

# Spleen neoangiogenesis in patients with myelofibrosis with myeloid metaplasia

Giovanni Barosi,<sup>1</sup> Rosti Vittorio,<sup>2</sup> Massa Margherita,<sup>3</sup> Viarengo G. Luca,<sup>4</sup> Pecci Alessandro,<sup>5</sup> Necchi Vittorio,<sup>6</sup> Ramaioli Isabella,<sup>7</sup> Campanelli Rita,<sup>2</sup> Marchetti Monia,<sup>1</sup> Bazzan Mario<sup>8</sup> and Magrini Umberto<sup>6</sup>

<sup>1</sup>Laboratory of Medical Informatics, <sup>2</sup>Transplant Research Area, <sup>3</sup>Laboratory of Biotechnology, <sup>4</sup>Clinical Immunology and Immunohematology and Transfusion Service, <sup>5</sup>Unit of Internal Medicine and Medical Oncology, <sup>6</sup>Institute of Pathology, <sup>7</sup>Unit of Internal Medicine III, IRCCS Policlinico S. Matteo, Pavia, Italy, and <sup>8</sup>Unit of Hematology, Ospedale Evangelico Valdese, Torino, Italy

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Correspondence: Giovanni Barosi, M.D., Laboratorio di Informatica Medica, IRCCS Policlinico S. Matteo, Viale Golgi 19, 27100 Pavia, Italy. E-mail: barosig@smatteo.pv.it

## Summary

Neoangiogenesis is an integral component of bone marrow myeloproliferation in patients with myelofibrosis with myeloid metaplasia (MMM). As extramedullary haematopoiesis is a constitutive feature of MMM, we studied spleen neoangiogenesis by a computerized image analysis in MMM patients. Compared with five normal subjects, spleen CD34-staining capillary vascular density (CVD) was 2.1–3.03 times higher than the upper range of normal in six of the 15 (40%) MMM patients. CD8-staining sinusoidal vascular density (SVD) was constantly normal or lesser than normal and was inversely correlated with CVD ( $R = -0.53$ ;  $P = 0.04$ ). In MMM patients who did not receive cytoreductive or radiation therapy in the month before splenectomy ( $n = 9$ ), the CVD was a significant determinant of spleen size ( $R = 0.88$ ;  $P = 0.04$ ). In MMM patients, the number of spleen CD34<sup>+</sup> haematopoietic stem cells was increased from 1.2 to 98 times the upper limit of normal, and predicted the expansion of CVD ( $R = 0.57$ ;  $P = 0.03$ ). A population of cells expressing the CD34<sup>+</sup>/CD133<sup>+</sup>/VEGFR-2<sup>+</sup> angiopoietic phenotype was present in the blood and spleen of five of seven patients. These results document that neoangiogenesis is an integral component of spleen re-localization of haematopoietic stem cells and suggest a cellular mechanism for spleen neoangiogenesis.

**Keywords:** myelofibrosis, myeloid metaplasia, angiogenesis, CD34<sup>+</sup> cells, spleen angiogenesis.

Neoangiogenesis, or the formation of new vessels, has been documented in the bone marrow of patients with myelofibrosis with myeloid metaplasia (MMM), and microvessel density has been reported to be higher than in other chronic myeloproliferative disorders (CMPDs) (Mesa *et al*, 2000). Plasma over-expression of angiogenic factors, such as vascular endothelial growth factor (VEGF) (Bellamy *et al*, 1999; Di Raimondo *et al*, 2001), and evidence that the myeloid clone is capable of producing such cytokines (Bellamy *et al*, 1999), has favoured the interpretation of neoangiogenesis in MMM as an integral reaction to myeloproliferation (Mesa *et al*, 2000).

As extramedullary haematopoiesis is a constitutive component of MMM, it is conceivable that neoangiogenesis could also occur in the spleen. This hypothesis is strengthened by the observed reduction of spleen volume with anti-angiogenic therapy (Barosi *et al*, 2002; Piccaluga *et al*, 2002; Mesa *et al*,

2003), and by the observation that responders to the treatment were patients with a documented increase in bone marrow angiogenesis (Piccaluga *et al*, 2002).

Spleen vasculature is a complex network of arterioles, capillaries and sinuses, with different functions and different staining properties (Ruck *et al*, 1994; Balazas *et al*, 2001; Korkusuz *et al*, 2002). In this study, we used two different antibodies, namely anti-CD34 and anti-CD8, to immunohistochemically measure the capillary vascular density (CVD) and sinusoidal vascular density (SVD), respectively, in the spleens of patients with MMM. We also measured the blood and spleen CD34<sup>+</sup> cells by cytofluorimetric analysis in order to assess the extent of spleen re-localization of haematopoietic stem cells. Moreover, as the expression of CD133 and VEGF receptor 2 (VEGFR-2) identifies a subpopulation of bone marrow stem cells that have a high potential to directly cause angiogenesis (Peichev *et al*, 2000; Reyes *et al*, 2002), these

surface antigens were also investigated in the peripheral blood and spleen cells among the patients.

