

Circulating vascular endothelial growth factor (VEGF) and its soluble receptors in patients with chronic lymphocytic leukemia

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ABSTRACT. The vascular endothelial growth factor (VEGF) transduction pathway may be very active in B-cell chronic lymphocytic leukemia (B-CLL) cells and contributes to their enhanced survival. Vascular endothelial growth factor receptor-1 (VEGFR-1) and receptor-2 (VEGFR-2), are the high-affinity VEGF receptors, which play an important role in *de novo* blood vessel formation and hematopoietic cell development. The aim of our study was to compare the concentration of VEGF, VEGFR-1 and VEGFR-2 in the serum of 83, never-treated B-CLL patients in different stage of disease according to Rai classification, and 20 healthy volunteers. Of all the cytokines only the serum concentration of VEGF was found to be significantly higher in the CLL group when compared to the control group (median 468.2 pg/mL and 246.9 pg/mL, respectively) ($p = 0.01$). In the group of CLL patients, the serum concentrations of VEGF and VEGFR-2 were significantly higher in patients in Rai stage III and IV (median 890.0 pg/mL and 4680.4 pg/mL respectively) than in patients in Rai stage 0-II (347.8 pg/mL and 2411.6 pg/mL respectively) ($p < 0.0001$). In the entire group of CLL patients, we have found a strong, positive correlation between the serum level of VEGF and VEGFR-2 ($p = 0.00001$, $R = 0.46$). We have also found a positive correlation between the number of lymphocytes in the peripheral blood of CLL patients and the level of VEGF ($p = 0.05$, $R = 0.24$) and VEGFR2 ($p = 0.02$, $R = 0.29$). In conclusion: VEGF and VEGFR-2, but not VEGFR-1, may have an important influence on the course of B-CLL.

Keywords: angiogenesis, VEGF, VEGFR-1, VEGFR-2, chronic lymphocytic leukemia

Many studies indicate that angiogenesis may play an important role in the pathogenesis of hematological malignancies, including lymphoma, myeloma and leukemia [1-4]. Evidence of the role of this process in chronic lymphocytic leukemia (CLL) originates from the finding that increased vessel density was observed in the bone marrow of CLL patients [5]. However, this observation has not been confirmed by Aguayo *et al.* [6]. Angiogenesis is regulated by many substances with pro-angiogenic activity, and a number of inhibitors. One of the most important is vascular endothelial growth factor (VEGF), which is a potent angiogenic, mitogenic and vascular permeability stimulator [7]. It is known that VEGF plays an important role in CLL. The VEGF transduction pathway may be very active in B-cell chronic lymphocytic leukemia (B-CLL) cells and contributes to their enhanced survival. Three receptor tyrosine kinases have been described as putative VEGF receptors: VEGFR-1 or Flt-1 (fms-like tyrosine kinase), VEGFR-2 or Flk-1/KDR (kinase-insert-domain-containing receptor) and VEGFR-3 or Flt-4 (fms-like tyrosine kinase). They are all high-affinity VEGF receptors, playing an important role in *de novo* blood vessel formation and hematopoietic cell development [8-10]. It is known that all three receptors are expressed in CLL cells

[11, 12]. To the best of our knowledge, the serum concentrations of soluble VEGFR-1 and VEGFR-2 in CLL patients have not been investigated to date.

The aim of our study was to compare the serum concentrations of VEGF and its receptors VEGFR-1 and VEGFR-2, between healthy blood donors and a group of never-treated CLL patients. We also wanted to know if the levels of these factors correlate with the stage of the disease and probability of positive treatment response.

PATIENTS AND METHODS

Patients

The study involved 83, consecutive B-CLL patients and 20 healthy volunteers. All CLL patients fulfilled the National Cancer Institute – Sponsored Working Group diagnostic criteria for CLL [13]. There were 52 male patients and 31 female patients with a mean age of 68 years (range 43-78). According to Rai's classification [14], 25 patients were in 0 stage, 15 in I, 10 in II, 20 in III and 13 in IV stage of the disease. All patients included in this study were previously untreated. Characteristics of the patients at the time of sampling are shown in *table 1*. Thirty of the 83 patients

Table 1
Patient characteristics

Number of patients	83
Age in years: median (range)	68 (43-78)
Sex (male/female)	52/31
Rai stage	
0	25
I	15
II	10
III	20
IV	13
Hemoglobin (g/l): median (range)	125 (75-145)
WBC ($\times 10^9/L$): median (range)	105.0 (15.0-211.0)
Lymphocytes (%): median (range)	92.0 (10.5-199.7)
PLT ($\times 10^9/L$): median (range)	180.0 (40.0-280.0)
BM lymphocytosis (%): median (range)	80 (40-91)
Disease duration (months): median (range)	72 (10-140)

WBC – white blood cells, PLT – platelets, BM – bone marrow.

received treatment after the serum had been collected. Characteristics of this group of patients are shown in table 2.

