Prognostic value of enhanced bone marrow angiogenesis
in early B-cell chronic lymphocytic leukemia

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SUMMARY
Since tumor progression is angiogenesis-dependent, angiogenesis density was investigated by immunohistochemistry and computed image analysis in bone marrow (BM) biopsies of 45 newly-diagnosed patients with Binet stage A B-cell chronic lymphocytic leukemia (B-cell CLL) and correlated to upstaging and progression-free survival during a 40-month follow-up. Their microvessel areas and counts were significantly higher than those of patients with anemia due to iron or vitamin B12 deficiencies. A cutoff value of 0.90 mm$^2 \times 10^{-2}$ or greater of the microvessel area identified patients with earlier upstaging and shorter progression-free survival. When the cutoff was applied to the Rai subclassification, both Rai 0 and Rai I-II patients who upstaged and shortened the progression-free survival were classified correctly. Information of this type was not given by the microvessel counts. The cutoff did not correlate with other predictors representative of tumor mass or disease progression. The microvessel area correlated with the expression of angiogenic vascular endothelial growth factor (VEGF) by tumor tissue, and serum levels of VEGF were found to be of prognostic value. A causal relationship between risk of progression and BM angiogenesis in B-cell CLL is suggested. A risk stratification inside Rai is proposed. The prognostic usefulness of BM angiogenesis in patients with B-cell CLL is envisaged.

INTRODUCTION
Tumor progression in the form of growth, invasion and metastasis depends on angiogenesis,$^1$ whose increase is thus indicative of poor prognosis in solid tumors.$^2$ The expression of several angiogenic growth factors, including vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) is of comparable prognostic value.$^3$ Knowledge on these relations in hematological tumors, however, is circumstantial. A tumor’s microvessel density predicts a risk of progression in multiple myeloma.$^4$ It is correlated with progression stages in both B-cell non-Hodgkin’s lymphomas$^5$ and mycosis fungoides,$^6$ and agrees with the growth of acute leukemias$^7$ and myelodysplastic syndromes.$^8,9$
Angiogenesis is involved in the pathogenesis of B-cell chronic lymphocytic leukemia (B-cell CLL).\textsuperscript{10,11} VEGF, present in both the patients’ serum and leukemic cells,\textsuperscript{12-14} is related to poor prognosis. FGF-2 expression by leukemic cells is associated with advanced disease and resistance to fludarabine.\textsuperscript{15} Studies on two series of B-cell CLL bone marrow (BM) angiogenesis carried out in limited and heterogeneous groups of patients provided conflicting results on its density and no prognostic information.\textsuperscript{11,16}

This paper presents an investigation of microvessel density and VEGF expression in the BM of a homogeneous series of 45 B-cell CLL patients with early disease. Relationships between angiogenesis, VEGF and FGF-2 serum levels and other markers of disease activity were assessed and clinical and prognostic implications were sought.

PATIENTS AND METHODS

Patients

Forty-five patients (25 M, 20 F) with Binet stage A\textsuperscript{17} B-cell CLL aged 42-81 (median 64) were studied. They were newly-diagnosed and had no prior history of B-cell CLL. Routine laboratory data, beta-2 microglobulin (β-2m) and LDH were assessed at diagnosis by standard procedures. Peripheral blood (PB) mononuclear cells were analysed by immunophenotyping\textsuperscript{18} to establish the diagnosis of typical B-cell CLL. Physical examination, chest x-ray, and abdominal ultrasound were always performed.

Simultaneously with Binet stage, patients were substaged according to Rai et al:\textsuperscript{19} stage 0, 27 patients (60%); stage I, 5 patients (11%); stage II, 13 patients (29%). BM biopsies were performed at diagnosis, and the histology pattern (nondiffuse or diffuse) was evaluated according to Rozman et al.\textsuperscript{20} Lymphocyte doubling time was assessed in 38 patients (84%) according to Montserrat et al.\textsuperscript{21} Control subjects were 12 patients (7 M, 5 F), aged 48-82 (median 67), with anemia due to iron or vitamin B\textsubscript{12} deficiencies.
The study was approved by the local ethics committee and all patients gave their informed consent.

