis was based on Italian Consensus Conference criteria [19]. Thrombotic episodes were objectively documented by: venography or ultrasonography (deep venous thrombosis); arteriography (peripheral arterial thrombosis); computed tomography scanning, magnetic resonance or angiography (intracerebral vessels thrombosis). Acute myocardial infarction required acute clinical presentation, typical electrocardiograph features and elevated creatine-kinase MB fraction. Cerebral transient ischemic attack (TIA) required neurological symptoms and/or signs lasting less than 24 hours.

### Assessment of JAK2$^{V617F}$ mutational status

Total RNA was extracted from peripheral blood mononuclear cells using RNAeasy MiniKit (QIAGEN). RNA was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using 5μM of random examers and 200 U of M-MLV Reverse Transcriptase

(Invitrogen, Paisley, UK) according to the manufacturer's recommendations. The cDNA was amplified using forward and reverse primers (5'-gatgagcaagctttctcacaagc-3' and 5'-aaacagttggcatgggccatgc-3'); the amplified product (185bp) was digested with Bsa XI (5U) (New England Biolabs, Germany) over-night at 37℃ [20]. After digestion, 20 µl of amplified PCR products was electrophoresed on a 2% agarose gel and stained with ethidium bromide.

**Statistical Methods**

Risk factors for thrombosis were tested using a Cox proportional hazards model. All multivariable models have been fitted after adjusting for sex, standard risk factors (age>60 years and/or previous thrombotic event), and quartile distributions of laboratory parameters measured at diagnosis (hemoglobin, hematocrit, leukocytes and platelet count). Overall survival (OS) was calculated by the product-limits method of Kaplan and Meier from the date of ET diagnosis to the date of death or last contact, whichever came first, with 95% confidence interval (95% CI). All p values were two-sided and the significance level for all statistical tests was 0.05.

## RESULTS

### Patients

From January 1978 to December 2008, 532 consecutive patients received a diagnosis of ET at the Institute of Hematology and Oncology “L. and A. Seràgnoli”, Bologna, Italy.

General characteristic of the patients are summarized in Table I. Median age at diagnosis was 64 years (range, 16-95); 307 were 60 years or older (58%). In 350 (66%) patients, one or more additional risk factors for thrombosis were present at diagnosis.

According to standard risk factors (age≥60 years and/or history of vascular events), patients were classified as low risk (n=186; 35%) and high risk (n=346; 65%). However, during the years, indications for cytotoxic and antiplatelet therapy were also represented by extreme thrombocytosis (platelet count higher than $1000x10^9$/L), presence of additional cardiovascular risk factors and/or symptoms due to microvessel disturbances. Consequently, 86% of patients received cytotoxic therapy (mainly, Hydroxyurea) and 92% received an antiplatelet agent (mainly, aspirin). More specifically, during up to 32.5 years of follow-up (median, 7.6 years), 130 out of 186 low-risk patients (70%), and 330 out of 346 high-risk patients (95%) received at least one cytoreductive drug.

Overall, 64 (12%) patients (15/186 low-risk patients, 8%, and 49/346 high-risk patients, 14%) developed 95 thrombotic complications. Arterial events (including stroke, TIA, acute coronary syndromes and peripheral arterial occlusions) constituted the majority (61%) of events.

### Risk factors for thrombosis

Multivariable analysis of risk factors for thrombosis confirmed the value of the high-risk condition. By dividing patients in quartile distribution of leukocytes and platelet number, hemoglobin concentration and hematocrite, also a WBC value above $11x10^9$/L,

corresponding to the fourth percentile value, significantly correlated with a higher thrombotic risk, together with the high risk condition (p=0.033 and p=0.003, respectively) (Table II). When arterial thrombosis were analyzed separately, only high risk condition correlated significantly with a higher risk of such events (HR=2.2, 95% CI 1.07 – 4.85, p=0.032). The presence of the JAK2$^{V617F}$ mutation was not found to be an independent risk factor for thrombotic events (11 thrombotic episodes in 121 patients without the mutation *versus* 17 thrombotic episodes in 202 mutated patients, p=0.84).