## Materials and methods

### Patients

Between June 2000 and June 2003, 15 consecutive patients with MMM, in whom splenectomy was indicated because of refractory anaemia or symptomatic splenomegaly, entered the study. There were eight males and seven females with a median age of 63 years (range, 35–70 years). The diagnosis of MMM was established according to the Italian Consensus Conference criteria (Barosi *et al*, 1999). The median duration of disease from the diagnosis of MMM to spleen removal was 60 months (range, 5–204 months). The patients had received a number of previous treatments, mainly corticosteroids, androgens and chemotherapy, and six patients were on hydroxyurea treatment at the time of splenectomy. One patient had also received spleen radiation therapy in the month before splenectomy.

Patients were assigned a risk class according to the Dupriez score, based on haemoglobin level and white blood cell count (Dupriez *et al*, 1996). 'High risk' patients were classified as such by a haemoglobin concentration of less than 10 g/dl (or transfusion dependent), and a leucocyte count of less than  $4 \times 10^9/l$  or greater than  $30 \times 10^9/l$ . 'Intermediate risk' patients had either a haemoglobin concentration of less than 10 g/dl, or a leucocyte count lesser than  $4 \times 10^9/l$  or greater than  $30 \times 10^9/l$ . 'Low risk' patients presented with a haemoglobin concentration of more than 10 g/dl and a white blood cell count between  $4 \times 10^9/l$  and  $30 \times 10^9/l$ .

At the time of splenectomy, the patients had a complete blood count and a peripheral blood smear examination performed. White blood cell count was corrected for the number of circulating erythroblasts. Circulating nucleated cells were classified as immature myeloid cells, erythroblasts and blasts. Blasts were defined as undifferentiated cells with an immature nucleolated nucleus and basophilic cytoplasm with or without azurophilic granules.

The size of the spleen was measured by ultrasonography by using the spleen index calculated by multiplying the length of the longitudinal axis by that of the transverse axis, the latter defined as the maximal width of the organ (Goulis *et al*, 1999). Liver enlargement was measured as the distance from the right costal margin in cm.

Circulating levels of VEGF were measured in seven of the 15 patients before the removal of the spleen. The detection of VEGF in the plasma was performed by an enzyme-linked immunosorbent assay (ELISA) method (R & D System, Minneapolis, MN, USA), according to the manufacturer's instructions. Samples were assessed in duplicate and detectable ranges of concentration were 15–2000 pg/ml. Twenty normal individuals were used as controls.

Five normal individuals (males, aged from 21 to 69 years) in whom a splenectomy was performed for traumatic lesions, served as controls for spleen cellularity, neovascularization and stem cell phenotype.

### Circulating and spleen stem cells

*Peripheral blood and spleen samples.* Permission for obtaining blood and spleen samples from the patients with MMM and spleen samples from the normal controls was obtained from the Institutional Ethical Committee. Spleen tissue was obtained immediately after spleen removal. Spleen samples, 4–5 cm long, were obtained randomly from the spleen avoiding the necrotic areas, and cut in to smaller pieces with sterile scissors. After careful rinsing, using a circular motion, each single piece was pressed between two sterile slides on a 100-mm Petri dish until only fibrous tissue remained. The slides were washed with Roswell Park Memorial Institute (RPMI) 1640 medium (Eurobio, France) supplemented with 2% fetal calf serum and the medium containing the cells was collected in the Petri dish. Finally, to eliminate small cell clumps, the suspension was passed up and down several times through a 5-ml syringe with a 23 G needle. After checking by microscopic examination that a single cell suspension was obtained, cells were washed twice, counted in a Burker chamber and re-suspended in the appropriate medium for flow cytometry analysis. To calculate the cellularity per gram of spleen tissue, each sample of spleen that was subsequently reduced to a single cell suspension was weighed and the total number of nucleated cells obtained was divided by the weight of the spleen sample.

Cytospin preparations of cell suspensions were stained by May-Grünwald-Giemsa for standard morphological examination. In order to identify mature endothelial cells, slides were also double-stained for CD34 antigen and von Willebrand factor. After fixation with acetone, specimens were stepwisely incubated with a mouse monoclonal antibody (MoAb) to CD34 (Exalpha, Watertown, MA, USA), an Alexa Fluor 594-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR, USA), a rabbit polyclonal antibody to von Willebrand factor (Dako, Carpinteria, CA, USA), and an Alexa Fluor 488-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, USA). Hoechst 33258 pentahydrate (Molecular Probes, Eugene, OR, USA) was used for nuclear counterstaining.