**Measurement of bone marrow angiogenesis**

Vessels were detected in 6-µm sections of 4% paraformaldehyde-fixed paraffin-embedded biopsies by red-staining endothelial cells with an anti-factor VIII-related antigen (FVIII-RA) rabbit antibody (Dako, Glostrup, Denmark) and a three-layer biotin-streptavidin-peroxidase system described previously. Megakaryocytes also stained with FVIII-RA and were easily distinguishable by their morphology and size. Angiogenesis was measured as microvessel area and number without knowledge of the stage and substage. Microvessels (capillaries and small venules) were selected as endothelial cells, single or clustered in nests or tubes and clearly separated one from another, and either without or with a lumen not exceeding 10 µm, though larger neovessels were found in some patients. A double-headed photomicroscope (Axioplan 2, Zeiss, Oberkochen, Germany) was used in the simultaneous identification by two of us (A.V., D.R.) of the microvessel, and each identification was agreed upon in turn. The microvessel area was measured on 4 to 6 x250 fields covering the whole of each of two sections per biopsy within a superimposed square reticle. This was drawn out by a KS-300 software (Zeiss) and formed of 22 lines per side giving 484 intersection points. At x250 it defined an area of 12.5 x 10^{-2} mm^2 (reference area), whereas each point covered an area of 72.15 µm^2.

The area occupied by microvessels was estimated by using the direct planimetric method of "point counting" with slight modifications for the computed image analysis (same software) as described, according to which the microvessel area equals the sum of point areas that hit microvessels. Because cellular areas are vascularized and noncellular areas (fat, dense connective tissue, necrotic and hemorrhagic foci, bone lamellae) are not, and because the latter hampered comparison between sections, they were always omitted from the reference area. Thus, the point areas that hit noncellular areas were subtracted from the reference area. Residual point areas defined
the cellular area only and the microvessel area was measured inside it. Basically, the measurement of the microvessel and cellular areas fitted the following equations:

- microvessel area \( y \) = sum of points that hit microvessels \( x \) \( \cdot 72.15 \, \mu m^2 \);
- cellular area = \( 12.5 \times 10^{-2} \, mm^2 \) – (sum of points that hit possible noncellular areas \( p \) \( \cdot 72.15 \, \mu m^2 \)).

Values of the microvessel area were normalized to those of the cellular area by the equation:

\[
x/(484 - p) = y/100.
\]

Microvessels were counted at x250 within a computed square reticle of \( 12.5 \times 10^{-2} \, mm^2 \) and formed of 34 lines per side giving 1156 intersection points, which were distant 10.7 \( \mu m \) each other. Counts were done by the planimetric point-count method as the total number of the reticle intersection points occupied by transversely cut microvessels, whose small size (diameter \( \leq 10 \, \mu m \)) and the sufficient distance between two adjacent points (10.7 \( \mu m \)) meant that a point could be occupied by only one microvessel. Vessels transversely cut that touched two or more adjacent points (diameter larger than 10.7 \( \mu m \)) were counted as 1. Those placed on the inside or on the sides of a given small square of the reticle (ie, that did not cross the points) were not counted. Longitudinally or tangentially cut microvessels were not counted, regardless of their position. It was thus sufficiently certain that a given microvessel was counted only once, even if several of its section planes were present. The counting method makes allowances for the heterogeneous distribution of microvessels in tissue. Indeed, in line with others and our own observations, BM involved by hematological malignancies shows areas of more intense neovascularization, defined as “hot spots”. As the whole section was evaluated, microvessels not included in the hot spots were also counted. Because the whole of each of two sections per biopsy was evaluated, and the transversely cut microvessels hit the points randomly, the methods produced objective counts.
Analysis of serial sections (n = 6 to 10) from 3 biopsy samples revealed an intra-biopsy variability of ≤ 10% (± 1.8) in both the microvessel area and number. The variability between the investigators checking neovessels separately was ≤ 5.0% (± 3.2) for both the area and number. The area and number assessments were highly inter-correlated (Pearson’s $r = 0.95; P < 0.0001$). The microvessel area and number were expressed as mean ± 1 standard deviation (SD) for each section and biopsy and groups of biopsies.