An assessment of history and a physical examination was performed as initial diagnostic procedures. The laboratory tests included: complete blood count, immunoglobulin level, liver and renal function tests, bone marrow aspiration for morphology and immunophenotyping. Surface marker analysis was performed to confirm B-cell origin and monoclonal proliferation, including immunoglobulins heavy- and light-chains, CD5, CD10, CD19, CD20 and CD23.

The controls included 11 men and 9 women, with a mean age of 53 years (range 39-70).

This project was performed in accordance with the Helsinki Declaration. Informed consent was obtained from all patients participating in the study. The local Ethics Committee approved the project.

Table 2
Treated patients characteristics

Number of patients	30
Rai stage	
II	2
III	18
IV	10
Chemotherapy regimen	
Chlorambucil	12
2-CdA	8
CC	10
Treatment response	
CR	8
PR	12
NR	10
Follow up (months)	
Median (range)	13 (8-20)

2-CdA- Cladribine (2-chlorodeoxyadenosine), CC-cyclophosphamide and 2-CdA, CR – complete remission, PR – partial remission, NR – no remission.

Treatment schedule

Patients were treated with 2-chlorodeoxyadenosine (Cladribine, 2-CdA) alone or in combination with cyclophosphamide or with chlorambucil and prednisone [15]. 2-CdA was given at a dose of 0.12 mg/kg in 2-hour intravenous infusion for 5 consecutive days. Patients treated additionally with cyclophosphamide received this drug on the first day of the cycle, at a dose of 650 mg/m², intravenously. Chlorambucil was given orally, at a dose of 12 mg/m² and prednisone at 30 mg/m², for 7 consecutive days. All types of cycles were repeated over 28 days, up to six cycles. In patients in whom treatment induced hematological complications (thrombocytopenia < 50x10⁹/L) or severe infections developed, the drug was readministered at time intervals longer than 1 month, ranging from 2 to 4 months, until the increase of haematological parameters or recovery from infections were noted. Patients were treated until maximal response or prohibitive toxicity. If no response or progression of the disease was observed after 3 courses, the treatment was discontinued.

Response criteria

Response criteria were those recommended by the NCI Sponsored Working Group [13]. Complete response (CR) required the absence of symptoms and organomegaly, and the return to normal of the blood count with a granulocyte count greater than 1.5x10⁹/L, thrombocyte count greater than 100x10⁹/L, and hemoglobin concentration greater than 11.0 g/dL, as well as a bone marrow lymphocyte percentage less than 30% in aspiration and biopsy material, for at least 2 months. Partial response (PR) was indicated by a more than 50% decrease in the size of lymph nodes, liver and spleen, and peripheral blood findings either identical to those of CR or improved over pretherapy values by at least 50%.

Serum sampling and cytokine determination

The serum obtained was stored at – 80°C until assayed for cytokines. The cytokines serum concentrations were assayed by specific, commercially available, enzyme linked (ELISA) assay kits. The kits for VEGF, VEGFR-1 and VEGFR-2 were the products of R&D Systems Inc., Minneapolis, U.S.A. Standards and samples were assayed as duplicates, and interassay variations were within the range given by the manufacturer. Assay sensitivity was 5.0 pg/mL for VEGF and VEGFR-1, 4.6 pg/mL for VEGFR-2. The procedure has been previously described in detail [16].

Statistical analysis

Comparisons of values were made with Mann-Whitney U tests. The linear correlations between serum cytokine or receptors levels with each other or with the other factors were evaluated using the Spearman rank-sum correlation method. To verify whether cytokine or receptor concentration impact remission achievement, the patients were divided into “high-expressers” (concentrations ≥ median) and “low-expressers” (concentrations < median). The Fisher test was used to compare proportions of remissions in these groups. Comparison and correlation were considered significant when p < 0.05.

