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# Prognostic Significance of VEGF and bFGF in Egyptian CLL Patients

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**ABSTRACT**
*Though an impressive number of prognostic factors have been identified in chronic lymphocytic leukemia (CLL), which exhibits a high variability in its clinical presentation and course, many of them have not yet been applied in routine clinical practice. Among these factors are the angiogenic factors, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), previously reported as prognostic markers for disease progression. The objective of the present study was to assess the impact of serum VEGF and bFGF levels at diagnosis in Egyptian CLL patients and their relationship with disease prognostic parameters and patients' outcome, aiming to apply them in routine clinical practice. For this purpose, 45 newly diagnosed CLL patients and 25 healthy controls were recruited. VEGF and bFGF were found to be significantly higher in the CLL group when compared to the control group (P <0.001). Moreover, the serum concentrations of VEGF and bFGF were significantly higher in CLL patients in progressive disease state (Rai stage III and IV) and in patients with positive ZAP70 and CD38 expression. A cutoff point of >320 pg/ml for VEGF and >145 pg/ml for bFGF was able to predict poor prognosis (treatment failure) in CLL patients with prognostic accuracies of 96.7% and 89.10% respectively.*
*In conclusion: VEGF and bFGF represent powerful prognostic markers in B-CLL, partially because of the simplicity of obtaining their samples and their assays procedure, the reliability of their assessment besides being optimal targets for potential novel therapies in conjunction with classical therapies.*
**Keywords**: *VEGF, bFGF, CLL, Prognostic factors, angiogenesis.*

## INTRODUCTION

As its clinical course is markedly heterogeneous, novel prognostic factors are being considered in order to identify high-risk chronic lymphocytic leukemia (CLL) patients as early as possible in a trial to optimize their treatment. CLL is characterized by the accumulation of monoclonal small CD5 and CD19 positive small lymphocytes, which is mainly attributed to delayed senescence, prolonged life span, and resistance to apoptosis of these cells [1], [2].

Dysregulation of angiogenesis, the production of new micro vessels, is one of the hallmarks of many malignant neoplasms. Emerging evidences suggest that angiogenic signaling pathways play an important role in the pathophysiology of CLL probably through abnormal neovascularization of the bone marrow microenviroment [3]. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have been identified as two of the most potent pro-angiogenic factors involved in CLL[1],[4],[5],[6]. VEGF was reported to stimulate angiogenesis and vascular permeability through its interaction with vascular endothelial growth factor receptors[3]. Meanwhile, bFGF has been implicated as a survival factor for a variety of cells, mediating both proliferation and cell survival, probably through the inhibition of some proapoptotic proteins. Thus, it has been postulated that both VEGF and bFGF are implicated in promoting the survival of CLL cells, conferring resistance to treatment and poor prognosis [7],[8]. Therefore, we aimed to investigate the impact of assessing serum VEGF and bFGF levels at diagnosis in Egyptian CLL patients and their relationship with disease prognostic parameters and patients' outcome.

## SUBJECTS AND METHODS

This study included 48 newly diagnosed adult B-CLL patients admitted to and following up at the clinical hematology, oncology and bone marrow transplant unit, Ain Shams University hospitals in the period from January 2012 to February 2015. Patients' diagnosis, management and follow up were performed according to the 2008 International workshop on CLL update of 1996 National Cancer Institute guidelines [9]. All enrolled patients had symptomatic and\or active disease necessitating therapy. Patients' characteristics were evaluated at diagnosis by history, physical examination, complete blood count using Coulter LH 750 analyzer, examination of Leishman-stained peripheral blood and bone marrow aspiration smears, Routine diagnostic flowcytometry panel for Lymphoproliferative disorders including CD5, CD19, CD20, CD23, CD25, CD79b, CD10, CD38, sIg, FMC7, κ and λ light chains, in addition to ZAP70 expression (by flowcytometry) (Cut off for positivity ≥20% of cells expressed that marker), and assessment of serum VEGF and bFGF levels using Enzyme Linked immunosorbant assays (RayBio Human VEGF and RayBio Human bFGF ELISA Kits, Ray Biotech, Inc.) according to the manufacturer's instructions.

**Treatment protocols:**

**Indications of treatment:**

1- Advanced Rai classification (Rai III or Rai IV)
2- Symptomatic patients (Rai I or Rai II)

**Chemotherapy Protocols:**

Patients were treated with one of the following regimens:

(1) FC- FC/R:

Fludarabine plus cyclophosphamide ± Rituximab repeated for up to six cycles.

