

# Chronic idiopathic myelofibrosis: independent prognostic importance of bone marrow microvascular density evaluated by CD105 (endoglin) immunostaining

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**Microvascular density (MVD) is substantially increased in bone marrow biopsies of patients with chronic idiopathic myelofibrosis (CIMF). CD105, a useful molecule for assessing MVD in various malignancies, is preferentially expressed by recently formed microvessels. Increased serum-soluble CD105 in patients with chronic myeloproliferative disorders, including CIMF, was documented. CD105 MVD has not so far been investigated in CIMF: to this end, the results in 55 patients with CIMF and 21 controls were compared with the conventional CD34 immunostaining as well as traditional histological and clinical disease features. The MVD mean values estimated by both CD105 and CD34 were significantly higher in CIMF patients than in controls ( $P < 0.00001$ ). In addition, the proportion of CD105-positive megakaryocytes was significantly higher in CIMF than in controls ( $P < 0.0001$ ). A degree of reticulin fibrosis  $> 2$  correlated with increased CD105 MVD ( $P = 0.05$ ). A multivariate analysis confirmed that CD105-positive MVD was an independent adverse prognosticator. This study demonstrates that while MVD, as assessed by both CD34 and CD105 immunostaining, is significantly increased in CIMF, only CD105-determined MVD correlates with the degree of fibrosis and is prognostically relevant. These findings provide a rationale for the investigational use of anti-CD105-targeted drugs in CIMF. *Modern Pathology* (2004) 17, 1513–1520, advance online publication, 23 July 2004; doi:10.1038/modpathol.3800224**

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Chronic idiopathic myelofibrosis (CIMF) is a clonal myeloproliferative disease, which is characterized by mainly megakaryocytic and granulocytic proliferation, reactive deposition on bone marrow connective tissue and extramedullary hematopoiesis.<sup>1</sup> The disease shows a progressive accumulation of fibrosis and an irreversible fatal course of variable length. The current therapy for CIMF is mostly palliative, although some limited success has been obtained by bone marrow transplantation.<sup>2,3</sup> No

clearcut prognostic factors have been identified so far.

Angiogenesis, which is characterized by the recruitment of new blood vessels,<sup>4</sup> has been found to be crucial for the growth and proliferation of many solid tumors. There is evidence that, similar to other hematopoietic disorders,<sup>5,6</sup> angiogenesis is involved in the pathogenesis and progression of CIMF. Serum levels of the proangiogenic cytokine VEGF and microvessel density (MVD) in the bone marrow are significantly increased in CIMF patients.<sup>7,8</sup> Moreover, thalidomide, an antiangiogenic drug, is effective in combination with prednisone in counteracting cytopenia.<sup>9</sup> Finally, CD34-positive MVD has been reported to be an independent predictor of survival in CIMF patients.<sup>8</sup>

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Angiogenesis has been most commonly assessed by immunostaining in routinely processed bioptic tissue with antibodies reactive with endothelial cells, including CD31, factor VIII, and CD34. In most studies, CD34 has been found to be the most useful antigen for assessing the number of microvessels (termed MVD) in both hematopoietic and nonhematopoietic malignancies.

The more recently introduced antiendoglin (CD105) monoclonal antibody appears to be superior to CD34 in the detection of new blood vessels in some solid tumors, such as non-small-cell lung carcinoma.<sup>10</sup> Preliminary evidence has also shown that CD105-assessed MVD may be a better predictor of adverse outcome than CD34-assessed MVD in solid tumors, such as breast carcinoma.<sup>11</sup> It has been recently demonstrated that serum levels of soluble CD105 are increased in patients with chronic myeloproliferative disorders, including one patient with CIMF.<sup>12</sup>

In the present study, we have compared for the first time the utility of CD105 and CD34 in the assessment of MVD in CIMF. We have correlated these measurements with known disease features such as degree of fibrosis and peripheral blood count values, as well as overall patient survival.

