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### Original article

## Increased angiogenesis in primary myelofibrosis: latent transforming growth factor- $\beta$ as a possible angiogenic factor



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#### ABSTRACT

**Objective:** The aim of this work was to demonstrate a possible relationship between anti-latency-associated peptide human latent transforming growth factor beta 1 (latent TGF- $\beta$ 1) expression in megakaryocytes and microvascular density in bone marrow biopsies from patients with essential thrombocythemia and primary myelofibrosis.

**Methods:** Microvascular density was evaluated by immunohistochemical analysis and the expression of latent TGF- $\beta$ 1 in samples (100 megakaryocytes per bone marrow sample) from 18 essential thrombocythemia and 38 primary myelofibrosis (19 prefibrotic and 19 fibrotic) patients. Six bone marrow donor biopsies were used as controls. Fibrosis in the bone marrow biopsies was evaluated according to the European Consensus.

**Results:** The average fibrosis grade differed between essential thrombocythemia and primary myelofibrosis groups when compared to the control group. Latent TGF- $\beta$ 1 expression differed significantly between the fibrotic primary myelofibrosis (PMF) group and the control group ( $p$ -value < 0.01). A high degree of neo-angiogenesis (demonstrated by analysis of CD34 expression) was detected in patients with myelofibrosis. There were correlations between latent TGF- $\beta$ 1 expression and microvascular density ( $r = 0.45$ ;  $p$ -value < 0.0009) and between degree of microvascular density and fibrosis grade ( $r = 0.80$ ;  $p$ -value < 0.0001). Remarkable differences for neo-angiogenesis were not observed between patients with essential thrombocythemia and controls.

**Conclusion:** Angiogenesis participates in the pathogenesis of primary myelofibrosis, in both the prefibrotic and fibrotic stages, while latent TGF- $\beta$  is differentially expressed only in the prefibrotic stage.

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## Introduction

Chronic myeloproliferative neoplasms (MPNs) were categorized for the first time in 1951 by William Dameshek, who noticed phenotypic similarities and characteristics common to chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF).<sup>1</sup>

PMF is a clonal stem cell disorder that is characterized by bone marrow myeloproliferation associated with a stromal reaction, including fibrosis, osteosclerosis and angiogenesis.<sup>2</sup>

Of the different types of MPN, PMF is associated with the worst prognosis; the primary causes of death are thrombohemorrhagic events, bone marrow failure and leukemic transformation. The median survival ranges from two to over 15 years and varies according to the stage: prefibrotic or fibrotic.<sup>3</sup>

According to the World Health Organization (WHO), the major differential criterion between ET and PMF is fibrosis, which is either minimal or absent in ET patients and present to various degrees in PMF patients.<sup>4</sup>

The classification of bone marrow fibrosis by the European Consensus involves an assessment of the stroma and bone characteristics on a scale that ranges from 0 to 3. Grade zero corresponds to few linear reticulin, Grade 1 to a few fiber intersections in focal areas around vessels, Grade 2 to a diffuse increase in reticulin, many intersections and some foci of collagen or osteosclerosis and Grade 3 to conspicuous and diffuse increases in reticulin fibers, many fiber intersections, collagenization and osteosclerosis.<sup>5</sup>

Special stains are necessary to identify and classify bone marrow fibrils. Mallory and Masson stains are used for collagen I while silver (Gomori) is used for reticulin fibers.<sup>6-9</sup>

Another histological feature observed in PMF is increased angiogenesis. Angiogenesis is the generation of new blood vessels from the pre-existing vasculature.<sup>2</sup>

In PMF, angiogenesis seems to be associated with stromal fibrosis triggered by the release of several cytokines produced by megakaryocytes, including transforming growth factor-beta (TGF- $\beta$ ).<sup>10</sup>

TGF- $\beta$  is a multifunctional cytokine with three isoforms: TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3, with TGF- $\beta$ 1 being the most abundant.<sup>11</sup> TGF- $\beta$  is synthesized as an inactive form (latent TGF- $\beta$ ) by various cells (epithelial, endothelial, hematopoietic, neuronal cells and connective tissue).<sup>12</sup> Platelets and megakaryocytes are an abundant source of TGF- $\beta$ 1 in humans.<sup>11</sup>

For TGF- $\beta$  to signal through its receptors, it must be converted into the active state; once activated, this cytokine acts on target cells, thereby producing different effects depending on the cell type and on the degree of differentiation of the cell.<sup>13-15</sup>

Of the several functions of TGF- $\beta$ , the stimulation of extracellular matrix production and angiogenesis should be highlighted.