**Cumulative risk of thrombosis by baseline leukocytosis**

Previous reports have shown that the cumulative risk of thrombosis was significantly higher in low-risk patients with leukocyte number over a median value (8.7x10$^9$/L) [13], and also identified as best leukocyte cut-off for predicting thrombotic events the value of 9.4 x10$^9$/L [14]. We performed an equivalent analysis by using both the median cut-off value of our cohort of patients (8.9x10$^9$/L) (Figure 1A) and the cut-off value of 9.4 x10$^9$/L (Figure 1B). While the latter cut-off value did not allow to recognize different incidences of thrombosis between risk categories, when the median cut-off value was utilized, the presence of leukocytosis >8.9x10$^9$/L identified a category of high risk patients with a thrombotic risk significantly higher than all the other patients (52% vs 17% at 20 years, p<0.0001), while high-risk patients with low WBC count and low-risk patients had the same cumulative thrombotic risk.

We also tested the WBC value of 11x10$^9$/L (corresponding to the fourth percentile value). As shown in Figure 1C, the cumulative risk of thrombosis was significantly higher in high-risk patients with WBC>11x10$^9$/L ("very" high risk category) as compared to the group with lower leukocytes count (p=0.002) ("standard" high risk category). The cumulative risk of thrombosis in low-risk patients was comparable to high-risk patients with lower leukocyte count (18% vs 21.5% at 20 years from diagnosis, p=0.24); also, low-risk patients with

leukocytosis had the same thrombotic risk than low-risk patients without leukocytosis (18% vs 13% at 20 yrs, p=0.92).

**Overall survival**

One hundred and twenty-three (23%) patients died at a median age of 81 years (range 51-100); the median survival was 24 years. Causes of death were: thrombotic/haemorrhagic events (8 patients, 6.5%), cardiac disease (13 patients, 10.5%), disease transformation (21 patients, 17%), second neoplasia (12 patients, 10%), senility (26 patients, 14.5%) or other causes unrelated to the haematological disease (not including cardiovascular events and development of second neoplasia) (43 patients, 27%). Thrombocytosis (>1500x$10^9$/L) and leukocyte count above the median value (8.9x$10^9$/L) resulted associated with higher mortality, but only in univariate analysis (p=0.01 and p=0.04, respectively). Conversely, neither a history of thrombosis nor the presence of the JAK2$^{V617F}$ mutation were significantly associated with poorer survival (p=0.45 and p=0.57, respectively).

Three independent parameters were noted as prognostic factors for survival also in multivariate analysis: age≥60 year, leukocytosis >11x$10^9$/L and haemoglobin level below normal values (<12.5 g/dL in females and <13.5 g/dL in males). Taking into account these parameters, a model for survival was constructed in order to define three groups of risk: high (2 or 3 risk factors, 130 patients), medium (1 risk factor, 265 patients) and low (no risk factors, 137 patients). Median survivals were 13 years, 28 years and yet undefined in the three groups, respectively (P<0.0001) (Figure 2).

## DISCUSSION

Many recent papers have investigated the possible association between leukocytosis and thrombosis in patients with myeloproliferative disorders, and particularly in ET, with controversial results [3,11-15]. In the current study, we used the database from a cohort of 532 ET patients, homogeneously followed at a single Institution for a median time of 7.6 years, with the purpose to investigate the prognostic relevance of baseline leukocytosis for subsequent thrombotic events and survival. This analysis confirms the importance of the classical high-risk profile (age>60years and/or previous thrombosis), which resulted strongly associated with the thrombotic risk. Conversely, extreme thrombocytosis was not found to correlate with thrombotic complications, and rather showed a paradoxical trend towards a protection from thrombosis (HR 0.67). These findings are consistent with previous retrospective studies [13], suggesting an association between thrombocytosis and bleeding diathesis, partly explained by the occurrence of AVWS in some patients.