RESULTS

The serum concentrations of VEGF, VEGFR-1 and VEGFR-2 were measurable in all normal individuals and all CLL patients. The results of the measurements of VEGF and its receptors are shown in *table 3*.

The serum concentration of VEGF was found to be significantly higher in the CLL group when compared to the control group (median 468.2 pg/mL and 246.9 pg/mL, respectively) ($p = 0.01$). The serum concentration of both VEGFR-1 and VEGFR-2 did not differ significantly in the CLL group (median 41.3 pg/mL and 2975.4 pg/mL respectively) when compared to the control group (median 48.7 pg/mL and 2471.4 pg/mL, respectively) ($p = 0.6$ and $p = 0.2$). In the group of CLL patients, the serum concentrations of VEGF and VEGFR-2 were significantly higher in patients in Rai stage III and IV (median 890.0 pg/mL and 4680.4 pg/mL respectively) than in patients in Rai stage 0-II (347.8 pg/mL and 2411.6 pg/mL respectively) ($p = 0.001$ and $p = 0.0002$). The serum concentration of VEGFR-1 did not differ between patients in Rai stage 0-II and patients in Rai stage III-IV (median 47.2 pg/mL and 40.1 pg/mL respectively) ($p = 0.06$). In the entire group of CLL patients, we found a strong, positive correlation between the serum concentrations of VEGF and VEGFR-2 ($p = 0.00001$, $R = 0.46$) (*figure 1*). We also found a positive correlation between the number of lymphocytes in the peripheral blood of CLL patients and the level of VEGF ($p = 0.05$, $R = 0.24$) and VEGFR-2 ($p = 0.02$, $R = 0.29$) (*figure 2*).

In this study, 30/83 CLL patients (36.2%) received chemotherapy. Characteristics of this group of patients are shown in *table 2*. Twenty patients (67%) have responded to treatment and achieved complete (CR) or partial (PR) response. To verify whether cytokine or receptor concentration impact achievement of remission, the patients were divided into “high-expressers” (concentrations \geq median) and “low-expressers” (concentrations $<$ median). There were 15 high and 15 low expressers of VEGF (median 705.2 pg/mL), 12 high and 18 low expressers of VEGFR-1 (median 38.5 pg/mL), 14 high and 16 low-expressers of VEGFR-2 (median 4520.5 pg/mL). No significant differences in the proportion of patients who achieved response to treatment (PR or CR) were detected as regards high and low expression of VEGF, VEGFR-1 and VEGFR-2 ($P = 0.37$, $p = 0.51$ and $p = 0.35$ respectively).

DISCUSSION

In this study we have shown that soluble VEGF receptors (both VEGFR-1 and VEGFR-2) are detectable in the serum of B-CLL patients. The results of our study have revealed that the concentration of VEGFR-2, but not VEGFR-1, is significantly higher in the group of patients with more advanced disease (Rai III and IV) as compare to patients with Rai 0, I and II.

Most studies have shown that VEGFR-2 is the critical receptor for transmitting cellular signals for the proliferation and differentiation of endothelial cells, whereas VEGFR-1 may be more important for vascular remodeling and monocyte migration [17-19]. The up-regulation of VEGF receptors have been observed in many solid tumors [20, 21]. Recently, several studies have shown that VEGF receptors are expressed by certain human leukemias [11, 22]. Bellamy *et al.* revealed that VEGF receptors expressed on leukemic cells are functional and convey their signals such as increasing proliferation, metalloproteinase activation and transbasement membrane migration [23]. Aguayo *et al.* and Ferrajoli *et al.* first determined, using western blot analysis, that VEGFR-1 and VEGFR-2 are present in peripheral blood CLL cells [11, 24]. Bairey *et al.* confirmed these data using flow cytometry analysis [12]. Ferrajoli have found that elevated VEGFR-2 protein levels were associated with higher lymphocyte counts, severe anemia, elevated beta2 microglobulin, advanced-stage disease and statistically significantly shorter survival, which is partly (lymphocyte count and stage of the disease) in accordance with our results regarding serum levels of soluble VEGFR-2. In contrast, Wierzbowska *et al.* revealed that the plasma concentration of VEGFR-1, but not VEGFR-2, is higher in acute leukemia patients and correlates with tumour burden and poor prognosis [25].