Fibroblasts display TGF- $\beta$  receptors, which are stimulated to produce structural proteins, primarily collagen.<sup>12,14</sup> TGF- $\beta$  also inhibits the synthesis of extracellular matrix proteases and is therefore involved in the pathogenesis of diseases associated with the over deposition of connective tissue.<sup>10</sup>

Endothelial cells (ECs) signal via two different type I receptors, also known as activin receptor-like kinases (ALK), with opposite effects. While the activation of ALK5 by TGF- $\beta$  results in the inhibition of migration and proliferation, TGF- $\beta$ -induced ALK1 activation results in the increased migration and proliferation of ECs. Both ALK1 and ALK5 are functional TGF- $\beta$  type I receptors in ECs. The ratio of TGF- $\beta$  signals via ALK1 versus ALK5 determines whether TGF- $\beta$  exerts pro- or anti-angiogenic effects.<sup>16</sup>

TGF- $\beta$  is also an indirect angiogenic factor promoting angiogenesis via the downstream induction of other cytokines, such as vascular endothelial growth factor (VEGF).<sup>17</sup> VEGF is a potent, direct-acting regulator of angiogenesis that is detectable in various types of malignancies.<sup>18,19</sup>

Angiogenesis is essential in the pathogenesis of PMF, while it is less pronounced in PV and ET.<sup>18</sup> The increase in microvascular density (MVD) in PMF correlates with cellularity and megakaryocytes clusters. Clonal proliferation of megakaryocytes in PMF is accompanied by an abnormal release of cytokines, including angiogenic factors, resulting in an excessive stromal reaction and an increase in bone marrow vascularity.<sup>20</sup>

Because megakaryocytes and platelets are a major source of TGF- $\beta$ 1, which is able to enhance collagen synthesis and vessel proliferation, it is thought to be one of the key cytokines involved in the development of primary myelofibrosis.<sup>9</sup>

The aim of this work was to demonstrate a possible relationship between latent TGF- $\beta$ 1 expression in megakaryocytes and MVD as assessed by immunohistochemical analysis of bone marrow biopsies from ET and PMF patients. As fibrosis is used to differentiate between ET and PMF,<sup>3</sup> possible associations between MVD, fibrosis and latent TGF- $\beta$ 1 expression were evaluated.

## Methods

The study was conducted using bone marrow biopsy samples collected from 56 patients with either ET or PMF. Samples from six bone marrow donors were used as normal controls. Patients previously evaluated for the relationship between TGF- $\beta$ 1 and bone marrow fibrosis<sup>21</sup> were the participants in this study. All patients were examined in the Hematology and Pathology Departments of Hospital São Paulo, Universidade Federal de São Paulo between 1992 and 2010. Participants were included in the study after informed consent was obtained according to the guidelines of the Institutional Ethics Committee.

The clinical, cytological and histological aspects of all cases were reviewed and classified according to WHO criteria<sup>3</sup> before being selected for the study. The cases were selected based on the diagnosis by biopsy and before the patient had started any type of therapy.

Subjects were divided into four groups: 18 cases of ET; 38 cases of PMF, which included 19 cases at each phase (prefibrotic PMF and fibrotic PMF); and six controls. The controls were six bone marrow biopsies from patients who were disease-free and were on the bone marrow donor registry. Two cases of reactive thrombocytosis were used as controls for the immunohistochemistry test.

The bone marrow biopsy tissue was fixed in 10% buffered formalin, processed according to conventional histological techniques and embedded in paraffin. Two-micrometer thick sections were submitted to the following staining protocols: hematoxylin-eosin (HE), Giemsa (GM) and Gomori's silver impregnation (reticulin). Fibrosis was evaluated in accordance with the European Consensus Criteria.<sup>5</sup> The histological slides were independently analyzed by two pathologists, with cases for which agreement was not consensual being discussed.