In addition to older age and previous thrombosis, also leukocytosis (cut-off, $11x10^9$/L) had a prognostic impact on thrombosis. After patients' stratification in high and low-risk according to the standard definition, baseline leukocytosis identified a cohort of "very" high risk group with leukocytosis (Figure 1c). Indeed, "standard" high-risk patients without leukocytosis presented a thrombotic risk fully comparable to low risk patients carrying leukocytosis. This result is in keeping with a recent analysis by Carobbio et al [14], who reported that leukocytosis (median value) per se could discriminate, within the low-risk category, two subgroups of patients at different risk of future vascular complications. On the other hand, in our analysis the thrombotic risk was not significantly different among the two subgroups of low-risk patients (p=0.92) and the WBC cut-off was higher ($4^{th}$ percentile). The role of baseline leukocytosis in the low risk category might have been masked by the fact that as many as 70% of these low-risk patients received cytotoxic therapy (plus aspirin), configuring them as high-risk from the point of view of prevention of thrombosis. The administration of cytotoxic therapy might have prevented an increase in

leukocyte count over time, which has been reported to correlate with a higher thrombotic risk [21]. Moreover, the impact of leukocytosis might have been confounded by several other variables, including the co-existence or the appearance over the time of other morbidities, influencing both the thrombotic risk and long-term survival.

Our analysis also confirmed a direct correlation between survival and baseline leukocytosis and haemoglobin level below normal values, as previously reported [3,12,13,22].

Patients were accordingly stratified in three categories, projected to a significantly different survival (Figure 2). To build this model, we verified that the proportion of treated patients was the same in the three groups (P>0.05) and that there were no myelodysplatic or fibrotic features at bone marrow examination, in the 115 patients with low haemoglobin level at diagnosis. In addition, baseline leukocytosis (quartile distribution) did not correlate with disease evolution (p=0.81), which occurred in 28 patients (acute leukemia in 6 cases and myelofibrosis in 22 cases). However, the possibility that some of these patients with "ET" characterized by older age, anemia and leukocytosis may actually have an early stage Primary Myelofibrosis (PMF-0) [23] cannot be entirely ruled out.

In conclusion, with the limitations in the interpretation of the data due to the fact that this is a retrospective study, baseline leukocytosis seemed to be associated with higher thrombotic risk, especially in high-risk category, and worse survival.

Prospective studies are needed to confirm or to rule out the accuracy of these stratifying models and to identify a WBC value which could be a widely reliable cut-off to define a significant leukocytosis.

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**Table I. Patients characteristics.** Abbreviations: ET, essential thrombocythemia; WBC, white blood cells; PLT, platelet; DVT, Deep venous thrombosis**.** High risk: age older than 60 yr and/or history of vascular events

| Characteristics | no. | percentage |
|---|---|---|
| No. of patients | 532 | |
| Sex | | |
| Male | 205 | 39% |
| Female | 327 | 61% |
| Median age at diagnosis, years | 64 | |
| Range | 16-95 | |
| Average WBC $x10^9$ / L (S.D.) | 9.5 (3.2) | |
| Average hemoglobin, g/dL (S.D.) | 14 (1.5) | |
| Average Hematocrite, % (S.D.) | 42.6 (4.6) | |
| Average PLT $x10^9$/ L (S.D.) | 852 (323.5) | |
| High risk patients, no. | 346 | 65% |
| JAK2 V617F mutation | | |
| No. of patients evaluated | 324 | 61% |
| Unmutated | 122 | 38% |
| Heterozygous | 200 | 61.5% |
| Homozygous | 2 | 0.5% |
| No. of patients with thrombotic events | | |
| Prior to or at ET diagnosis | 90 | 17% |
| During follow-up | 64 | 12% |
| Thrombotic events during follow-up | 95 | |
| Acute myocardial infarction / angina | 21 | 22% |
| Stroke / Transient ischemic attack | 31 | 33% |
| Peripheral arterial thrombosis | 6 | 6% |
| Venous thromboembolism / DVT | 28 | 29.5% |
| Peripheral phlebitis | 9 | 9.5% |
| Treated patients, no | | |
| never treated | 13 | 2.5% |
| Chemotherapy | 460 | 86% |
| Aspirin | 493 | 92% |
| Median follow-up of living patients, yrs | 7.6 | |
| Range | 0.5-32.5 | |