## Materials and methods

### Patients

The study population included 55 patients with CIMF. Patients with postpolycythemic or postthrombocytopenic myelofibrosis and other myelofibrotic disorders were carefully excluded. There were 32 male and 23 female patients, with a median age of 72 (range 18–89) years. Peripheral blood values were available at diagnosis for 42 patients. In these subjects, the median hemoglobin, white cell and platelet counts were 11.5 g/dl,  $12.6 \times 10^9/l$  and  $448.5 \times 10^9/l$ , respectively. The control group consisted of 21 patients with bone marrow biopsies obtained for staging purposes for non-Hodgkin's lymphomas or for miscellaneous non-neoplastic conditions and found to be free of neoplasia and other abnormalities at histological examination. We deliberately did not include in the control group bone marrow biopsies taken from patients staged for Hodgkin's lymphoma, due to the increased microvascularity often seen in this setting (G Pruneri and M Ponzoni, personal communication).

### Pathologic Material and Immunohistochemistry

Bone marrow biopsies (BMB) were collected from the Department of Pathology files of Columbia University, New York ( $n=39$ ) and S Raffaele Hospital, Milan ( $n=16$ ), during the period 1990–2000. Tissues were fixed with formalin ( $n=17$ ) or Bouin's solution ( $n=38$ ), decalcified with an EDTA-

based solution or nitric acid, paraffin embedded and cut at 4  $\mu$ m thick sections. Control group included BMB fixed either with formalin or Bouin's solution. Each BMB was stained using hematoxylin and eosin and reticulin silver impregnation. Reticulin content was evaluated according to a scoring system previously published;<sup>13</sup> cases were subdivided according to the degree of fibrosis '0–2' and '3–4', respectively. Immunohistochemistry was performed by the avidin–biotin peroxidase complex (ABC) method and the staining reaction revealed by 3,3'-diaminobenzidine tetrahydrochloride chromogen method, using a DAKO automated immunostainer. For antigen retrieval, the slides were placed in a 0.01 M EDTA buffer at pH 8 and underwent three 4-min 780 W cycles at 90° in a microwave oven before immunostaining.

### Bone Marrow Microvessel Staining and Estimation of MVD

For CD105 and CD34 MVD assessment, 49 and 55 cases of CIMF were evaluable, respectively. Staining of microvessels was performed using anti-CD34 (QEnd/10, Novocastra, Newcastle upon Tyne, UK; working dilution 1:100) and anti-CD105 (4G11, Novocastra; working dilution 1:400) monoclonal antibodies (MoAbs). The negative controls were similarly processed bone marrow biopsies in which nonimmune mouse serum was substituted for the primary antibody. For immunohistochemistry, proper internal controls were present in each sample and represented by blasts and arterioles for CD34 and by erythroid precursors and few sinusoids for CD105.

The measurement of MVD was performed according to a previously published method:<sup>14</sup> any positively stained endothelial cell or endothelial cell cluster that was clearly separated from adjacent microvessels was considered as a single, countable microvessel. Vessel lumens were not a prerequisite to define a structure as microvessel. Sinusoid-like structures, as opposed to arterioles, were included in the definition of microvessel. Large vessels spanning two adjacent fields were counted only once. We modified our previous experience in visual microvessel scoring<sup>14</sup> as follows: instead of choosing an arbitrary number of fields as previously published, all fields of the whole section were counted at  $\times 200$  microscopic magnification and a final average MVD value for the whole section was registered. The described modification was introduced in order to avoid the bias of selecting areas with more abundant and/or more intensely stained microvessels. This is particularly important when dealing with CIMF, which is characterized both in early and in advanced stages by the presence of areas of greatly variable cellularity. In addition, in view of the CD105 reactivity with megakaryocytes (M Ponzoni and G Pruneri, unpublished observation), the presence of CD105-positive megakaryo-

cytes was determined. The measurements were taken independently by two observers (MP and AO), without the knowledge of any clinical data.