Goat IgG polyclonal anti-LAP human TGF- $\beta$ 1 (R&D Systems - AF-246-NA), and mouse IgG1 kappa monoclonal anti-CD-34 Class II antibodies (Dako-M7165 - QBEnd 10) were used for immunohistochemistry. The bone marrow sections were processed in xylene and alcohol for deparaffinization, and were blocked for endogenous peroxidase activity (five times). The samples were incubated in a 1:100 dilution of the primary antibodies for 18 h at 4°C, and Biotin Link was used as a secondary antibody for 40 min followed by streptavidin-peroxidase treatment. Visualization was achieved with diaminobenzidine chromogen and counter-staining using Harris hematoxylin.

Latent TGF- $\beta$ 1 antibody expression was evaluated via light microscopy of 100 megakaryocytes per case to determine positivity and intensity.

Positivity was assessed using a grading system ranging from 0 to 3 based on the percentage of positive cells (Table 1A). Immunoreaction intensity was designated as negative, weak, moderate, or strong on a scale from 0 to 3 (Table 1B). A final score for latent TGF- $\beta$ 1 immunostaining was obtained by multiplying the two analyzed variables (positivity and intensity), the values of which ranged from 0 to 9s<sup>21-23</sup> (Table 1 and Fig. 1).

Angiogenesis was assessed in 14 cases of ET, 32 cases of PMF (18 prefibrotic and 14 fibrotic phases), and five controls.

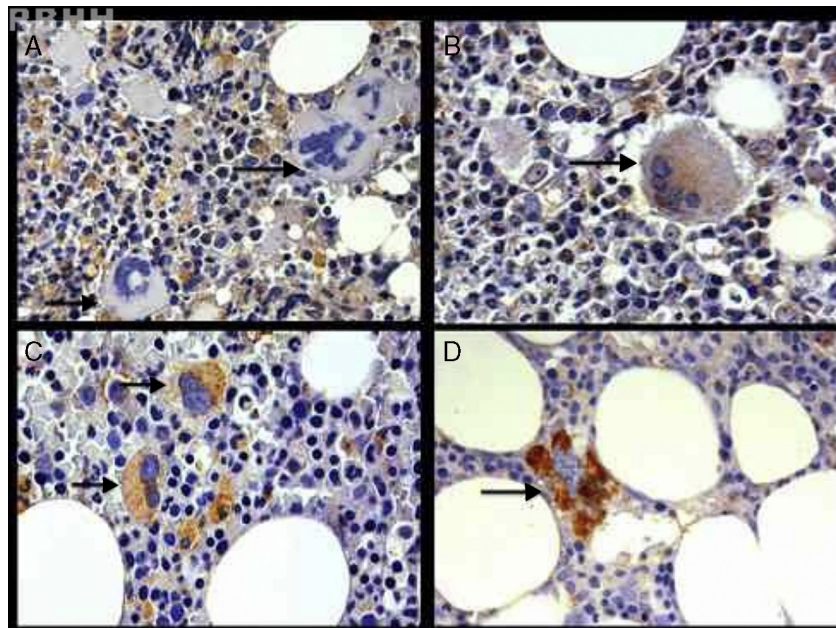
**Table 1 – Immunohistochemical evaluation of latent TGF- $\beta$ 1 antibodies in the control, essential thrombocythemia and primary myelofibrosis samples.**

A	B
Positivity (%)	Intensity
0 Negative (0–10)	0 – negative
1 Slight (11–25)	1 – weak
2 Intermediate (26–50)	2 – moderate
3 Extensive (over 50)	3 – strong