It is known that CLL marrow vascularity is enhanced and positively related to advancing stages of the disease [5, 26]. It is also known that CLL is characterised by the accumulation of mature B-cells, which are unable to undergo apoptosis. Therefore, the interactions between VEGF and its receptors are of special interest in this disease [27].

We have found that the level of VEGF was significantly higher ($p = 0.01$) in the group of CLL patient as compared to the healthy control group. Moreover, among the CLL patients, the level of this cytokine was significantly higher in more advanced stages of the disease ($p = 0.0001$). In

Table 3

Comparison of serum levels of VEGF and its receptors VEGFR-1 and VEGFR-2 in the healthy control group and 83 untreated CLL patients

Cytokine/Receptor	Control (n=20)	All CLL patients (n=83)	Rai 0-II (n=50)	Rai III-IV (n=33)	Statistical signification: p value
	A	B	C	D	
VEGF pg/mL median (range)	246,9 (48,7-1087,4)	468,2 (50,2-2937-4)	347,8 (68,4-2937,4)	890,0 (54,9-1700-0)	AvsB: 0,01; CvsD: 0,001
VEGF-R1 pg/mL median (range)	48,7 (24,1-214,8)	41,3 (20,5-90,7)	47,2 (20,5-82,3)	40,1 (21,3-90.7)	AvsB: 0,6; CvsD: 0,06
VEGF-R2 pg/mL median (range)	2471,4 (1676,5-5888,0)	2975,4 (1369,5-6251,5)	2411,6 (1369,5-4981,0)	4680,4 (1928,2-6251,5)	AvsB: 0,2; CvsD: 0,0002

The difference is significant for $p < 0.05$.