(2) COP- COP/R

vincristine, cyclophosphamide and, prednisone ± rituximab repeated for up to eight cycles.

(3) Daily oral chlorambucil

chlorambucil for 3- 6 weeks or more according to clinical situation.

**Patients' follow up:**

The elapsed time between diagnosis and treatment initiation ranged from 0 to 3 months. Restaging was performed at 3 months intervals.

The patients were followed up after 1 year from start of therapy to assess response to treatment.

**Control group**

Twenty five age and sex matched normal individuals were chosen as control group for whom serum VEGF and bFGF levels were also measured.

Informed consent was obtained from all participant individuals. The study was conducted in accordance with the stipulations of the local ethical and scientific committees of Ain shams University and the procedures respected the ethical standards in Helsinki declaration of 1964.

**STATISTICAL ANALYSIS:**

Statistical analysis performed using SPSS V17.

Comparisons of quantitative and qualitative variables were conducted between groups using the student-t test and Chi-square tests respectively. In addition, correlations between quantitative variables within groups were performed using the Pearson correlation coefficient.

$p<0.05$ and $<0.001$ were set as statistically significant and highly significant respectively.

An operator characteristic (ROC) curve was constructed to establish clinically relevant cut off values for studied parameters with calculation of sensitivity, specificity, positive predictive value, negative predictive value and, diagnostic/prognostic accuracy.

## RESULTS

### Baseline characteristics

Out of the 48 CLL patients; 3 patients did not show up during the follow up period so they were excluded from the statistical analysis which was done on 45 patients. The mean age of the patients and controls was 58 ± 11 years and 49 ± 9 years respectively. All Patients enrolled in the study were classified according to modified Rai Clinical staging [9],[10], where group I included earlier disease stage (stage I, II Rai), and group II included advanced disease stage (stages III, IV Rai).

Concerning ZAP-70 and CD38 expression, 32/45 (71.1%) and 23/45 (51.1%) of the patients were positive, while 13/45 (28.9%) and 22/45 (48.9%) of the patients were negative respectively.

Patients were classified after 1 year of follow up according to response to treatment into clinically beneficial response group (those with partial or complete remission) and treatment failure group (those with stable disease, non response, progressive disease or death from any cause) (Table 5).

### Comparative analysis

There was a statistically significant higher levels of VEGF and bFGF in patients compared to controls (P-value<0.001) (Table1). In addition, higher VEGF and bFGF levels were found in group II patients, positive ZAP 70 and positive CD38 expression patients, as well as in treatment failure patients (P-value <0.001 for all) (Table 2, 3).

There was a highly statistically significant positive correlation between bFGF level and VEGF level (Figure 1), also VEGF showed a negative correlation with hemoglobin level and platelet count whereas bFGF showed a negative correlation with hemoglobin level (Table 4).

### Relation of outcome to studied parameters

There was a significant association of treatment failure state and higher VEGF and bFGF serum levels. Treatment failure was also found to be associated with lower hemoglobin level, group II patients, positive ZAP 70 and positive CD38 expression patients (Table 6)

### Prognostic performance characteristics of VEGF and bFGF using Receiver operator characteristics (ROC) curve analysis

A cutoff point of >320 pg/ml for VEGF and >145pg/ml for bFGF was able to predict treatment failure in CLL patients with prognostic accuracies of 96.7% and 89.10% respectively. (Table7) (Fig 2)

## DISCUSSION

CLL is characterized by an indolent course, with chronic lymphocytosis and progressive spleen and lymph nodes enlargement. CLL cells were shown to exhibit markedly distinct behaviours in the blood compared to those in the LNs, with proliferation occurring almost exclusively in the latter. These observations suggest that local microenvironments in the bone marrow, spleen or lymph nodes provide factors that support the growth and survival of the leukemic cells [1],[2],[3].

Pathological angiogenesis is reported to play a crucial role in the progression of solid as well as hematological malignancies, including CLL [1]. VEGF and bFGF have been postulated as the main pro-angiogenic factors contributing to the pathophysiology of CLL via their roles in activation and regulation of blood vessel growth and formation, activation of fibroblasts, and regulation of multiple endothelial cell functions which thus will favor CLL cells growth, survival and may be involved in drug resistance [1],[4],[5].