To screen for patients who likely could progress, cutoffs of microvessel area and number corresponding to the highest Youden index$^{24}$ were chosen. The index combines information on sensitivity and specificity, giving equal weight to each, and measures the percentage gain in certainty of predicting the risk of progression. If microvessel area at a definite cutoff has an index of 0, it has no predictive power; if the cutoff has an index of 100%, progression is perfectly predicted. Microvessel area cutoffs set at the 25$^{\text{th}}$, 50$^{\text{th}}$ and 75$^{\text{th}}$ percentiles gave an index of 10%, 13% and 45% respectively. Thus, the highest cutoff was set. The microvessel number cutoff was set accordingly, since the 25$^{\text{th}}$, 50$^{\text{th}}$ and 75$^{\text{th}}$ percentiles paralleled an index of 0, 0 and 19% respectively. The decision to peak 75$^{\text{th}}$ percentile was arbitrary. The Youden index has already been applied in patients with B-cell CLL to compare CD38 expression with the risk of progression.$^{25}$

**Immunohistochemical staining of VEGF**

BM immunoreactivity to VEGF was investigated in 11 patients with a rabbit anti-VEGF antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) and the immunoperoxidase staining just described. Positive controls were anti-k+anti-λ chains and anti-CD20 rabbit antisera (used for immunophenotyping), and the negative control was a rabbit preimmune serum (all from Santa Cruz Biotechnology Inc). Two 6-µm sections per biopsy, adjacent to those examined for vascularity, were stained with each reagent by the same operator (L.T.).
The intensity of VEGF staining related to the degree of antigen expression. It was scored by the KS-300 software in 4 to 6 x400 fields per section, judged to be representative of the B-cell CLL tissue viewed from several x160 fields. In all patients, inflammatory mononuclear cells (fibroblasts, macrophages and polymorphs) also stained with VEGF. These cells were found as few, isolated or clustered elements throughout the stroma, and were clearly distinguishable from tumor cells both on morphological basis and because they usually displayed a different staining intensity (in plus or minus) from tumor cells. Care was taken to recognize these inflammatory cells and omit them from the evaluation.

The red-staining intensity of B-cell CLL tissue due to VEGF was fixed in each pixel, allocated on a gray intensity scale as density unit counts x 10^5, and expressed as the mean of the 4 to 6 fields per section, and finally as mean of the two sections. The background given by the negative control reagent in 4 to 6 x400 fields per section was also expressed as mean of the two sections and subtracted. The resulting value represented the mean gray density unit counts of VEGF intensity per biopsy. The staining technique was performed three times, on separate days, for each biopsy, and revealed that the intra-biopsy variability in the staining intensity was ≤ 20% (± 3.6) for all reagents. To overcome this variability for VEGF intensity and express it as a sole value, the final value per biopsy was the mean ± 1 SD of the three means per section, as described.26

Measurement of serum levels of VEGF (sVEGF) and FGF-2 (sFGF-2)
Sera from peripheral venous blood sampled at diagnosis were stored at −80°C. Their sVEGF and sFGF-2 levels were determined in duplicate by using the sandwich enzyme-linked immunosorbent assay (ELISA) (Quantikine human VEGF and Quantikine human FGF-2; R & D Systems, Minneapolis, MN, USA), according to the manufacturer’s instructions. Its sensitivity was < 5 pg/ml for VEGF and < 3 pg/ml for FGF-2. The coefficients of variation reported by the manufacturer for
inter-assay and intra-assay determinations vary from 6.2 to 8.8% and from 2 to 9% respectively for sVEGF, and from 7.4 to 9.1% and from 3 to 9.7% respectively for sFGF-2.

Patients were compared with 63 healthy blood donors (40 M, 23 F) aged 22-61 (median 51). Since measurement of sVEGF could be overestimated due to VEGF release by platelets during clotting, we correlated the sVEGF with the plasma VEGF in 30 randomly-chosen patients and found a close inter-relation (Pearson’s $r = 0.51$, $P < 0.0001$) and correlation of both with the platelet counts ($sVEGF\ r = 0.52$; plasma VEGF $r = 0.44$; $P < 0.0001$).

**Fluorescence in situ hybridization (FISH) studies**

Twenty-height patients with available cytogenetic pellet at diagnosis were characterized by FISH, using the 13q14.3 LSI D13S25 probe, the 17p13.1 LSI p53 probe, and the chromosome-12-centromeric probe CEP 12 (Vysis Co, distributed by Olympus, Milan, Italy), in dual color experiments with appropriate control probes. The lambda EMBL3 clones 19 and 65, spanning an area of approximately 40 kb within the middle portion of the ATM gene, were used to detect 11q22-23 deletions, as previously described. At least two-hundred interphase nuclei with well delineated signals were counted in each slide. The FISH procedure was repeated in those slides with low hybridization efficiency (ie, less than 80% cells with the expected two normal signals of the control probe).