*FACS analysis.* Cells in EDTA-anticoagulated blood or spleen-derived cell suspensions were stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- peridinin chlorophyll protein (PerCP)- and laser dye styryl (LDS751)-conjugated MoAbs for immunophenotypic analysis. The following antibodies were obtained from Becton Dickinson (San Jose, CA, USA): CD45FITC/CD34PE/LDS751, CD38-FITC/CD34-PE/CD45PerCP, and used for the analysis of haematopoietic progenitor cells. CD133-PE (Mytenyi Biotech, Germany), CD34-FITC, and VEGFR-2-PerCP (Sigma, St.

Louis, MO, USA) MoAbs, were used for the analysis of haemo-endothelial progenitor cells. The analyses were performed by a FACScalibur (Becton Dickinson); 20 000 events per sample were acquired and the percentage of positive cells was calculated based on the appropriate isotype control (FITC; Immunotech, Marseille, France).

### Spleen vessel density

**Histopathology.** Immediately after spleen removal, the spleens were weighed and measured. At least five randomly selected tissue blocks were prepared from the removed spleens. Parallel slices 1 cm thick were fixed in 10% neutral-buffered formalin and routinely processed. The paraffin-embedded tissue was cut into 4- $\mu$ m thick sections, mounted on glass slides and stained with haematoxylin and eosin, Giemsa, AS-D chloroacetate esterase (CAE) and Gomori for reticulin.

Two histopathologists reviewed the pathological features of each spleen and selected at least five representative paraffin blocks for immunostaining. Spleen capillaries were visualized by immunohistochemical staining for CD34, while sinusoids were visualized by immunohistochemical staining for CD8. Monoclonal antibodies to CD61, glycophorin, and myeloperoxidase (MPO) were also used to evaluate extramedullary haematopoiesis. A labelled streptavidin–biotin peroxidase detection system and an automated immunostainer (Dako, Glostrup, Denmark) were used. The primary antibodies (Ylem for CD34, Roma, Italy; Dako Cytomotion, Glostrup, Denmark for CD8, Glycophorin A, CD61 and MPO) were incubated with tissue sections in appropriate dilutions. Positive and negative controls were run with the study cases and stained appropriately.

**Vessel density measurement.** Spleen CVD and SVD were assessed by using ImageJ, a public domain downloadable Java computerized image analysis and processing program from the National Institutes of Health (Bethesda, MD, USA), which calculates area and pixel value statistics of user-defined selections. The slides of spleen tissue were scanned at low magnification and five random areas of approximately 1 mm<sup>2</sup> were determined. With computerized pixel counting, the number of vessel structures and the mean surface area of each vessel were also determined. The mean value of the counts of the five fields was utilized.

### Statistical analysis

Non-parametric statistical methods (Mann–Whitney *U*-test for unpaired data and Spearman rho for simple correlation analysis) were used in the primary analyses of the data. CVD and SVD within-spleen variability was assessed from the five readings per spleen by measuring means, standard deviation (SD), and coefficient of variation (CV) (CV = SD/mean  $\times$  100%). The CVD and SVD estimates were studied for possible correlations with various clinical or laboratory

variables obtained at the time of splenectomy. Multiple group comparison for CVD and SVD at baseline was performed by analysis of variance (ANOVA). All computations were performed with STATISTICA software (Statsoft, Tulsa, OK, USA). *P* values < 0.05 were considered significant.

## Results

### Patient characteristics

The clinical and haematological data of the 15 patients obtained during the course of hospitalization for splenectomy are summarized in Table I. Eleven patients (73.3%) were in the 'intermediate' or 'high' risk class. There was no evidence in any patient of blast transformation, however, three patients had more than 5% of blasts in peripheral blood at the time of splenectomy. Circulating CD34<sup>+</sup> cell number was correlated with the percentage of blasts in peripheral blood (*R* = 0.66; *P* = 0.009).

### Spleen Neoangiogenesis

The weight of the removed spleens in MMM patients ranged from 1600 to 5400 g (median, 2650 g) and were correlated with the spleen index as measured before splenectomy by sonography (*R* = 0.51; *P* = 0.048). Microscopic examination showed that the basic structure of the spleens was not greatly altered. In all the cases the follicular component of the white pulp was reduced to a few, sparsely distributed, small residual structures. Most of the splenic architecture was represented by

**Table I.** Baseline characteristics of the 15 patients with myelofibrosis with myeloid metaplasia (MMM) included in this analysis.