**Table II.** **Multivariate analysis of risk factors for thrombosis in the follow-up**

| Factor | HR | 95% CI | P |
|---|---|---|---|
| Sex (male vs female) | 0.9 | 0.59 to 1.70 | 0.99 |
| **High risk patients** | **2.45** | **1.35 to 4.43** | **0.003** |
| Haemoglobin, g/dL (<13) | 1 (ref) | | |
| 13 – 14.1 | 0.79 | 0.39 to 1.59 | 0.50 |
| 14.1-15.1 | 0.81 | 0.39 to 1.70 | 0.58 |
| >15.1 | 1.17 | 0.61 to 2.25 | 0.63 |
| Hematocrite, % (<39.4%) | 1 (ref) | | |
| 39.5 – 42.8 | 1.66 | 0.79 to 3.54 | 0.18 |
| 42.8 – 45.9 | 1.47 | 0.65 to 3.18 | 0.36 |
| >45.9 | 1.36 | 0.62 to 3.03 | 0.46 |
| PLT, x $10^9$ / L (<653) | 1 (ref) | | |
| 653 – 774 | 1.17 | 0.58 to 2.38 | 0.67 |
| 774 – 954 | 1.11 | 0.54 to 2.25 | 0.77 |
| >954 | 0.67 | 0.30 to 1.51 | 0.34 |
| WBC, x $10^9$ / L (<7.4) | 1 (ref) | | |
| 7.4 – 8.9 | 0.85 | 0.40 to 1.77 | 0.65 |
| 8.9 – 11 | 0.89 | 0.39 to 1.76 | 0.77 |
| **>11** | **1.76** | **1.05 to 2.97** | **0.033** |

**Figure 1.** Cumulative probability of thrombosis in the follow-up according to standard risk factors and categories of WBC count at diagnosis.

WBC cut-off values are as follows: $8.9 \times 10^9$/L (median WBC count) (Figure 1A), $9.4 \times 10^9$/L (Figure 1B), $11 \times 10^9$/L (4th percentile) (Figure 1C). High risk: age older than 60 yr and/or history of vascular events

**Figure 2.** Overall survival curves for ET patients stratified according to the risk including the three risk-factors: age>60yr, leukocytosis $>11 \times 10^9$/L and haemoglobin level below normal values. The risk was classified as low (no risk factors, line a, 137 patients), intermediate (1 risk factor, line b, 265 patients), high (2-3 risk factors; line c, 130 patients, with 23 patients having all the 3 risk factors). Median survivals were yet undefined, 28 yrs and 13 yrs in the three groups, respectively ($P<0.0001$).

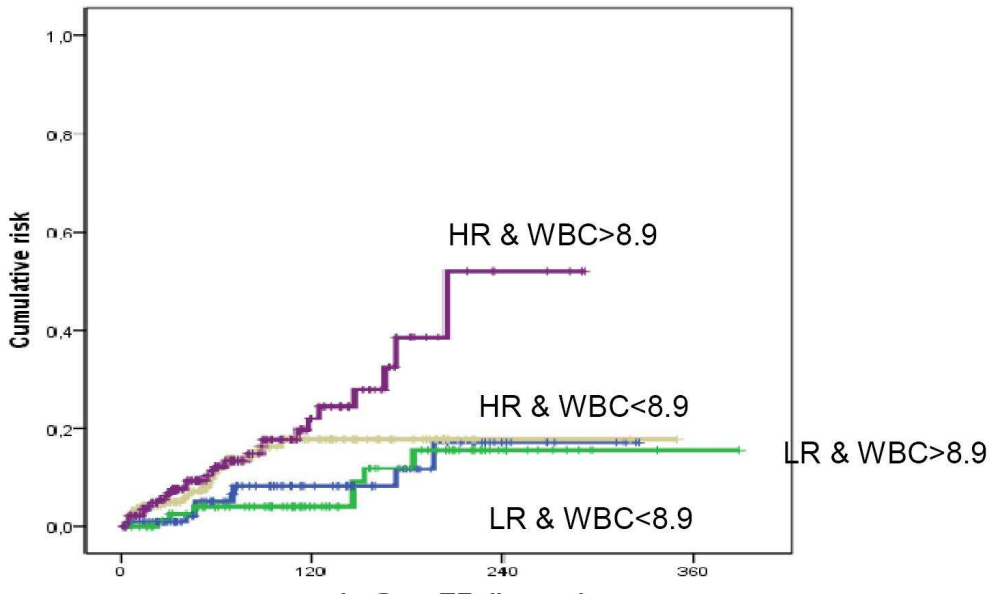
Cumulative risk
1,0
0,8
0,6
0,4
0,2
0,0
0
120
240
360
HR & WBC>8.9
HR & WBC<8.9
LR & WBC>8.9
LR & WBC<8.9