**Clinical studies and disease-progression evaluation**

The degree of BM angiogenesis was correlated with main clinical and hematological variables, namely Rai substages, BM histology, absolute PB lymphocytosis, lymphocyte doubling time, LDH, $\beta$-2m, cytogenetics, and with sVEGF and sFGF-2. The 75th percentile of the microvessel area and number were chosen as cutoffs which could act as predictors of clinical outcome by using an end-point disease-progression, defined as the appearance of Binet upstaging during the treatment-free period. The significant impact of this end-point on the overall survival of Binet A patients...
enables it to replace overall survival as a prognostic parameter and shortens their clinical studies. The cutoffs were chosen according to the Youden index just described.

RESULTS

Prognostic value of enhanced BM angiogenesis

Table 1 shows the BM microvessel area and number and the cellular area in patients and control subjects. The patients’ microvessel area was significantly higher. The counting gave similar results. Because of the large bias of microvessel area and number within the patients’ group, the 75th percentile of the area (0.90 mm² x 10⁻²) and number (6 /12.5 mm² x 10⁻² fields) were selected as cutoffs to discriminate between highly and poorly vascularized BM, and related to progression.

After a median follow-up of 13 months (range = 2-40), 18 out of 45 patients (40%) progressed to a more advanced Binet stage, the risk of progression being 55.5% at 36 months. As shown in Fig. 1, the median duration of progression-free survival was 19 months for 12 patients with the microvessel area ≥ 75th percentile (or ≥ 0.90 mm² x 10⁻²), and 40 months for those with a lower area. Neither the microvessel number ≥ 75th percentile (or ≥ 6 /12.5 mm² x 10⁻²) nor the 50th percentile of both parameters as cutoffs gave such a discrimination.

Histologically, the 12 patients with an area ≥ 0.90 mm² x 10⁻² had particular microvessel configurations: 8 of them (66%) showed the microvessels widely distributed throughout the tumor tissue, as tortuous, thin, arborised tubes and single or clustered endothelial cells closely interwoven with tumor cells (Fig. 2, A); the remaining patients (44%) showed these microvessels coexisting with enormously dilated neovessels (Fig. 2, B) or displaying microaneurysmatic dilations (Fig. 2, C). Because all patients had a nondiffuse pattern of BM histology, a correlation between histology and each vessel configuration was unfeasible. In sharp contrast with these patients, the 33 patients with a lower area uniformly showed straight, not branched microvessels lacking dilations (Fig. 2, D), as did controls (Fig. 2, F), and tumor nodules often without microvessels (Fig. 2, E).
The risk of progression as progression-free survival was evaluated according to known and putative prognostic parameters. As shown in Table 2, parameters found to be significant predictors of the progression-free survival by univariate analysis were the microvessel area, BM histology, LDH, β-2m, lymphocyte doubling time, and sVEGF, whereas only LDH and B-2m provided independent prognostic information by multivariate analysis. sFGF displayed no prognostic power.

Although the microvessel area was not an independent prognostic factor in multivariate analysis (Table 2), it might be incorporated into the Rai substaging of Binet stage A patients to allow a better assessment of their risk of progression. As shown in Fig. 3, when Rai 0 patients were split by the cutoff area, 6 of them with ≥ 0.90 mm$^2$ x 10$^{-2}$ had a 20 months median progression-free survival, whereas the remaining 21 with a lower area did not reach the median at 47 months. Similarly, the cutoff discriminated 7 Rai I-II patients with 4 month median, from 11 patients with 18 months.