### Statistical Analysis

Clinical characteristics and MVD among the study subgroups were compared using the  $\chi^2$  tests or Fisher's exact test for categorical variables, according to the sample size. The comparison of mean values was performed using the 't-test'. Survival curves were generated by the Kaplan–Meier method. Overall survival (OS) was calculated from the date of the diagnosis to death or to the last date of follow-up. The prognostic relevance of CD34-positive and CD105-positive MVD was assessed using the mean MVD values in CIMF cases as a cutoff. Survival rates were reported as 5-year OS  $\pm$  standard error. Impact on survival of clinical and immunohistochemical variables were evaluated by comparing the survival curves by the log-rank test. The independent prognostic value of the variables was analyzed using the Cox model. All the probability values were two-sided. Analyses were carried out using the Statistica 4.0 statistical package for Windows (Statsoft Inc., Tulsa, OK, USA).

## Results

### MVD Assessed by CD105 and CD34 Immunostains

The mean number of immunostained vessels per  $\times 200$  field that were evaluated was 11 (range 4–22) and 14 (3–38) for controls and CIMF subgroups, respectively ( $P=0.2$ ). The mean ( $\pm$ s.d.) MVD value for CD34 was  $2.7 \pm 1.4$  (range 0.8–5.5) for controls and  $37.7 \pm 24.7$  (6.3–100) for CIMF ( $P<0.00001$ ), respectively. The mean ( $\pm$ s.d.) MVD value for CD105 was  $8.2 \pm 2.8$  (range 2.5–14.1) in controls and  $30.8 \pm 7.8$  (range 15.3–47.2) in CIMF ( $P<0.00001$ ). The mean MVD values for CD34 and CD105 immunoreactivity in CIMF cases were used as cutoffs to divide subgroups in correlation and survival analyses. There was no correlation between MVD calculated on the whole section and overall cellularity.

Both MoAbs highlighted the irregular shape of CIMF vessels, although CD105 was detected on a greater number of 'sinusoid-like' vessels<sup>5,14</sup> than CD34 (Figure 1c,d). No difference in MVD was observed between formalin- and Bouin's solution-fixed specimens.

The percentage of cases with CD105-positive megakaryocytes was significantly increased in CIMF in comparison to the control biopsies: 36/49 (73%) vs 3/21 (14%), respectively ( $P<0.00001$ ). In most cases, the megakaryocytes displayed weak-to-moderate cytoplasmic staining without predilection for scattered (rather than clustered) megakaryocytes (Figure 1e,f). No CD34-positive megakaryocytes

were observed in the control group, while 2/35 (6%) CIMF cases showed this finding ( $P=0.29$ ).

### MVD and Reticulin Fibrosis

The control group did not show an increase in reticulin fibrosis. In the 50 valuable CIMF cases, reticulin content was '0–2' in eight (16%) cases and '3–4' in 42 (84%) cases. A significant correlation was observed between CD105-positive MVD and degree of fibrosis (ie degree 3–4) (mean  $\pm$  s.d. =  $31.6 \pm 7.8$ ) in comparison to a lesser degree of fibrosis (mean  $\pm$  s.d. =  $25.7 \pm 7.5$ ) ( $P=0.05$ ). A significant correlation between CD105 megakaryocytes and substantial fibrosis was also observed; all cases with low degree of fibrosis (ie  $\leq 2$ ) displayed CD105-positive megakaryocytes, which were found in 57% of cases with an high degree (ie 3–4) of fibrosis ( $P=0.05$ ) (Table 1).