Age, years, median (range)	63 (35–70)
Males, number (%)	8 (53.3%)
Haemoglobin, g/dl, median (range)	8.2 (7.1–15.5)
White blood cell count, $\times 10^9/l$ , median (range)	10.8 (0.9–48.4)
Circulating blasts, $\times 10^9/l$ , median (range)	0.03 (0–2.2)
Platelet count, $\times 10^9/l$ , median (range)	139 (19–400)
Spleen size, ultrasound measured, index (cm <sup>2</sup> ), median (range)	567 (325–900)
Time from diagnosis to splenectomy, months, median (range)	60 (5–204)
CD34 <sup>+</sup> cells in peripheral blood, $\times 10^6/l$ , median (range)	63.4 (0.49–6.630)
Dupriez risk score*, number (%)	
0	4 (27)
1	6 (40)
2	5 (33)

\*A Dupriez score of 0 (low risk) was assigned for haemoglobin level greater than 10 g/dl and a white blood cell count between  $4 \times 10^9/l$  and  $30 \times 10^9/l$ , score of 1 (intermediate risk) for either a haemoglobin level less than 10 g/dl or a white blood cell count greater than  $30 \times 10^9/l$  or less than  $4 \times 10^9/l$ , and a score of 2 (high risk) if both the haemoglobin level and white blood cell count were in the aberrant ranges.

the red pulp, which appeared to be expanded as a result of the presence of haematopoiesis, increased vascularity and congestion of vascular structures.

Haematopoiesis always appeared to be trilinear, but one cell line might predominate in a given case. Glycophorin-positive erythroid precursors were more frequently localized in sinuses. CD61-positive megakaryocytes exhibited dysplastic features and occurred in clusters both in cords and sinuses. CAE- and MPO-positive granulocyte precursors were recognizable in cords and sinuses.

A significant increase in silver reticulum was observed in two cases exhibiting post-transfusional haemosiderosis.

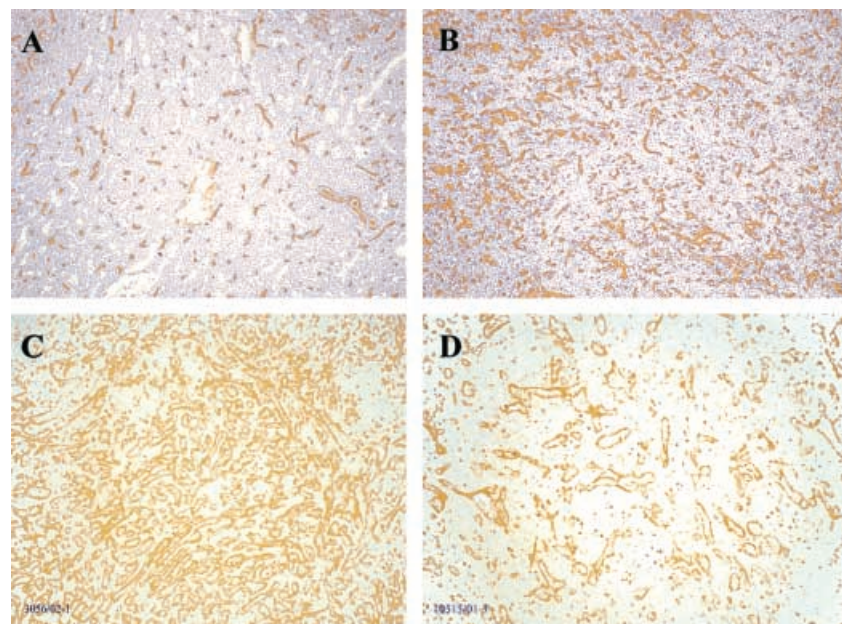
The weight of the removed spleens in normal control subjects ranged from 120 to 190 g.

At the immunohistochemical staining of spleen sections, both CD34<sup>+</sup> endothelial cells lining capillary vascular structures, and CD8<sup>+</sup> endothelial cells lining sinuses, were easily recognizable and clearly distinguishable from the simultaneously stained CD34<sup>+</sup> progenitor cells or CD8<sup>+</sup> lymphocytes. CD34-stained capillaries appeared to be of small calibre, while CD8 stained vessels were of varying calibre, mostly thin-walled

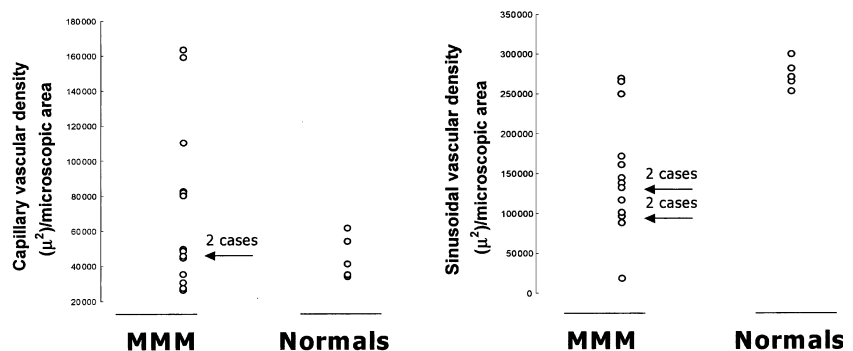
and markedly ectatic (Fig 1). In MMM patients, the number of slides with tissue sections of sufficient quality ranged from four to five per spleen. Vascular density was obtained by calculating the average of five spleen tissue sections per patient, and is given as  $\mu^2$  per microscopic area. The CV for the CVD was 1.6% and that of the SVD was 1.8%.