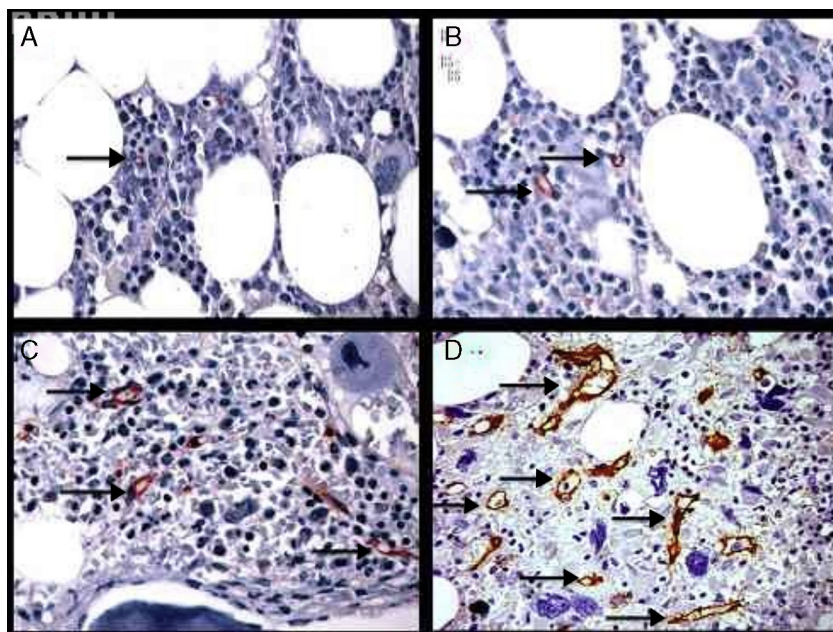
Score: (positivity)  $\times$  (intensity).

Two different methods were used for this assessment:

- (1) Estimating angiogenesis (microvessel grade)  
Angiogenesis was evaluated in each bone marrow sample by two independent reviewers using visual microvessel grading. Specifically, slides were visually scanned at 100 $\times$ , 200 $\times$  and 400 $\times$  magnification and were semi-quantitatively graded for vessels by CD34 staining using a light microscope. Each specimen was graded on a semi-quantitative scale: microvessel Grade 1, scarce vessels; Grade 2, diffuse slight increase in vessels; Grade 3, areas with marked increases in vessels; and Grade 4, extensive increases in vessels<sup>20,24</sup> (Fig. 2).
- (2) Microvascular density  
Immunostaining for the CD34 antigen was used to evaluate the vessels in the bone marrow biopsy sections. The absolute number of CD34-positive vascular structures was recorded in five randomly selected fields at 400 $\times$  magnification for each case by light microscopy. Microvessels were identified as ECs appearing as a single cell or cells clustered in the network or tubes either with or without lumen. Sinusoids were included in the count, while arterioles



**Figure 1 – Photomicrograph of latent transforming growth factor-beta 1 immunoreaction (400 $\times$ ) in the controls and patients (essential thrombocythemia and primary myelofibrosis) according to the intensity of the reaction: (A) negative (0), (B) weak (1), (C) moderate (2), and (D) strong (3) (arrow).**



**Figure 2 – Photomicrograph of the microvessel grade for CD34 immunopositivity (400×): (A) Grade 1, (B) Grade 2, (C) Grade 3, and (D) Grade 4 (arrow).**

were excluded. MVD was calculated as the median value of vascular structures and used for statistical analysis.<sup>25-27</sup>

The score assigned to the immunopositivity results of investigated antigens were compared between the different groups: control, ET, prefibrotic PMF and fibrotic PMF. The results were assessed using the Kruskal-Wallis non-parametric test followed by post hoc Dunn's test to identify significant differences between the groups. The correlation between the degree of fibrosis, latent TGF- $\beta$ 1 immunopositivity, angiogenesis by estimating microvessel grade, and MVD in the groups and all cases studied was analyzed with the Spearman test.  $p$ -values  $\leq 0.05$  were considered statistically significant.

## Results

The average age of the patients with ET and PMF was approximately 20 years greater than the average age of the subjects in the Control Group. There was no difference associated with gender among PMF cases, while there was a predominance of women in the ET group.

The degree of fibrosis and latent TGF- $\beta$ 1 immunopositivity in megakaryocytes based on the positivity and intensity criteria in each group and the final score obtained are presented in Table 2.

As shown in Table 2, the criterion positivity for latent TGF- $\beta$ 1 expression shows a tendency of extensive immunopositivity in all groups, while the intensity is stronger in the prefibrotic PMF group and varies from 'weak' in the control and ET groups to 'moderate' in the fibrotic PMF group.

The score was significantly lower in the controls compared to the prefibrotic PMF group ( $p$ -value  $< 0.01$ ). The analysis with the ET group showed no significant difference.

The reticulin graduation (fibrosis) for each group is listed in Table 2. There were significant differences in the grade of fibrosis between groups and in the latent TGF- $\beta$ 1 score between the control and prefibrotic PMF groups ( $p$ -value  $< 0.01$ ) (Fig. 3).