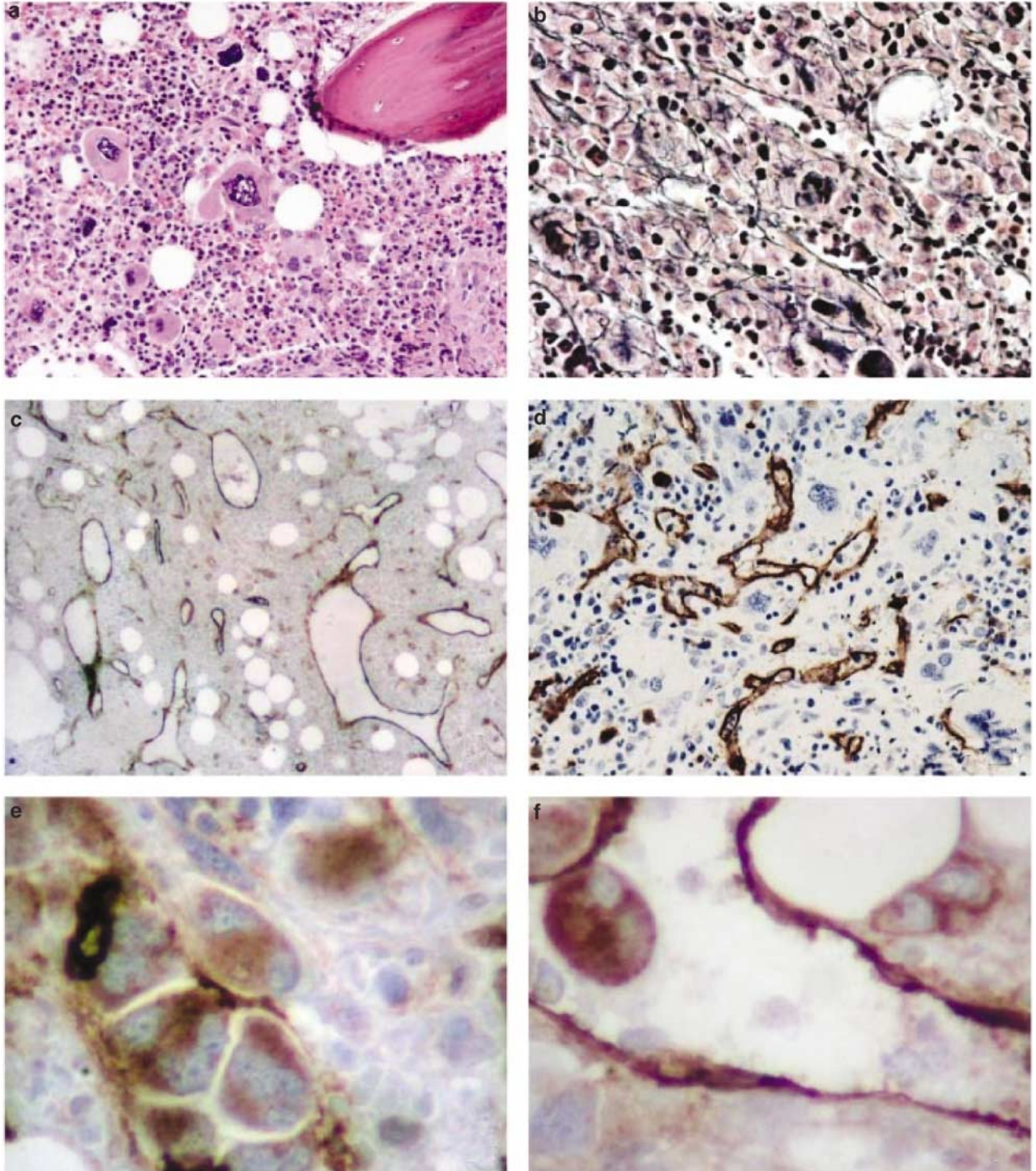
### Survival Analysis

A total of 33 patients are alive at a median follow-up of 32 months (range 1–241), with a 5-year OS of  $67 \pm 8\%$ . CD105-positive MVD (ie cases with CD105 MVD  $\geq$  mean value, 30.8 MVD) was an adverse predictor of survival ( $48 \pm 13\%$  vs  $86 \pm 8\%$ ,  $P=0.02$ ) (Figure 2a and Table 2). CD34 MVD (Figure 2b), sex, fibrosis, hemoglobin, WBC and platelets values were not associated with poor survival.