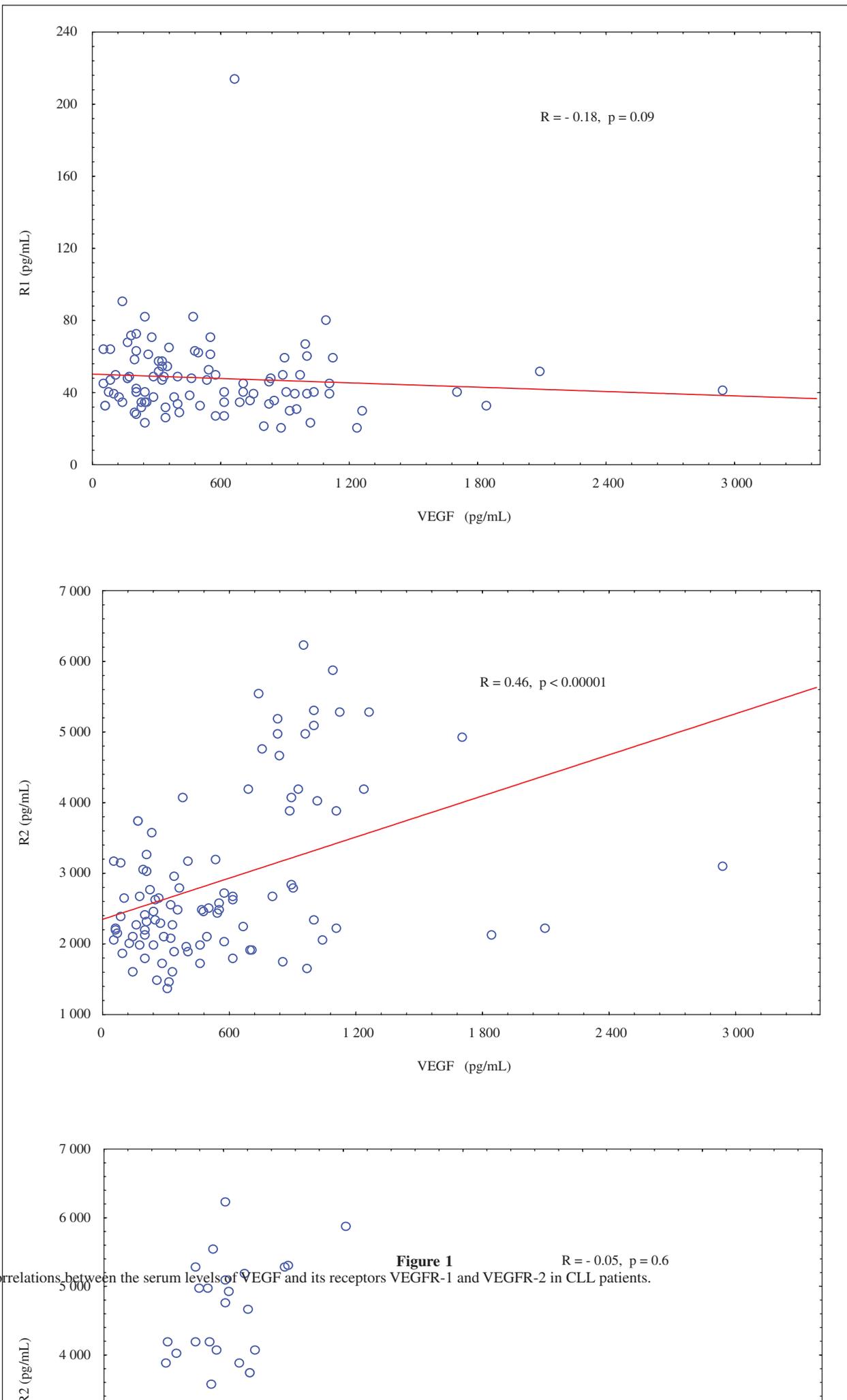


Figure 1

Correlations between the serum levels of VEGF and its receptors VEGFR-1 and VEGFR-2 in CLL patients.