The current study was conducted to evaluate the clinical value of serum levels VEGF and bFGF at initial diagnosis as prognostic factors for CLL via investigating their correlations with other well-

established prognostic factors of biologic relevance mainly the CD38, ZAP-70 expression as well as indicators of CLL tumour burden, as clinical staging. In agreement with other previous studies [4],[5],10],[11], our results showed a statistically significant higher serum levels of both VEGF and bFGF among CLL patients compared to healthy controls ($p< 0.001$), which in turn enforces previous reports about the role of both VEGF and bFGF in CLL development or pathology.

Regarding the source of high serum levels of VEGF and bFGF in CLL, there is evidence that the CLL cells themselves may be an important source of VEGF and bFGF as reported by Kay and his colleagues [12], who observed increased VEGF and bFGF in the supernatant of CLL cells grown in vitro in addition to upregulation of mRNA encoding VEGF and bFGF. Moreover, Wrober et al. [8] found an association between the VEGF gene polymorphism responsible for high VEGF levels and thus susceptibility for CLL. On the other hand, normal tissues as endothelial cells and fibroblasts could be the source of these pro-angiogenic factors [6]. Thus, the increased serum concentrations of both VEGF and bFGF in CLL patients is multifactorial and depend on several different sources.

As the size of the leukemic cell population is regulated by the balance between its rate of proliferation and its rate of cell death by apoptosis, thus, any factor that delays apoptosis may create a survival advantage for the leukemic population. Both VEGF and bFGF are reported to delay apoptosis and prolong CLL cells survival by increasing expression of the anti-apoptotic molecule Bcl2 [1],[7]. In addition, bFGF was reported to inhibit the apoptosis signaling kinase I (ASKI), a pro-apoptotic protein [13]. In this context, we found that among CLL patients, the levels of VEGF and bFGF were significantly higher in more advanced stages of the disease with higher tumor burden (Rai stage III and IV) ($p = 0.0001$) which is in accordance with other previous studies [11],[14]. Similarly, Bairey and his associates reported that serum bFGF in CLL patients was positively correlated to Bcl-2 levels as well as to high white blood cell counts and progressive stages of the disease [7]. Moreover, Raheem found an association of VEGF expression in the bone marrow sections by immunohisto-chemistry and advanced disease of CLL patients with significant replacement of bone marrow hemopoietic elements by lymphoid cells [15].

Likewise, both VEGF and bFGF levels in our current study showed a statistically significant association with both ZAP-70 expression and CD38 expression, which are known poor prognostic indicators in CLL. Thus, the increased levels of VEGF and bFGF may provide a rationale for CLL disease progression and poor clinical prognosis. This was in accordance with what was previously reported by Molica et al. [16], who found that high VEGF serum levels, but not bFGF levels, correlated positively with CD38 expression, ZAP-70 expression and mutational status of IgVH. However, on the contrary, Smolej et al [17] found significantly increased level of VEGF in untreated B-CLL patients negative for ZAP-70. These results could be influenced by many variables including limited number of patients and differences in the determination of either ZAP-70 expression, VEGF and/or bFGF levels.

On exploring the relation of VEGF and bFGF levels to the outcome of the CLL, a statistically significant association of high VEGF and bFGF levels was found among treatment failure group, with a cutoff points of >320 pg/ml for VEGF and >145pg/ml for bFGF. This is in context with the findings of Shanafelt et al., [18] who documented that a relationship exists between pretreatment serum angiogenic cytokine levels and response to chemoimmunotherapy in CLL patients. Specifically, a pro-angiogenic phenotype (higher pro-angiogenic factors and lower anti-angiogenic factors) at the time of treatment correlated with a poorer response to therapy and a lower likelihood of achieving a complete or partial remission. This finding also supports previous in vitro studies suggesting that angiogenic cytokines are important for the survival of CLL B cells and that these cytokines may up-regulate levels of anti-apoptotic proteins known to influence response to therapy [19].

In addition, both Smolej et al. [5] and Maffei et al. [20] reported a significant decrease in the levels of both

VEGF and bFGF in patient achieving remission after intensive fludarabine and lenalidomide based therapy regimens respectively. These observations suggest that therapeutic approaches targeting selected microenvironmental interactions in CLL could possibly disrupt the protective effect of angiogenic factors as VEGF and bFGF on malignant cells and thus overcome resistance mechanisms in high-risk and relapsed CLL patients.

In conclusion, this study shows that serum levels of both VEGF and bFGF can be considered as informative, useful tools for assessing disease activity and discriminating patients with high-risk CLL, which may thus encourage the use of anti-angiogenic factors in therapy of CLL patients and we highly recommend their application in routine clinical practice.