Serial studies were performed in 4 Rai 0 and 3 Rai I-II patients, and showed a trend for the microvessel area to increase in step with progression. Specifically, two Rai 0 patients (out of the 6 with area ≥ 0.90 mm$^2$ x 10$^{-2}$; Fig. 3) had 0.98 ± 0.61 and 1.15 ± 0.84 areas at diagnosis. Values rose to 1.27 ± 0.84 (+23%) and 1.77 ± 1.1 (+36%) after 30 and 37 months in parallel with progression. The other two Rai 0 patients (who belonged to the group with area < 0.90; Fig. 3) had 0.67 ± 0.25 and 0.52 ± 0.17 at diagnosis, and 0.49 ± 0.16 (-27%) and 0.58 ± 0.22 (+10%) after 25 and 28 months, when stable disease was recorded. Similarly, two Rai I-II patients (out of the 7 with area ≥ 0.90; Fig. 3) had 1.25 ± 1.62 and 2.21 ± 1.81 at diagnosis, and 2.62 ± 1.7 (+51%) and 2.94 ± 1.73 (+25%) after 10 and 12 months, when they progressed. The third patient with 0.77 ± 0.21 at diagnosis had 0.68 ± 0.16 (-12%) after 18 months in the presence of a stable condition. These data agree with those showing that lymph node angiogenesis in B-cell NHL enhances in step with progression.\textsuperscript{5}
Another approach was to correlate the microvessel area cutoff with the prognostic parameters representative of tumor mass (Rai substages, absolute PB lymphocytosis, BM histology, LDH, β-2m), or disease progression (lymphocyte doubling time, cytogenetics). As shown in Table 3, no correlations were found.

VEGF expression of the B-cell CLL bone marrow and sVEGF levels

VEGF expression evaluated immunohistochemically as an arbitrary intensity unit in the 11 BM increased in proportion with both microvessel area and number (Figs. 2 and 4). We were unable to correlate the BM histology pattern with the intensity of VEGF expression, since only 1 sample (the unique in our series) was diffuse and the remaining nondiffuse. The diffuse sample displayed neither the highest nor the lowest intensity, but was allocated on 4 x 10^5 intensity units, similarly to what was seen in two nondiffuse samples which displayed 3.3 and 3.7 x 10^5 units respectively. A higher number of diffuse samples is thus needed to reach safe conclusions.

The sVEGF was significantly higher in patients (median = 218 pg/ml, range = 9-2000) than controls (median = 142 pg/ml, range = 40-487; P< 0.02; Mann-Whitney’s test). As shown in Table 2, adoption of the median value of 218 pg/ml as a cutoff showed that sVEGF was a significant prognostic factor by univariate analysis. Since a high correlation between sVEGF and platelet counts had been previously observed (Pearson’s r = 0.52; P<0.0001), we wondered whether the counts also had prognostic power. No association was found between the counts and the risk of disease progression (P = 0.09). Thus, sVEGF levels are high in Binet stage A B-cell CLL patients and higher (≥ 218 pg/ml) in patients with poor outcome, irrespective of their platelet counts. Finally, we asked whether sVEGF could be used as an indicator of the density of BM angiogenesis. It was not, however, correlated with microvessel area and number (r = -196, P = 0.200; Spearman’s test).

Serial evaluation of sVEGF in 6 patients showed full agreement with outcome. Three patients whose average value at diagnosis was 412, 305 and 615 pg/ml displayed 741 (+45%), 455
(+53%) and 825 (+26%) after 22, 25 and 18 months when they progressed. The others who had 278, 145 and 340 at diagnosis gave 180 (-36%), 165 (+11%) and 270 (-21%) after 9, 15 and 8 months when stable disease was assessed. These data are in line with those showing that sVEGF levels in B-cell NHL increase with progression.31

Karyotype and angiogenesis

Since genomic aberrations are independent predictors of disease progression in early CLL,32 their correlation with microvessel area was sought. Twenty-height patients were available for comparison. Thirteen patients (46.4%) had normal karyotype, while 15 (53.6%) had aberrations. Of these, 8 patients displayed 13q (6 gave deletion as a sole aberration, and 2 gave 13q plus 12q trisomy), 6 patients displayed 12q trisomy as a sole aberration and 2 patients displayed 11q or 17p deletions. Patients were stratified into 4 groups according to major cytogenetic categories (normal karyotype, 13q as a sole aberration, 12q trisomy, 11q or 17p deletion) and aberrations were compared to microvessel area. No correlation was found (Fig. 5; Table 3).

DISCUSSION

Here we show a significant increase of BM angiogenesis (evaluated as FVIII-RA+ microvessel area and number) in patients with Binet stage A B-cell CLL, compared with control subjects. Kini et al16 obtained similar results with a CD34 monoclonal antibody (another endothelial cell marker) and found that microvessel number, BM cellularity and Binet stages were inter-correlated. In contrast, no increase in BM neovascularization and no dependence of vascularity on cellularity or stages were shown by Aguayo et al who used the FVIII-RA and counted in “hot spots”.11 A different counting system and a heterogeneous population of patients may account for this discrepancy.