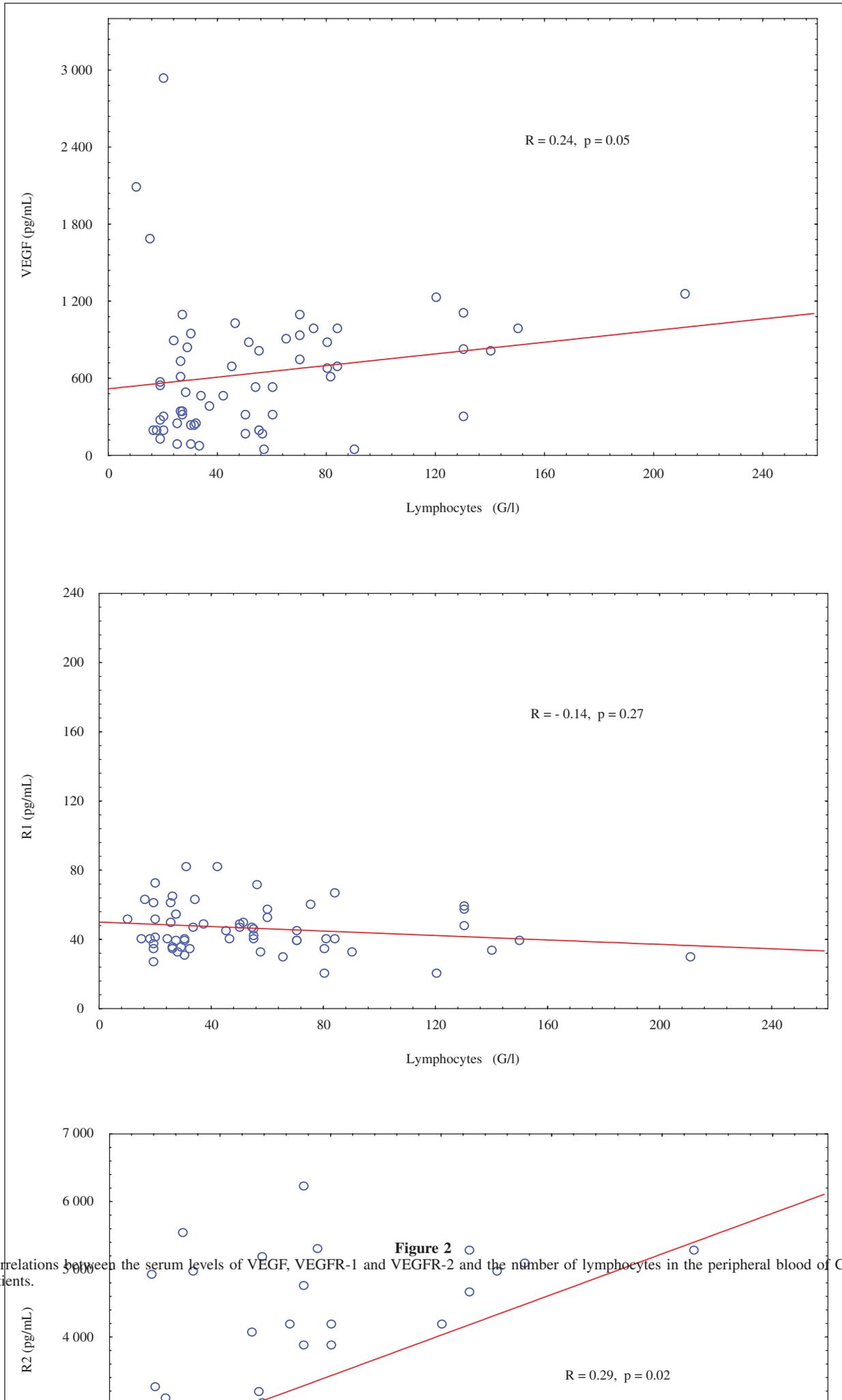


Figure 2

Correlations between the serum levels of VEGF, VEGFR-1 and VEGFR-2 and the number of lymphocytes in the peripheral blood of CLL patients.

contrast, Aquayo *et al.* revealed that increased levels of intracellular VEGF correlated with a less aggressive course of the disease in CLL [28]. There is evidence that the CLL cells themselves may be an important source of VEGF [29]. On the other hand, it is known that VEGF is produced by many normal tissues eg macrophages, fibroblasts, endothelial cells [7]. Therefore, the serum concentration of VEGF depends on all these different sources and it may not reflect intracellular concentration in a simple way. This could explain why our results are different from those reported by Aguayo *et al.* [28].

In our group of CLL patients we found a strong, positive correlation between the serum levels of VEGF and VEGFR2 ($p = 0.00001$, $R = 0.46$). Our results suggest that the signalling pathway enhanced by the VEGF/VEGFR-2 interaction is essential for the course of CLL, at least for more advanced stages of the disease. This could have important clinical implications. Dias *et al.* found, using an *in vivo* mice model of human leukemia xenografts, that inhibition of the VEGF/VEGFR-2 signalling pathway induced long-term remission [30].

In conclusion, the serum levels of VEGF and VEGFR-2, but not VEGFR-1, were significantly higher in more advanced CLL patients, and may have an important influence on the course of B-CLL.