The distribution of microvessels and the MVD values for both patients and controls are shown in Fig. 3 and Table 3.

Dunn's multiple comparison test demonstrated that patients with fibrotic PMF had significantly higher MVD values than those with ET ( $p$ -value  $< 0.001$ ) and the controls ( $p$ -value  $< 0.001$ ), and patients with prefibrotic PMF also had higher values for MVD compared to the ET ( $p$ -value  $< 0.01$ ) and control groups ( $p$ -value  $< 0.001$ ) (Fig. 3 and Table 3).

The Spearman correlation between MVD and the estimation of angiogenesis (microvessel grade) was demonstrated for each group (ET, prefibrotic PMF and fibrotic PMF).

It was impossible to assess the relationship for the control group because the estimation for angiogenesis in this group did not change. An intermediate correlation ( $r = 0.61$ ;  $p$ -value  $< 0.0181$ ) was observed for the ET group and strong correlations were observed for the prefibrotic PMF ( $r = 0.90$ ;  $p$ -value  $< 0.0001$ ) and fibrotic PMF groups ( $r = 0.83$ ;  $p$ -value  $< 0.0002$ ).

The correlation coefficient was also evaluated between each variable analyzed in this study considering the total number of cases.

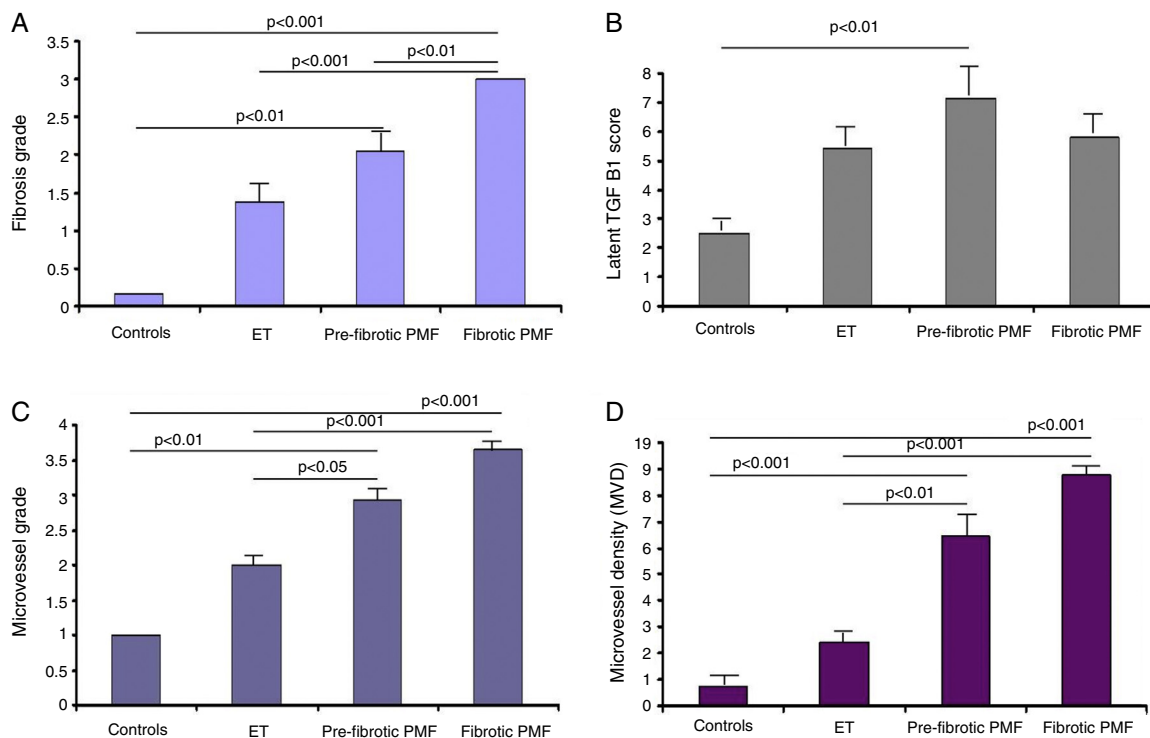
Strong correlations were found on comparing MVD with microvessel grade ( $r = 0.92$ ;  $p$ -value  $< 0.0001$ ), MVD with degree of fibrosis ( $r = 0.80$ ;  $p$ -value  $< 0.0001$ ) and microvessel grade with fibrosis grade ( $r = 0.77$ ;  $p$ -value  $< 0.0001$ ). The correlation of the latent TGF- $\beta$ 1 score with MVD ( $r = 0.45$ ;  $p$ -value  $< 0.0009$ ) and with the microvessel grade ( $r = 0.46$ ;  $p$ -value  $< 0.0006$ ) was also demonstrated.