Figure 1A

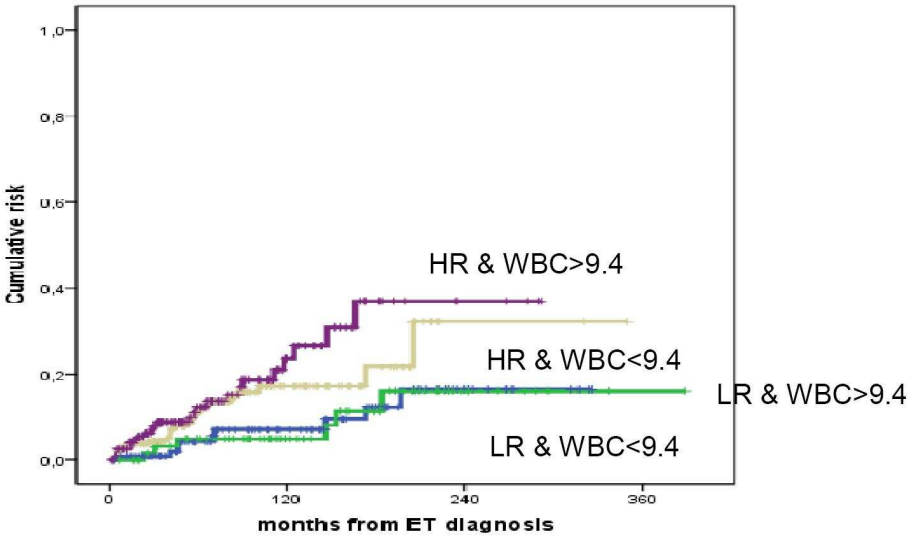
1,0
0,8
0,6
0,4
0,2
0,0
Cumulative risk
HR & WBC>9.4
HR & WBC<9.4
LR & WBC>9.4
LR & WBC<9.4
0
120
240
360
months from ET diagnosis

Figure 1B

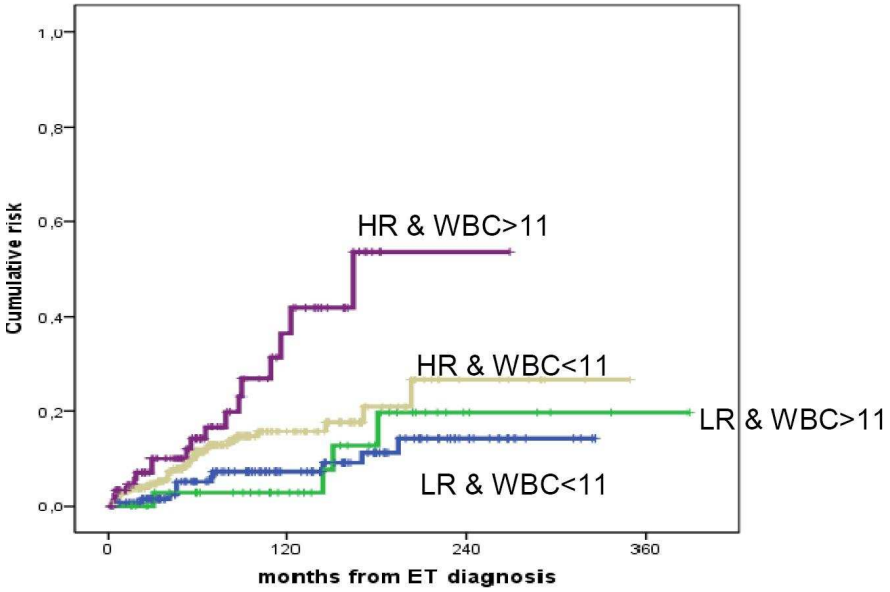
1,0
0,8
0,6
0,4
0,2
0,0
Cumulative risk
HR & WBC>11
HR & WBC<11
LR & WBC>11
LR & WBC<11
0
120
240
360
months from ET diagnosis