In patients with MMM, the density of CD34<sup>+</sup> capillaries (CVD) had a median value of 48 663  $\mu^2$  (range, 26 520–1 63 422  $\mu^2$ ), higher than in normal individuals in whom the median value was 38 197  $\mu^2$  (range, 34 082–53 997  $\mu^2$ ) (Fig 2). Six of 15 patients (40%) had a CVD value 2.15–3.03 times higher than the upper range of normal controls and 11 (73.3%) had a CVD value higher than the median value of normal controls. In these patients, the number of measured vessel structures and the per vessel surface area were also increased with respect to normal (data not shown).

The density of CD8<sup>+</sup> sinusoids (SVD) had a median value of 1 17 466  $\mu^2$  (range, 19 256–2 69,940  $\mu^2$ ), lower than in normal individuals, in whom the median value was 2 81 652  $\mu^2$  (range, 2 53 624–3 64 052  $\mu^2$ ) (Fig 2). Thirteen of 15 patients (86.6%) had a SVD value lower than the lower range of normal



**Fig 1.** (A) Visual CD34-stained capillary vascular density (CVD) in a normal subject; (B) Visual CD34-stained CVD in a patient with myelofibrosis with myeloid metaplasia (MMM). (C) Visual CD8-stained sinusoidal vascular density (SVD) in a normal subject. (D) Visual CD8-stained SVD in a patient with MMM. Original magnification  $\times 50$ .



**Fig 2.** Individual measurements of CD34-stained capillary vascular density and CD8-stained sinusoidal vascular density in patients with myelofibrosis with myeloid metaplasia ( $N = 15$ ) and normal subjects ( $N = 5$ ).

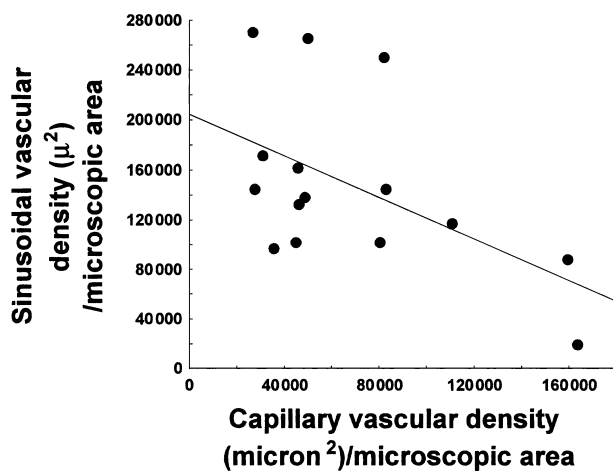


Fig 3. Relationship between CD34-stained capillary vascular density and CD8-stained sinusoidal vascular density in patients with myelofibrosis with myeloid metaplasia. Linear regression analysis demonstrated a significant inverse correlation ( $R = -0.53$ ;  $P < 0.048$ ).

controls. In these patients the number of measured vessel structures and the per vessel surface area were also decreased with respect to normal (data not shown). Patients with the lowest values of SVD had the highest values of CVD, and, in the whole population of patients, an inverse correlation between CVD and SVD was evidenced ( $R = -0.53$ ;  $P < 0.048$ ) (Fig 3).

The extent of spleen capillary and sinusoidal vasculature of the 15 patients with MMM was investigated for possible correlations with clinical and laboratory characteristics at the time of splenectomy. Neither spleen CVD nor SVD correlated with the weight of removed spleens or with the spleen index as measured by ultrasound before splenectomy, with the risk class of the disease or with any of the haematological parameters. However, patients who received chemotherapy or spleen radiotherapy in the month preceding splenectomy ( $N = 6$ ) had a significantly lower CVD than patients who were only under supportive therapy ( $N = 9$ ) ( $48,418 \mu^2$  vs.  $82,572 \mu^2$ ;  $P = 0.02$ ). By using only cases that did not receive chemotherapy or radiation therapy, a direct correlation was found between CVD and spleen index ( $R = 0.88$ ;  $P = 0.002$ ), while an inverse correlation was evidenced between SVD and spleen index ( $R = -0.68$ ;  $P = 0.04$ ), (Fig 4).