**Table 2 – Latent TGF- $\beta$ 1 evaluation and fibrosis grade in the control, essential thrombocythemia, prefibrotic and fibrotic primary myelofibrosis groups.**

	Control n = 6	ET n = 18	Prefibrotic PMF n = 19	Fibrotic PMF n = 19
<b>Latent TGF-<math>\beta</math>1 positivity – n (%)</b>				
Negative	–	–	–	–
Discrete	1 (16.67)	–	–	–
Intermediate	1 (16.67)	2 (11.11)	2 (10.52)	1 (5.27)
Extensive	4 (66.67)	16 (88.89)	17 (89.48)	18 (94.73)
<b>Latent TGF-<math>\beta</math>1 intensity – n (%)</b>				
Negative	–	–	–	–
Weak	6 (100.00)	8 (44.44)	4 (21.05)	6 (31.58)
Moderate	–	5 (27.78)	3 (15.79)	8 (42.11)
Strong	–	5 (27.78)	12 (63.16)	5 (26.31)
<b>Latent TGF-<math>\beta</math>1 score – n (%)</b>				
Zero	–	–	–	–
One	1 (16.67)	–	–	–
Two	1 (16.67)	2 (11.11)	2 (10.52)	1 (5.27)
Three	4 (66.67)	6 (33.33)	2 (10.52)	5 (26.31)
Six	–	5 (27.78)	3 (15.79)	8 (42.11)
Nine	–	5 (27.78)	12 (63.16)	5 (26.31)
Latent TGF- $\beta$ 1 score mean ( $\pm$ SD)	2.50 ( $\pm$ 0.83) <sup>a</sup>	5.39 ( $\pm$ 2.70)	7.15 ( $\pm$ 2.71) <sup>a</sup>	5.79 ( $\pm$ 2.41)
<b>Fibrosis grade – n (%)</b>				
Zero	5 (83.33)	–	–	–
One	1 (16.67)	12 (66.67)	1 (5.27)	–
Two	–	5 (27.78)	16 (84.21)	–
Three	–	1 (5.55)	2 (10.52)	19 (100.00)
Fibrosis grade mean ( $\pm$ SD)	0.17 ( $\pm$ 0.40) <sup>a</sup>	1.39 ( $\pm$ 0.60) <sup>a</sup>	2.05 ( $\pm$ 0.40) <sup>a</sup>	3.00 ( $\pm$ 0.00) <sup>a</sup>

ET: essential thrombocythemia; PMF: primary myelofibrosis; TGF- $\beta$ 1: transforming growth factor-beta 1; SD: standard deviation.

<sup>a</sup> p-value < 0.01.



**Figure 3 – Fibrosis grade (A), latent transforming growth factor-beta 1 score (B), microvessel grade (C) and microvessel density (D) for each group.**

**Table 3 – Microvessel grade and microvascular density in the control, essential thrombocythemia, prefibrotic and fibrotic primary myelofibrosis groups.**

	Control n = 5	ET n = 14	Prefibrotic PMF n = 18	Fibrotic PMF n = 14
Microvessel grade – n (%)				
One	5 (100.00)	1 (7.14)	–	–
Two	–	12 (85.72)	6 (33.33)	–
Three	–	1 (7.14)	7 (38.89)	5 (35.71)
Four	–	–	5 (27.78)	9 (64.29)
Microvessel grade Mean (±SD)	1.00 (±0.00) <sup>a</sup>	2.00 (±0.39) <sup>a</sup>	2.94 (±0.80) <sup>a</sup>	3.64 (±0.49) <sup>a</sup>
Microvessel density Mean (±SD)	0.72 (±0.17) <sup>a</sup>	2.42 (±0.69) <sup>a</sup>	6.46 (±3.53) <sup>a</sup>	8.78 (±1.94) <sup>a</sup>

ET: essential thrombocythemia; PMF: primary myelofibrosis; SD: standard deviation.