Figure 1C

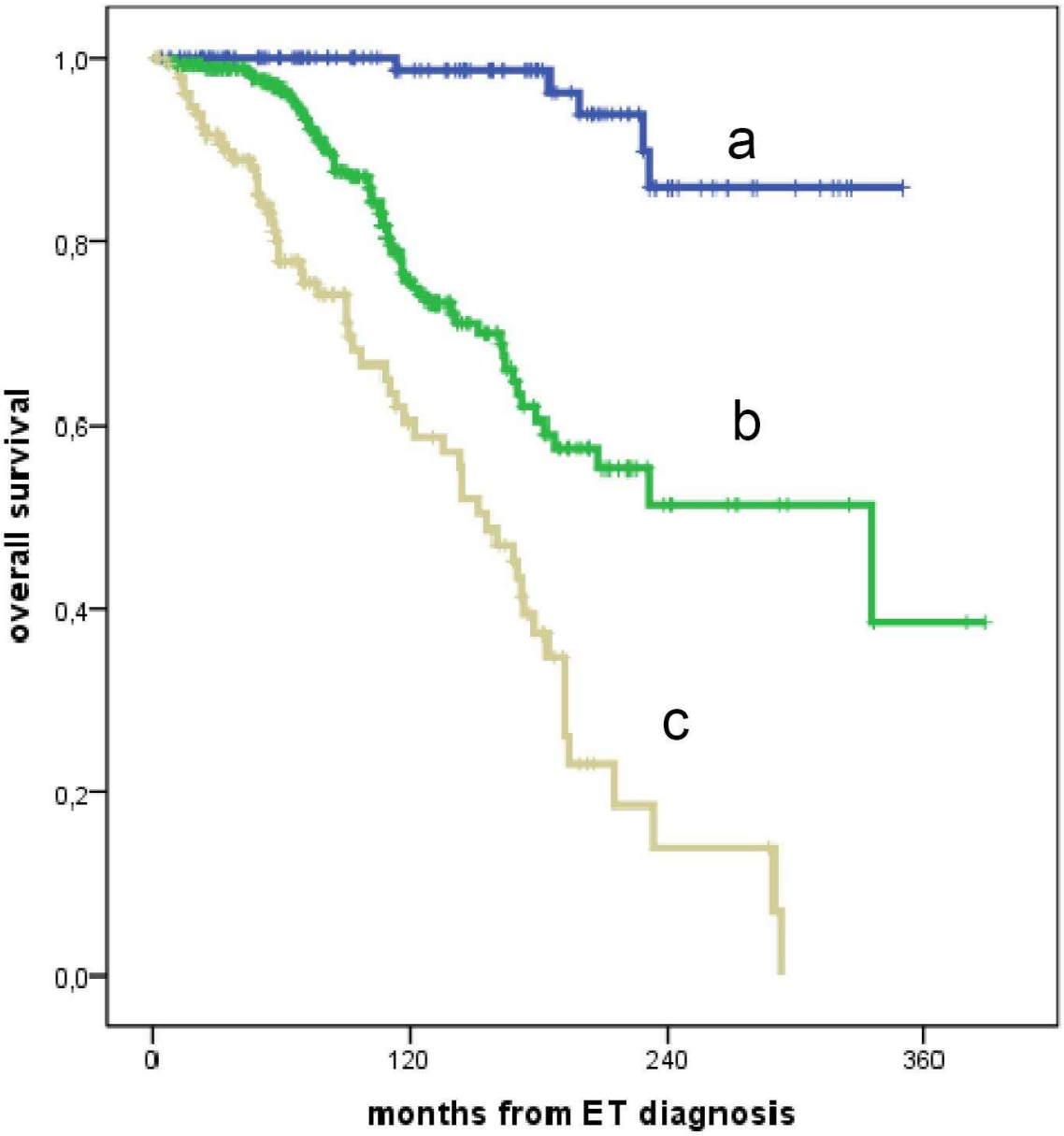
1,0
0,8
0,6
0,4
0,2
0,0
overall survival
a
b
c
0
120
240
360
months from ET diagnosis

Figure 2