We also show that the extension of microvessel area predicts the risk of progression of the Binet stage A. We calculated a cutoff of the 75th percentile or more of the area (≥ 0.90 mm² x 10⁻²), at or above which patients with an increased risk of Binet upstaging were identified. Information of
this type was not given by the microvessel number, suggesting that the microvessel area should be preferred for prognostic assessment. Indeed, assessment of the area implies that all neovessels, ie, those transversely, longitudinally or tangentially cut, irrespective of their diameter are precisely quantitated as the space they occupy in the BM. In contrast, the counting system we used scores only neovessels transversely cut, which hit the intersection points of the reticle. Also, it scores both neovessels with a diameter ≤ 10 µm and those larger as 1 each. Hence, although the system provides objective counts and is a useful index of BM vascularity in B-cell CLL (as in other tumors),\textsuperscript{2,5,7} it does not exactly quantitate the full vascular space in the tissue. Thus, the microvessel area provides better information on the degree of angiogenesis.

When the cutoff was applied to the Rai subclassification, the risk was greater for both Rai 0 and Rai I-II patients on or above the cutoff. Although such a finding concerned a relatively small series and in multivariate analysis the microvessel area was not an independent prognostic factor (different from serum β-2m and LDH), it seems of special interest for patients with early disease, since many prognostic factors such as BM histology, lymphocyte doubling time, sCD23, β-2m and serum thymidine kinase cannot be considered definitive markers.\textsuperscript{10,30} Also, as shown in patients with solid tumors,\textsuperscript{2} B-cell NHL\textsuperscript{5} and multiple myeloma,\textsuperscript{4,33} evaluation of angiogenesis density in tumor sites discriminates heterogeneous clinical outcomes within apparently homogeneous (same stage) groups of patients.

On the other hand, an accurate prognostic assessment of B-cell CLL patients with early disease is mandatory to optimise the timing of therapy and avoid exposure to the toxicity of treatment unlikely to be useful.\textsuperscript{34} One can also hypothesize that patients of this type who have enhanced BM angiogenesis (microvessel area ≥ 0.90 mm\textsuperscript{2} x 10\textsuperscript{-2} according to the present study) can be eligible for antiangiogenic treatments, such as thalidomide,\textsuperscript{35} anti-VEGF compounds,\textsuperscript{36} and 2-methoxy-estradiol,\textsuperscript{37} to delay their progression and improve prognosis.

Overall, the data could be interpreted as pointing to a threshold of BM neovascularization above which the risk of progression increases. This association is consistent with that found in solid
tumors, and agrees with the stimulatory effect of neovessels on tumor growth, since they convey oxygen and metabolites, and endothelial cells secrete growth factors for tumor cells, including those of B-cell lineage.\textsuperscript{38}

The level of BM angiogenesis in hematologic cancers (as in other tumors) is a complex process related to an interaction of an array of angiogenic and antiangiogenic factors released into the microenvironment and of cell populations of tumor and host stromal origin with angiogenic and antiangiogenic activities.\textsuperscript{39} It is the final product of these interactions. Our data showing that B-cell CLL expression of VEGF was correlated with microvessel area and number suggest that tumor VEGF prevails over antiangiogenic factors/activities and is a substantial angiogenic factor in BM of B-cell CLL. The expression of VEGF by B-cell CLL due to activation of some oncogenes including c-myc, c-fos, ets-1\textsuperscript{40-42} may switch the avascular to the vascular phase and thus contribute to disease progression and a poor prognosis. Also, some inflammatory stromal cells which stained with VEGF might be synergistic with B-cell CLL in the induction of the vascular phase.

Studies on the correlation between tumor angiogenesis and sVEGF are circumstantial.\textsuperscript{3} We found no correlation, in agreement with studies on multiple myeloma.\textsuperscript{43} Therefore, different from the tumor expression of VEGF in the BM, sVEGF levels cannot be a marker of BM angiogenesis. The presence of many cell sources of sVEGF, such as stromal cells, platelets, and other endothelia, may account for this observation. However, much in the same way as the microvessel area, the sVEGF had prognostic significance. sFGF-2 was not of prognostic interest.
Although genomic aberrations as evaluated by the FISH technique retain prognostic value in early CLL, we found no correlation with the microvessel area. We consider that this correlation is probably lacking because of the limited number of patients investigated. However, it may well be that aberrations and enhanced angiogenesis reflect different biological events which are not inter-related, thus providing a biological basis of clinical variability of early CLL as to the disease progression.