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REFERENCES

- Bertolini F, Mancuso P, Gobbi A, Pruneri G. The thin red line: angiogenesis in normal and malignant hematopoiesis. *Exp Hematol* 2000; 28: 993.
- Kay NE, Jelinek DF, Peterson L. Angiogenesis in B-chronic lymphocytic leukemia. *Leuk Res* 2001; 25: 709.
- Kumar S, Witzig TE, Timm M, *et al.* Expression of VEGF and its receptors by myeloma cells. *Leukemia* 2003; 17: 2025.
- Góra-Tybor J, Blonski J, Robak T. Circulating proangiogenic cytokines and angiogenesis inhibitor endostatin in untreated patients with chronic lymphocytic leukemia. *Mediat Inflamm* 2003; 12: 167.
- Kini AR, Kay NE, Peterson LC. Increased bone marrow angiogenesis in B-cell chronic lymphocytic leukemia. *Leukemia* 2000; 14: 1414.
- Aguayo A, Kantarjian H, Manshour T, *et al.* Angiogenesis in acute and chronic leukemias and myelodysplastic syndromes. *Blood* 2000; 96: 2240.
- Veikkola T, Karkkainen M, Claesson-Welsh L, Alitalo K. Regulation of angiogenesis via vascular endothelial growth factor receptors. *Cancer Res* 2000; 60: 203.
- Terman BI, Dougher-Vermazen M, Carrion ME, *et al.* Identification of KDR tyrosine kinase as a receptor for vascular endothelial growth factor. *Bioch Biophys Res Comm* 1992; 187: 1579.
- De Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 1993; 255: 989.
- Klagsbrun M, D'Amore PA. Vascular endothelial growth factor and its receptor. *Cytokine Growth Factor Rev* 1993; 7: 259.
- Aguayo A, Manshour T, O'Brien S, *et al.* Clinical relevance of Flt1 and Tie1 angiogenesis receptors expression in B-cell chronic lymphocytic leukemia (CLL). *Leuk Res* 2001; 25: 279.
- Bairey O, Boycov O, Kaganovsky E, Zimra Y, Shaklai M, Rabizadeh E. All three receptors for vascular endothelial growth factor (VEGF) are expressed on B-chronic lymphocytic leukemia (CLL) cells. *Leuk Res* 2004; 28: 243.
- Cheson BD, Bennett JM, Grever M, *et al.* National Cancer Institute – Sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. *Blood* 1996; 87: 4990.
- Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS. Clinical staging of chronic lymphocytic leukemia. *Blood* 1975; 46: 219.
- Robak T, Błoński JZ, Kasznicki M, *et al.* Cladribine with prednisone versus chlorambucil with prednisone as first-line therapy in chronic lymphocytic leukemia: report of a prospective, randomized, multicenter trial. *Blood* 2000; 96: 2723.
- Robak E, Sysa-Jędrzejewska A, Robak T. Vascular endothelial growth factor and its soluble receptors VEGFR-1 and VEGFR-2 in the serum of patients with systemic lupus erythematosus. *Mediat Inflamm* 2003; 12: 293.
- Dias S, Hattori K, Zhu Z, Heissig B, *et al.* Autocrine stimulation of VEGFR-2 activates human leukemic cell growth and migration. *J Clin Invest* 2000; 106: 511.
- Usui T, Ishida S, Yamashiro K, *et al.* VEGF164(165) as the pathological isoform: differential leukocyte and endothelial responses through VEGFR1 and VEGFR2. *Invest Ophthalmol Vis Sci* 2004; 45: 368.
- Karkkainen MJ, Petrova TW. Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis. *Oncogene* 2000; 1: 5598.
- Dales JP, Garcia S, Carpentier S, *et al.* Prediction of metastasis risk (11 year follow up) using VEGF-R1, VEGF-R2, Tie-2/Tek and CD105 expression in breast cancer. *Br J Cancer* 2004; 22: 1216.
- Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ, Dvorak HF. Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in kidneys and bladder carcinomas. *Am J Pathol* 1993; 143: 1255.
- Belgore FM, Lip GY, Bareford D, Wadley M, Stonelake P, Blann AD. Plasma levels of vascular endothelial growth factor (VEGF) and its receptor, Flt-1, in hematological cancers: a comparison with breast cancer. *Am J Hematol* 2001; 105: 412.
- Bellamy WT, Richter L, Frutiger Y, Grogan TM. Expression of vascular endothelial growth factor and its receptors in hematopoietic malignancies. *Cancer Res* 1999; 59: 728.
- Ferrajoli A, Manshour T, Estrov Z, *et al.* High levels of vascular endothelial growth factor receptor-2 correlate with shortened survival in chronic lymphocytic leukemia. *Clin Cancer Res* 2001; 7: 795.
- Wierzbowska A, Robak T, Wrzesień-Kuś A, Krawczyńska A, Lech-Marańda E, Urbańska-Ryś H. Circulating VEGF and its soluble receptors sVEGFR-1 and sVEGFR-2 in patients with acute leukemia. *Eur Cytokine Netw* 2003; 14: 149.
- Molica S, Vacca A, Ribatti D, Cuneo A, Cavazzini F, Levato D. Prognostic value of enhanced bone marrow angiogenesis in early B-cell chronic lymphocytic leukemia. *Blood* 2002; 100: 3344.
- Kay NE. The angiogenic status of B-CLL B cells: role of the VEGF receptors. *Leuk Res* 2004; 28: 221.
- Aguayo A, O'Brien S, Keating M, *et al.* Clinical relevance of intracellular vascular endothelial growth factor levels in B-cell chronic lymphocytic leukemia. *Blood* 2000; 96: 768.
- Kay NE, Bone ND, Tschumper RC, *et al.* B-CLL cells are capable of synthesis and secretion of both pro- and anti-angiogenic molecules. *Leukemia* 2002; 16: 911.
- Dias S, Hattoti K, Heissig B, *et al.* Inhibition of both paracrine and autocrine VEGF/VEGFR-2 signaling pathways is essential to induce long-term remission of xenotransplanted human leukemias. *Proc Natl Acad Sci USA* 2001; 98: 10857.