The median value of plasma VEGF in MMM patients was  $55.4 \text{ pg/ml}$  (range,  $15.6\text{--}968 \text{ pg/ml}$ ), significantly higher than in normal controls (median,  $30.16 \text{ pg/ml}$ ; range,  $15.6\text{--}130.6 \text{ pg/ml}$ ). There was no association between plasma VEGF concentration and spleen CVD, spleen SVD or spleen volume.

#### Spleen CD34<sup>+</sup> cells

The number of nucleated cells obtained by spleen fragmentation in MMM patients ranged from 14,8 to  $466 \times 10^6/\text{g}$  of

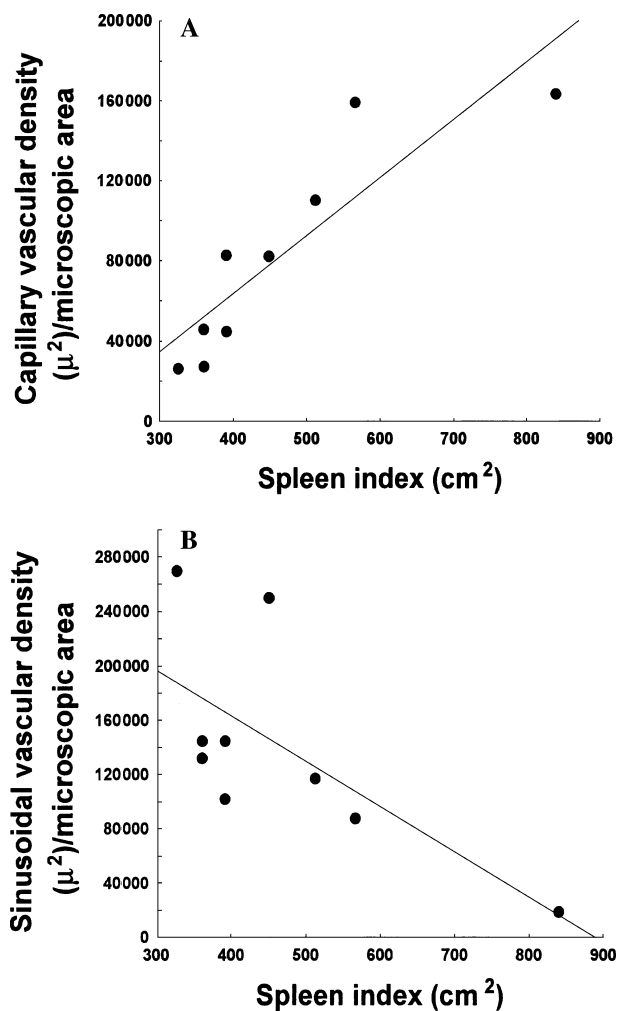


Fig 4. Relationship between spleen index ( $\text{cm}^2$ ) and capillary vascular density (CVD; panel A), or sinusoidal vascular density (SVD; panel B) in patients with myelofibrosis with myeloid metaplasia who did not receive chemotherapy or spleen radiotherapy during the month before splenectomy. Linear regression analyses demonstrated that the spleen index was directly correlated with CVD ( $R = 0.88$ ;  $P = 0.002$ ) but inversely correlated with SVD ( $R = -0.68$ ;  $P = 0.04$ ).

spleen tissue (median,  $35 \times 10^6/\text{g}$ ). Although the percentage of different cell types, as identified by microscopic examination, varied from one spleen to the other, the erythroid precursors, granulocytic precursors and megakaryocytes accounted for  $65.9\text{--}91\%$  (median,  $75.5\%$ ) of the spleen cell population. Mature endothelial elements, as identified by double-labelling for CD34 and von Willebrand factor, accounted for  $0.2\text{--}0.6\%$  of spleen cells. The remaining cells were lymphocytes and macrophages.

In the overall series of MMM patients, the median value of immunophenotypically measured spleen CD34<sup>+</sup> cells was  $1.89\%$  (range,  $0.18\text{--}14.7\%$ ) of the number of nucleated cells in the cell suspension. By comparing this figure with that of the normal controls (median,  $0.11\%$ ; range,  $0.09\text{--}0.15$ ), the MMM patients had a CD34<sup>+</sup> cell count of  $1.2\text{--}98$  times higher than

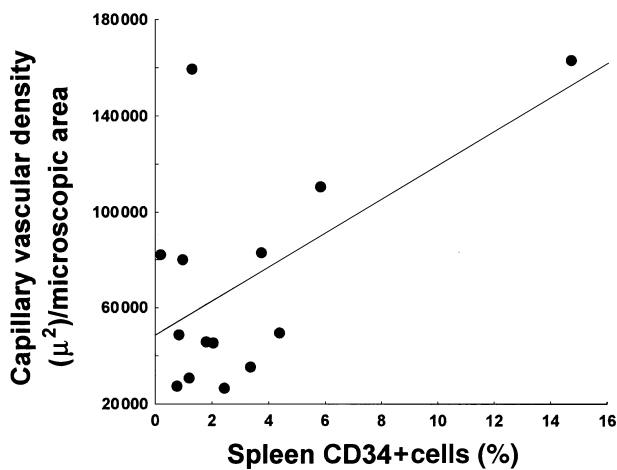


Fig 5. Relationship between the content of CD34<sup>+</sup> cells in the spleen (as % of the total spleen cell population) and capillary vascular density. Linear regression analysis demonstrated a significant direct correlation ( $R = 0.57$ ;  $P = 0.03$ ).