In conclusion, our study tentatively suggests that angiogenesis is both a sizeable component of the BM in B-cell CLL and a marker of the risk of progression in early disease. Longitudinal studies are warranted to know whether the biological evolution of early B-cell CLL is prevascular and vascular. This will provide a rationale for the fast use of conventional therapy and/or antiangiogenic agents in patients with early B-cell CLL who are likely to progress and finally die of their disease.

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REFERENCES


**Table 1. Bone marrow microvessel areas and counts**

<table>
<thead>
<tr>
<th>Subjects (no.)</th>
<th>Microvessel area as mm$^2 \times 10^{-2}$</th>
<th>Microvessel number in 12.5 mm$^2 \times 10^{-2}$ fields</th>
<th>Cellular area as mm$^2 \times 10^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± 1 SD (median, range)</td>
<td>Mean ± 1 SD (median, range)</td>
<td>Mean ± 1 SD (median, range)</td>
</tr>
<tr>
<td>Patients (45)</td>
<td>0.96 ± 0.83*° (0.70, 0.45 – 4.12)</td>
<td>7.39 ± 7.11* (5, 3 – 35)</td>
<td>10.8 ± 1.7 (8.6, 8.7 – 11.5)</td>
</tr>
<tr>
<td>Controls (12)</td>
<td>0.09 ± 0.01° (0.09, 0.06 – 0.12)</td>
<td>0.66 ± 0.98 (0, 0 – 3)</td>
<td>9.7 ± 0.9 (7.8, 6.8 – 10.7)</td>
</tr>
</tbody>
</table>

P < 0.001 (analysis of variance by Fisher and Kruskal-Wallis tests followed by paired Duncan [t], Bonferroni [t] and Wilcoxon tests), or P < 0.0001 (Mann-Witney test) vs. controls.

°Microvessel area normalized to the cellular area: patients, 8.9% ± 7.6 (7.4%, 4.1 – 35.8%); controls, 0.92% ± 0.1 (0.89%, 0.88 – 1.52%).
Table 2. Risk of progression as progression-free survival

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cutoff</th>
<th>Relative risk</th>
<th>P-value</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>(95% confidence interval)</td>
<td>Univariate</td>
<td>Multivariate</td>
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<tr>
<td>Age</td>
<td>65 yrs*</td>
<td>0.86</td>
<td>n.a.</td>
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<tr>
<td>Sex</td>
<td>M/F</td>
<td>0.49</td>
<td>n.a.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microvessel area</td>
<td>0.90 (mm$^2 \times 10^{-2}$)**</td>
<td>0.03</td>
<td>n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microvessel number</td>
<td>6 (per 12.5 mm$^2 \times 10^{-2}$ fields)**</td>
<td>0.83</td>
<td>n.a.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB lymphocytes</td>
<td>21.3 (x 10$^9$/L)*</td>
<td>0.73</td>
<td>n.a.</td>
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<tr>
<td>BM histology</td>
<td>nondiffuse/diffuse</td>
<td>0.05</td>
<td>n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDH</td>
<td>478 (U/L)*</td>
<td>3.303</td>
<td>0.01</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.22-8.88)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-2m</td>
<td>2.7 (ng/mL)*</td>
<td>2.5751</td>
<td>0.04</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.96-6.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocyte doubling time</td>
<td>12 months</td>
<td>0.006</td>
<td>n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SVEGF</td>
<td>218 (pg/mL)*</td>
<td>0.04</td>
<td>n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sFGF-2</td>
<td>36.6 (pg/mL)*°</td>
<td>0.25</td>
<td>n.a.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The cutoffs are the median (*) or the 75th percentile (**).

n.a. = not applied; n.s. = not significant.

°The sFGF-2 levels were significantly higher than in controls (median = 2 pg/mL, range = 2-37; P < 0.00001, Mann-Whitney’s test).