Multivariate analysis, adjusted for the above-mentioned parameters, confirmed the independent prognostic value of CD105-positive MVD for OS (Table 3).

## Discussion

Previous research supports the view that tumor growth depends on angiogenesis.<sup>4</sup> In addition to the well-established role of angiogenesis in solid tumors,<sup>4</sup> the increase in tumor vascularity has been previously investigated in a variety of hematological neoplasms, including acute lymphatic<sup>15</sup> and myeloid<sup>16,17</sup> leukemias, myelodysplastic syndromes,<sup>14,18</sup> Hodgkin's disease,<sup>19</sup> myeloma,<sup>20–25</sup> lymphoproliferative disorders,<sup>26–31</sup> systemic mastocytosis<sup>32</sup> and chronic myeloproliferative disorders,<sup>5,14</sup> including CIMF.<sup>8</sup> The number of small vessels in a tumor, or MVD, is considered a surrogate indicator of angiogenesis.<sup>4,22,24,33</sup> The prognostic impact of MVD in hematological malignancies has been investigated by a number of groups, with conflicting results.<sup>15,16,20,21,23–25,30,31</sup> In these studies, bone marrow microvessels are visualized by immunostaining of vascular endothelial cells; the stained microvessels are then counted and MVD determined. Commonly used antigens for assessment of MVD include CD34, CD31 and von Willebrand Factor (vWF). Of these, CD34 appears preferable to CD31 and vWF in



**Figure 1** Increased MVD in chronic idiopathic myelofibrosis. A case of CIMF stained with hematoxylin and eosin (**a**), showing an increase in reticulin fibers when stained by silver impregnation (**b**); CD105 MoAb (**c**) preferentially highlights the 'sinusoid-like' morphology of increased microvessels when it is compared to CD34 immunostaining (**d**); clustered (**e**) and intrasinusoidal (**f**) megakaryocytes are immunoreactive for CD105.

patients with myeloid disorders because of its strong immunoreactivity with endothelial cells and its limited nonvascular expression.<sup>14,21,22</sup> The value of CD34 for assessment of MVD in myeloproliferative

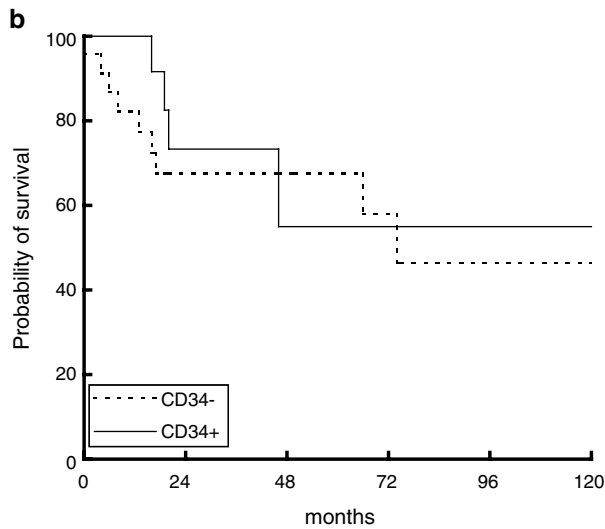
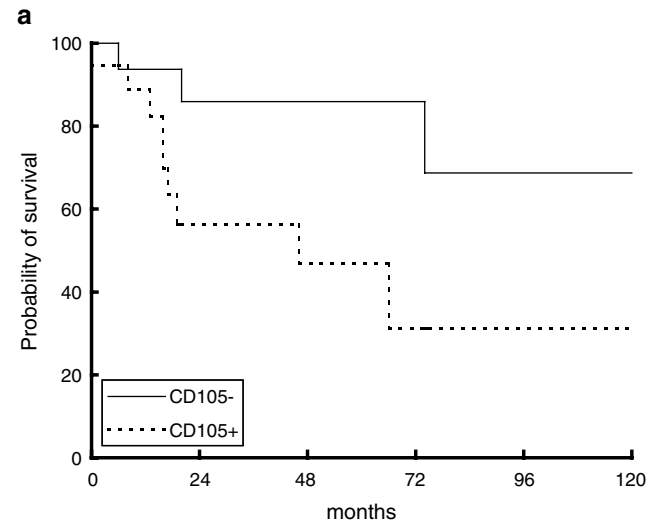
disorders, including CIMF, has been demonstrated in recent studies.<sup>8,33</sup>