the upper range of normal. Patients with the highest rates of CD34<sup>+</sup> in the spleen had the highest rates of CD34<sup>+</sup> cells in the peripheral blood ( $R = 0.94$ ;  $P = 0.001$ ). Moreover, there was a clear association between the spleen CD34<sup>+</sup> cell content and the spleen CVD ( $R = 0.57$ ;  $P = 0.03$ ), as shown in Fig 5.

For characterizing the phenotype of CD34<sup>+</sup> cells in the spleen, we also measured their expression for two differentiation markers, (i.e.) CD38 and CD133. When cells from the spleens of MMM patients were double-stained with anti-CD34 and anti-CD38, the median proportion of CD34<sup>+</sup> cells that also were CD38<sup>+</sup> was 54% (range, 7.5–82%). The median percentage of CD34<sup>+</sup> cells in peripheral blood that also expressed CD38 was 34% (range, 14–88%). Patients with the highest expression of CD38 in peripheral blood had the highest expression in the spleen ( $R = 0.68$ ;  $P = 0.029$ ). When cells from the spleens of MMM patients were double-stained with anti-CD34 and anti-CD133, the median proportion of CD34<sup>+</sup> cells that also were CD133<sup>+</sup> was 33% (range, 6–75%). The median percentage of CD34<sup>+</sup> cells in peripheral blood that also expressed CD133 was 40% (range, 0–65%).

In order to identify whether cells with an angiopoietic phenotype resided in the spleen, we searched for the presence of cells with immunostaining characteristics of CD34<sup>+</sup>, CD133<sup>+</sup> and VEGFR-2<sup>+</sup>. CD34<sup>+</sup>/CD133<sup>+</sup>/VEGFR-2<sup>+</sup> cells were found in the spleen of 5 out of 7 patients in whom they were measured. These patients also had cells that had that phenotype in the peripheral blood, but the percentage of the cells was higher in the spleen (median, 11.5%; range, 0–23.6% of the CD34<sup>+</sup> cells) than in the peripheral blood (median 2.5%; range, 0–13.1% of the CD34<sup>+</sup> cells). Thus, cells with an angiopoietic phenotype represented a median of 0.08% of all the spleen cell population, with a range from 0.04% to 0.17%. This subset of cells was not detectable in the spleen of normal subjects. We also sought to identify whether the presence or

extent of the angiopoietic cells predicted the extent of neoangiogenesis: the two patients with no detectable angiopoietic cells had the lowest degree of CVD in their spleens.

## Discussion

Angiogenesis is crucial in tumourigenesis, and the bone marrow of MMM patients is characteristically occupied by a highly vascular myeloid tissue. Starting from the consideration that in MMM, haematopoietic stem cells move from bone marrow to extramedullary organs (Barosi *et al*, 2001) and that bone marrow neoangiogenesis is an integral part of myeloproliferation (Mesa *et al*, 2000), in the present work we investigated spleen microvasculature in patients with MMM. As data indicate that the endothelia of spleen sinusoidal and capillary vessels immunostain differently (Ruck *et al*, 1994; Balazas *et al*, 2001; Korkusuz *et al*, 2002), we used two antibodies that differentiate between the endothelial cells of capillaries, which are reactive for CD34, and the endothelial cells of the sinusoidal vascular system, which are reactive for CD8.

The immunohistochemical staining of spleen sections of 15 patients with MMM produced results that were mostly different from that of the spleen of normal subjects. By grading the vascular area by a computerized approach, the median values of CD34 staining capillary density in MMM was higher than that in normal subjects and 40% of MMM patients had a spleen sectional area occupied by capillary vascular structures that was up to three times larger than the upper value of normal subjects.

In contrast to CD34<sup>+</sup> endothelial cells, CD8<sup>+</sup> endothelial cells, which line large sinusoidal structures, produced a vascular density area smaller than normal in most of the MMM patients studied. The lack of knowledge on the mechanisms of vessel patterning of normal spleen angiogenesis precludes any interpretation of this observed diverging growth for the two vascular structures in the spleen of patients with MMM. However, there is evidence from animal studies (Stalmans *et al*, 2002) that endothelial cells from different vessel structures are molecularly distinct and that they can express different molecules, like VEGF receptors, that may have effect on angiogenesis.