<sup>a</sup> p-value < 0.05.

## Discussion

The increase in stromal fibrosis is evident in chronic myeloproliferative neoplasms albeit with a variable course depending on the specific subtype.<sup>3</sup> Fibrosis seems to be associated with abnormalities in the number and function of megakaryocytes and platelets.<sup>9</sup>

The primary histological feature common to PMF and ET cases is megakaryocytic hyperplasia with nuclear atypia, while fibrosis represents the major differential criteria between the two diseases.<sup>3</sup> Megakaryocytes and platelets produce high levels of TGF-β1.<sup>9</sup>

It has been postulated that defective or abnormal megakaryocytes with higher immunoreexpression of the latent form of TGF-β1 are involved in the development of myelofibrosis and angiogenesis.<sup>10,21</sup>

In the present study, we showed that the mean latent TGF-β1 immunoreexpression levels and degree of fibrosis were lower in the control group than in the prefibrotic PMF group (*p*-value < 0.01). The differences in the microvessel grade and MVD between these groups are also significant (*p*-value < 0.01), suggesting that TGF-β1 is a common factor in both situations.

Panteli et al.<sup>18</sup> and Steurer et al.<sup>26</sup> assessed MVD in chronic myeloproliferative neoplasms by examining CD34 immunoreexpression and demonstrated that neo-angiogenesis is increased in both phases of PMF (prefibrotic and fibrotic) compared to cases of PV, and ET and controls. The outcome of these studies is in agreement with our findings.

In this study, the use of two methods to assess angiogenesis, estimating angiogenesis by examining microvessel grade<sup>20,24</sup> and MVD,<sup>25-27</sup> aimed to improve the credibility of the results.

The two approaches were in agreement with similar results of the differentiation of the groups (control, ET, prefibrotic PMF and fibrotic PMF) with *p*-values ≤ 0.05 (Fig. 3) and a strong correlation coefficient between microvessel grade and MVD with *p*-value < 0.0001 and *r* = 0.92. This overlap of the results shows that estimating angiogenesis (microvessel grade) in bone marrow biopsies can be as accurate as evaluating the MVD, thereby validating this simple method for use in the routine practice.

In the development of scar fibrosis, neo-angiogenesis promotes the deposition of collagen,<sup>28</sup> with TGF-β1 being one cytokine involved in these events.<sup>29</sup> Our study showed a clear

correlation between angiogenesis and fibrosis, even though the association between the expression of TGF-β1 and vascularization was only moderate.

In our histological evaluation of fibrotic PMF cases, there was very low cellularity including decreased numbers of megakaryocytes, resulting in decreased latent TGF-β1 immunoreexpression in the fibrotic phase which may explain the absence of any correlation between this cytokine and fibrosis. On the other hand, induction of angiogenesis and fibrogenesis by TGF-β1 acting in the prefibrotic stage becomes obvious in the histological features of the fibrotic phase.<sup>21</sup>

Moreover, latent TGF-β1 immunoreexpression correlated with angiogenesis as assessed using the two methods, MVD (*r* = 0.45; *p*-value < 0.0009) and microvessel grade (*r* = 0.46; *p*-value < 0.0006). These results may be associated with the dynamics of the healing process, in which the involvement of inflammatory cytokines and growth factors, particularly TGF-β1, prior to the neo-angiogenesis stage represents an intermediate phase in the establishment of interstitial fibrosis.

Moreover, we believe that there are other special features involved in the development of stromal fibrosis associated with primary myelofibrosis, such as other growth factors (platelet-derived growth factor, fibroblast growth factor) or cytokines (interleukin-1, tumor necrosis factor), which require further investigation.

## Conclusions

A correlation was observed between latent TGF-β1 immunoreexpression and neo-angiogenesis, which precedes the establishment of bone marrow stromal fibrosis. A highly reliable method to estimate angiogenesis (microvessel grade) was also demonstrated; this technique is easier than MVD and as accurate and can be used to assess microvasculature in bone marrow biopsies.

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## Conflict of interest

The authors declare no conflicts of interest.

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