Our study shows that CD105 is also an effective tool for staining bone marrow microvessels and

**Table 1** Relationship between CD105 MVD, CD105-positive megakaryocytes and degree of fibrosis in CIMF

Parameter	Fibrosis 1–2	Fibrosis 3–4	P-value
CD105 MVD	25.7 ± 7.5	31.6 ± 7.8	0.05
CD105+ve meg	8 (100%)	24 (57%)	
CD105–ve meg	0 (0%)	18 (43%)	0.05

MVD = microvascular density; meg = megakaryocytes.



**Figure 2** CD105-positive microvascular density is an adverse predictor of survival in chronic idiopathic myelofibrosis. Overall survival curves for patients grouped according to CD105 (a) and CD34 (b) immunoreactivity.

assessing angiogenesis in bone marrow biopsies of patients with CIMF. In these patients, similar to what has been observed for CD34, the density of CD105 stained microvessels is significantly higher than in the marrow of control patients. Although CD34 immunostaining can be used to assess MVD in

**Table 2** Correlation between CD105 and CD34 MVD and overall survival in CIMF patients

Parameter	Subgroup	5-year OS ± s.d.	P-value
CD105 MVD	Negative vs positive	86 ± 8 vs 48 ± 13%	0.02
CD34 MVD	Negative vs positive	68 ± 10 vs 55 ± 18%	0.22

MVD = microvascular density; OS = overall survival. Cases were defined as positive for CD105 MVD when exhibited ≥ 30.8 MVD and negative when < 30.8 (mean value). Cases were defined as positive for CD34 MVD when exhibited ≥ 37.7 MVD and negative when < 37.7 (mean value). Sex, fibrosis, intravascular hemopoiesis, hemoglobin, white cell, and platelet counts did not correlate with survival.

**Table 3** Multivariate analysis

Parameter	Subgroup	Odds ratio	95% CI	P-value
CD105 MVD	Negative vs positive	6.6	1.51–9.41	0.02
CD34 MVD	Negative vs positive	0.4	0.11–8.03	0.28
Fibrosis	1–2 vs 3–4	1.4	0.17–2.53	0.73
Age	Continuous var.	1.0	0.97–1.15	0.20
Hemoglobin	Continuous var.	0.8	0.77–1.24	0.11
WBC	Continuous var.	1.01	0.94–1.08	0.81
Platelets	Continuous var.	0.9	0.9–1.01	0.82

Cases were defined as positive for CD105 MVD when exhibited ≥ 30.8 MVD and negative when < 30.8 (mean value). Cases were defined as positive for CD34 MVD when exhibited ≥ 37.7 MVD and negative when < 37.7 (mean value); var. = variable. CD105 MVD and CD34 MVD showed similar results when analyzed as continuous variables.

CIMF,<sup>8</sup> our multivariate analysis results demonstrate that only CD105 can provide prognostically relevant information in this disorder (Figure 2). CD34-assessed MVD was not prognostically helpful (Table 3). Other parameters, including age, fibrosis, and peripheral blood values also lacked prognostic significance (Table 3).