P-value by the Cox model.
Table 3. Characteristics of the patients grouped according to the extent of bone marrow microvessel area

<table>
<thead>
<tr>
<th>Variable*</th>
<th>Microvessel area</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 0.90 mm$^2 \times 10^{-2}$</td>
<td>≥ 0.90 mm$^2 \times 10^{-2}$</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>65 (42-74)</td>
<td>62 (45-81)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>21/12</td>
<td>8/4</td>
</tr>
<tr>
<td>Rai substages 0</td>
<td>21</td>
<td>6</td>
</tr>
<tr>
<td>I-II</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>PB lymphocytes (x 10$^9$/L)</td>
<td>20.7 (5-100)</td>
<td>22.5 (5-75)</td>
</tr>
<tr>
<td>BM histology (nondiffuse/diffuse)</td>
<td>32/1</td>
<td>12/0</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>431 (129-953)</td>
<td>486 (36-931)</td>
</tr>
<tr>
<td>β-2m (ng/mL)</td>
<td>2.66 (1.6-8.0)</td>
<td>3.20 (1.8-7.25)</td>
</tr>
<tr>
<td>Lymphocyte doubling time ≥ 12 months</td>
<td>72.7%</td>
<td>83.3%</td>
</tr>
<tr>
<td>sVEGF (pg/mL)</td>
<td>218 (9-1120)</td>
<td>168 (36-2000)</td>
</tr>
<tr>
<td>sFGF-2 (pg/mL)</td>
<td>35.9 (6-206)</td>
<td>36.8 (2-142)</td>
</tr>
<tr>
<td>Cytogenetics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal karyotype</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>13q as a sole aberration</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>12q trisomy</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>11q or 17p deletion</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

*Variables were assessed in all 45 patients except those of lymphocyte doubling time and cytogenetics, which were assessed in 38 and 28 patients respectively.
LEGENDS

**Fig. 1** - Progression-free survival according to the microvessel area cutoff. Curves were plotted with the Kaplan-Meier method and statistically compared with the log-rank test. (hazard risk = 0.419; 95% confidence interval = 0.111-0.983). Number of patients between brackets.

**Fig. 2** - Staining of B-cell CLL bone marrow with FVIII-RA (x250 fields). A), B), C) Patients with microvessel area and number ≥ 75th percentile. Note in B) an enormously dilated neovessel, and in C) numerous microaneurysmatic dilations of neovessels (arrowheaded). D), E) Patients with lower area and number. Note in D) straight, not branched neovessels (arrowheaded), and in E) a tumor nodule devoid of neovessels. F) A control subject with anemia due to vitamin B12 deficiency. In D), E), F), megakaryocytes strongly stained with the FVIII-RA are recognizable. G) Staining of bone marrow B-cell CLL with VEGF (x250 fields) in the same patient as in B), and H) in the same patient as in D).

**Fig. 3** - Progression-free survival of Rai substaged patients according to the microvessel area. Curves were plotted with the Kaplan-Meier method and statistically compared with the log-rank test. Rai 0 curves: hazard risk = 0.22; 95% confidence interval = 0.0181-0.740. Rai I-II curves: hazard risk = 0.373; 95% confidence interval = 0.048-1.024. Number of patients between brackets.

**Fig. 4** - Bone marrow angiogenesis, evaluated as microvessel area and number, in comparison with VEGF staining intensity, evaluated as a gray score (mean value per biopsy). Significance by Pearson’s regression analysis.

**Fig. 5** - Bone marrow angiogenesis, evaluated as microvessel area, in comparison with karyotypic aberrations. The box plots compare median values of microvessel area in 4 cytogenetic categories.
(P = .80 by Kruskal-Wallis test). Vertical lines express the 95% confidence interval. Number of patients between brackets.
Fig. 1

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FIGURE 3
FIGURE 4
Microvessel area

\( \text{mm}^2 \times 10^{-2} \)

Normal karyotype
\((n=13)\)

13q deletion
\((n=6)\)

12q trisomy
\((n=8)\)

11q deletion or 17p deletion
\((n=2)\)

\( P=0.80 \)

(Kruskal-Wallis test)

Fig 5

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Prognostic value of enhanced bone marrow angiogenesis in early B-cell chronic lymphocytic leukemia

Stefano Molica, Angelo Vacca, Domenico Ribatti, Antonio Cuneo, Francesco Cavazzini, Domenico Levato, Gaetano Vitelli, Luigi Tucci, Aldo M Roccaro and Franco Dammacco