Table (1): Comparison between patients and controls regarding VEGF and bFGF serum levels

| Parameter | Patients mean ±SD | Controls mean ±SD | T-Test | P value | Significance |
|---|---|---|---|---|---|
| VEGF (pg/ml) | 376.6±161.9 | 143.6 ± 40.8 | t= 4.858 | <0.001 | HS |
| bFGF (pg/ml) | 163.7 ± 35.98 | 38.76 ± 20.40 | t= 5.966 | <0.001 | HS |

S.D: standard deviation ; HS: Highly significant difference

Table (2): Comparison between different patients' groups regarding bFGF serum level

| Items | | N | bFGF | | | T-test | |
|---|---|---|---|---|---|---|---|
| | | | Mean | ± | SD | t | p-value |
| Stage | Group I | 19 | 127.421 | ± | 23.078 | -5.444 | <0.001* |
| | Group II | 26 | 173.577 | ± | 33.762 | | |
| Patients' outcome | Clinically beneficial response | 18 | 126.263 | ± | 19.799 | 5.966 | <0.001* |
| | Treatment failure | 27 | 174.423 | ± | 34.031 | | |
| ZAP70 | Negative | 13 | 117.231 | ± | 18.144 | -6.790 | <0.001* |
| | positive | 32 | 169.063 | ± | 32.470 | | |
| CD38 | Negative | 22 | 129.091 | ± | 21.837 | -5.837 | <0.001* |
| | positive | 23 | 178.000 | ± | 33.411 | | |

N: number; S.D: standard deviation; Group I: stage I, II Rai; Group II: stage III, IV Rai; *;significant p value

Table (3): Comparison between different patients'groups regarding VEGF serum level

| Items | | N | VEGF | | | T-test | |
|---|---|---|---|---|---|---|---|
| | | | Mean | ± | SD | t | P-value |
| Stage | Group I | 19 | 244.211 | ± | 55.611 | 6.551 | <0.001* |
| | Group II | 26 | 473.423 | ± | 144.523 | | |
| Patients' outcome | Clinically beneficial response | 18 | 272.842 | ± | 67.915 | -4.363 | <0.001* |
| | Treatment failure | 27 | 452.500 | ± | 169.380 | | |
| ZAP70 | Negative | 13 | 250.231 | ± | 66.747 | -3.816 | <0.001* |
| | positive | 32 | 428.000 | ± | 161.544 | | |
| CD38 | Negative | 22 | 277.727 | ± | 86.041 | -4.969 | <0.001* |
| | positive | 23 | 471.261 | ± | 162.084 | | |

N: number; S.D: standard deviation; Group I: stage I, II Rai; Group II: stage III, IV Rai;*; significant p value

Table (4): Correlation studies of VEGF and bFGF levels with studied parameters

| Prognostic factor | bFGF | | VEGF | |
|---|---|---|---|---|
| | r | P-value | r | P-value |
| VEGF pg/ml | 0.597 | <0.001* | | |
| Age (years) | -0.104 | 0.495 | -0.079 | 0.605 |
| Hb (g\dl) | -0.575 | <0.001* | -0.567 | <0.001* |
| PLT ( $x10^9$ /L) | -0.257 | 0.088 | -0.356 | 0.016* |
| TLC ( $x10^9$ /L) | 0.269 | 0.074 | 0.257 | 0.089 |
| PBL( $x10^9$ /L) | 0.267 | 0.076 | 0.249 | 0.099 |
| BML(%) | 0.190 | 0.211 | .239 | 0.1140 |

Hb: Haemoglobin level; PLT: Platelets count; TLC: Total leucocytic count; PBL: Peripheral blood absolute lymphocyte count; BML%: Bone marrow lymphocyte percentage;*: significant pvalue.