The lack of prognostic significance for CD34 MVD values in CIMF are at variance with another recent study, in which increased MVD, as detected by CD34 (clone HPCA-1) immunostaining, was linked to a worse outcome.<sup>8</sup> There may be several reasons for this discrepancy. Variations in immunostaining technique, such as the use of different primary antibodies among different laboratories, may be one factor. In contrast with Mesa *et al*,<sup>8</sup> who employed the clone HPCA-1 for CD34 immunostaining, we used the QBEND/10 clone, which has been extensively utilized by our group and other investigators to assess MVD<sup>14,16,24,34</sup> In addition, in our study, a different approach for MVD evaluation was used. Instead of numbering semiquantitatively vessels as done by Mesa *et al*,<sup>8</sup> we performed a complete count of all vessels in each section.<sup>24</sup> This approach avoids the possible bias of selectively choosing areas with higher vascular density, and produces quantitative data without introducing predetermined arbitrary cutoffs or 'hot-spots'. It is noteworthy that the

prognostic value of CD105 persisted when this parameter was analyzed as a continuous variable. Finally, Mesa *et al*<sup>6</sup> included in their study a substantial number of patients with 'postpolycythemic' or 'post-thrombocytopenic' myeloid metaplasia and performed multivariate analysis without adjusting for these separate clinicopathological entities.

The differences observed in our study may be due to the different endothelial specificity of the two antibodies. In contrast to CD105, which is preferentially expressed by recently formed vessels,<sup>11</sup> CD34 is not a selective marker for newly formed vessels. Alternatively, our study might have failed to detect a prognostic role for CD34, at least in part, due to the bias intrinsic to retrospective investigations.

The significant correlation between CD105-assessed MVD and degree of fibrosis may not be fortuitous, since it has been shown that CD105, which interacts with receptors for transforming growth factor beta (TGF- $\beta$ ), is capable of modulating several of the cellular responses to this fibrogenetic cytokine.<sup>35,36</sup> In CIMF, TGF- $\beta$  is strongly expressed by megakaryocytes,<sup>37,38</sup> a large proportion of which express CD105.

Taken together, the fibrogenetic role for endoglin as well as its abnormally increased expression in megakaryocytes may be relevant to the currently accepted pathogenetic model for CIMF in which the megakaryocytes play a primary role by their production of fibrogenetic cytokines, which in turn affect the bone marrow microenvironment through autocrine or paracrine mechanisms.<sup>39-45</sup>

With regard to therapeutic aspects, clinical improvement offered by treatment with the antiangiogenic drug thalidomide has been recently reported in CIMF, although the efficacy of this drug in reducing MVD is still uncertain.<sup>9,46</sup> It is noteworthy that a recent preliminary report focused on CD105 suggested the efficacy of anti-CD105 MoAb therapy, in synergy with cyclophosphamide, in reducing tumor size in human skin/SCID (severe combined immunodeficiency) mouse chimeras bearing human breast cancer.<sup>47</sup> CD105 may represent a target for antiangiogenic therapy;<sup>48</sup> this belief is also supported by the concept that endothelial cells of tumor microvessels may be more susceptible to killing effects of anti-CD105 immunoconjugates than the vascular endothelium of normal tissues.<sup>49</sup>

The prognostically significant increase of CD105-positive megakaryocytes in CIMF patients, in contrast to the absence in the marrows of lymphoproliferative disorders such as myeloma,<sup>24</sup> suggests a potential role for this MoAb as a target for abnormal cells of the megakaryocytic lineage. To confirm this hypothesis, CD105 expression needs to be studied in a wider range of myeloid disorders characterized by abnormal megakaryocytopoiesis, such as various leukemic conditions (eg acute megakaryoblastic leukemia) as well as in chronic myeloproliferative disorders and myelodysplastic syndromes.

In conclusion, our study shows that CD105-assessment represents an improved method for MVD determination in CIMF. CD105 shares with CD34 the advantage of having fewer positive cells of nonendothelial nature, thus facilitating evaluation of MVD. CD105 is preferentially expressed by recently formed vessels,<sup>11,49</sup> although nonspecific.<sup>49,50</sup> The presence of CD105-positive MVD appears to worsen the prognosis of patients with CIMF. The potential therapeutic use of anti-CD105 immunotherapy in CIMF deserves investigation, particularly in the group of CIMF patients with highest CD105-MVD values.

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