By immunostaining spleen vessels, patients with the highest values of capillary density had the lowest values of sinusoidal density. A competitive mechanism, which produces a differential expansion of the two vascular structures, may be hypothesized. However, the observed inverse relationship between sinusoidal vascular density and spleen volume also indicates a possible dilution effect affecting non-growing sinusoidal structures intermingled in an area of increased capillary and cell density.

The result of an up to three-times increased capillary area in MMM patients suggests a significant contribution of neoangiogenesis to the spleen volume expansion. Thus, we explored whether the increased capillary structures had a role in

increasing the spleen volume in our MMM patients. While the whole population of patients did not show an association between CVD and spleen volume, considering only patients who were not receiving chemotherapy at splenectomy or had not received spleen radiation in the month before splenectomy, the association was strong. According to the correlation coefficient, CVD justified 77% of the variance of spleen size. However, as this analysis was forcedly restricted to nine patients, the result needs to be confirmed with larger numbers of cases to be accepted as a clinically meaningful conclusion.

We explored the possible determinants of increased capillary structures in the spleen of patients with MMM. Patients with increased CVD could be identified neither by their disease duration or severity, nor by their haematological phenotype. Furthermore, we did not find any correlation between spleen CVD and the concentration of plasma VEGF, the major angiogenetic growth factor. Thus, we could not support the interpretation that elevation of VEGF and angiogenesis are associated reactions. However, this association, even though biologically meaningful, was rarely documented in recent *in vivo* studies (Choi *et al*, 2002).

Significantly, we documented that the spleen content of CD34<sup>+</sup> haematopoietic cells in MMM was increased with respect to normal spleens, and that the patients with the highest extent of spleen localization of haematopoietic progenitors had the highest extent of CVD. This result is in line with that obtained by bone marrow and spleen immunohistochemical analysis of patients with MMM, in which CD34<sup>+</sup> cells were found to accumulate in the spleen in a higher proportion than in the bone marrow (Thiele *et al*, 1992). These results, in conjunction with the result that the number of spleen CD34<sup>+</sup> haematopoietic cells paralleled that of peripheral blood, are in keeping with the concept that a tight equilibrium exists between the blood and spleen haematopoietic cell compartments and that spleen myeloid metaplasia and capillary neoangiogenesis are correlated mechanisms.

To elucidate better the differentiation profile of the CD34<sup>+</sup> haematopoietic stem cells in the spleen, the CD38 and CD133 expression of CD34<sup>+</sup> cells was measured in all patients. CD38 is a transmembrane molecule that is expressed heterogeneously during haematopoietic cell differentiation. Most human immature haematopoietic cells with a high potential for self-renewal express low or no detectable levels of CD38 (Novelli *et al*, 1998). CD133 is a 120 kd glycosylated polypeptide, expressed on a subset of bone marrow derived stem cells, with the capacity to differentiate into hematopoietic cells, but also to form endothelial cells (Handgretinger *et al*, 2003). We demonstrated that our patients with advanced MMM had relatively low expression of CD38 and high expression of CD133 in their spleen CD34<sup>+</sup> cells. These results indicate that a highly undifferentiated stem cell phenotype dominates the spleen progenitor cells, and that there are patients with a consistent population of cells with potential angiopoietic phenotype. As it has recently become apparent that circulating

bone marrow-derived endothelial progenitor cells are involved in promoting physiological and pathological neovascularization, such as wound healing, tumour growth and tissue repair (Hill *et al*, 2003), we also studied the spleen content of a more restricted population of cells, namely CD34<sup>+</sup>/AC133<sup>+</sup>/VEGFR2<sup>+</sup> (Peichev *et al*, 2000; Reyes *et al*, 2002). The surface expression of these three antigens enabled us to document the presence of progenitor cells that are able to generate endothelial lineage. We found this phenotypically-characterized population of cells was consistently present in the spleen of patients with MMM, constituting up to 13% of CD34<sup>+</sup> cells and that their presence was associated with a sustained spleen neoangiogenesis.

These results are unique in the domain of CMPDs. The only report of a cell line with combined haematopoietic and endothelial features established from a patient with essential thrombocythaemia (Fiedler *et al*, 2000) sustains the possibility of spontaneous dual differentiation potential of stem and progenitor cells in CMPDs. Although it is tempting to speculate that progenitor cells with an angiopoietic phenotype could have a role in newly forming blood vessels in the spleen of patients with MMM, the nature and size of our study do not permit us to determine whether cells with an angioblastic phenotype behave *in vivo* as endothelial progenitor cells and whether they are able to transdifferentiate in spleen endothelium. Future studies will therefore be needed to determine whether cell trafficking in MMM is a factor in the pathogenesis of spleen neoangiogenesis.

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