Table (5): Patients' outcome and response to treatment after one year from start of treatment

| Patient stage at diagnosis | Patients 'response to treatment and outcome | | State of life | |
|---|---|---|---|---|
| | Clinically beneficial response N (%) | Treatment Failure N (%) | Alive N (%) | Dead N (%) |
| Group I N(19) | 14/19 (73.6%) | 5/19 (26.3%) | 19/19 (100%) | ___ |
| Group II N (26) | 4/26 (15.3%) | 22/26 (84.6%) | 21/26 (80.7%) | 5/26 (19.2%) |
| TOTAL | 18/45 (40%) | 27/45 (60%) | 40/45 (88.8%) | 5/45 (11.1%) |

N: number of patients; Group I: stage I, II Rai; Group II: stage III, IV Rai;

Table (6): Relation of patients' outcome and response to treatment to studied parameters

| Prognostic factors | | Patients 'response to treatment and outcome | | Test of significance | |
|---|---|---|---|---|---|
| | | Treatment failure N (27) | Clinically beneficial response N (18) | | |
| | | Mean ± SD | Mean ± SD | t | p-value |
| bFGF (pg/ml) | | 174.423 ± 34.031 | 126.263 ± 19.799 | 5.514 | 0.000* |
| VEGF (pg/ml) | | 452.500 ± 169.380 | 272.842 ± 67.915 | 6.551 | 0.000* |
| Age (years) | | 57.21 ± 11.66 | 59.77 ± 10.22 | -0.764 | 0.449 |
| Hb (g\dl) | | 9.97 ± 2.19 | 13.08 ± 1.70 | -5.151 | 0.000* |
| PLT ( $x10^9$ /L) | | 127.58 ± 70.31 | 161.95 ± 48.59 | -1.832 | 0.074 |
| TLC ( $x10^9$ /L) | | 68.41 ± 50.78 | 47.43 ± 28.95 | 1.616 | 0.113 |
| PBL( $x10^9$ /L) | | 52.18 ± 42.81 | 36.12 ± 23.98 | 1.472 | 0.148 |
| BML(%) | | 73.27 ± 12.13 | 66.79 ± 11.85 | 1.787 | 0.081 |
| Total N (%) | | N (%) | N (%) | $X^2$ | p-value |
| Stage | Group I 19(42.22%) | 5(18.6) | 14(77. 8) | 13.933 | <0.001* |
| | Group II 26(57.78%) | 22(81.4) | 4(22. 2) | | |
| ZAP70 | Negative 13(28.89%) | 2(7.5) | 11(61.2) | 14.138 | <0.001* |
| | Positive 32 (71.11%) | 25(92.5) | 7(38.8.) | | |
| CD38 | Negative 22(48.89%) | 5(18.5) | 17(94.5) | 24.117 | <0.001* |
| | Positive 23 (51.11%) | 22(81.5) | 1 (5..5) | | |

N: number; Hb: Hemoglobin level; PLT: Platelets count; TLC: Total leucocytic count; PBL: Peripheral blood absolute lymphocyte count; BML%: Bone marrow lymphocyte percentage;*: significant P value

Table (7): Best cutoff value for VEGF and bFGF for prediction of poor prognosis (treatment failure) in B- CLL patients

| ROC curve between clinically beneficial response and treatment failure patients as regards VEGF and bFGF | | | | | |
|---|---|---|---|---|---|
| Cutoff | Sensitivity. | Specficity. | PPV | NPV | Accuracy |
| VEGF >320 (pg/ml) | 89.5 | 92.3 | 89.5 | 92.3 | 96.7 |
| bFGF >145 (pg/ml) | 89.5 | 84.6 | 81.0 | 91.7 | 89.10 |

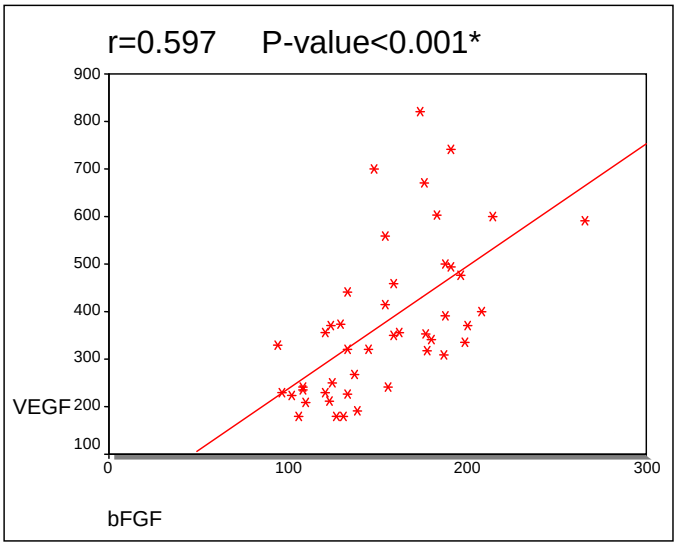


**Figure (1):** Correlation study between VEGF and bFGF showing statistically significant positive correlation between them

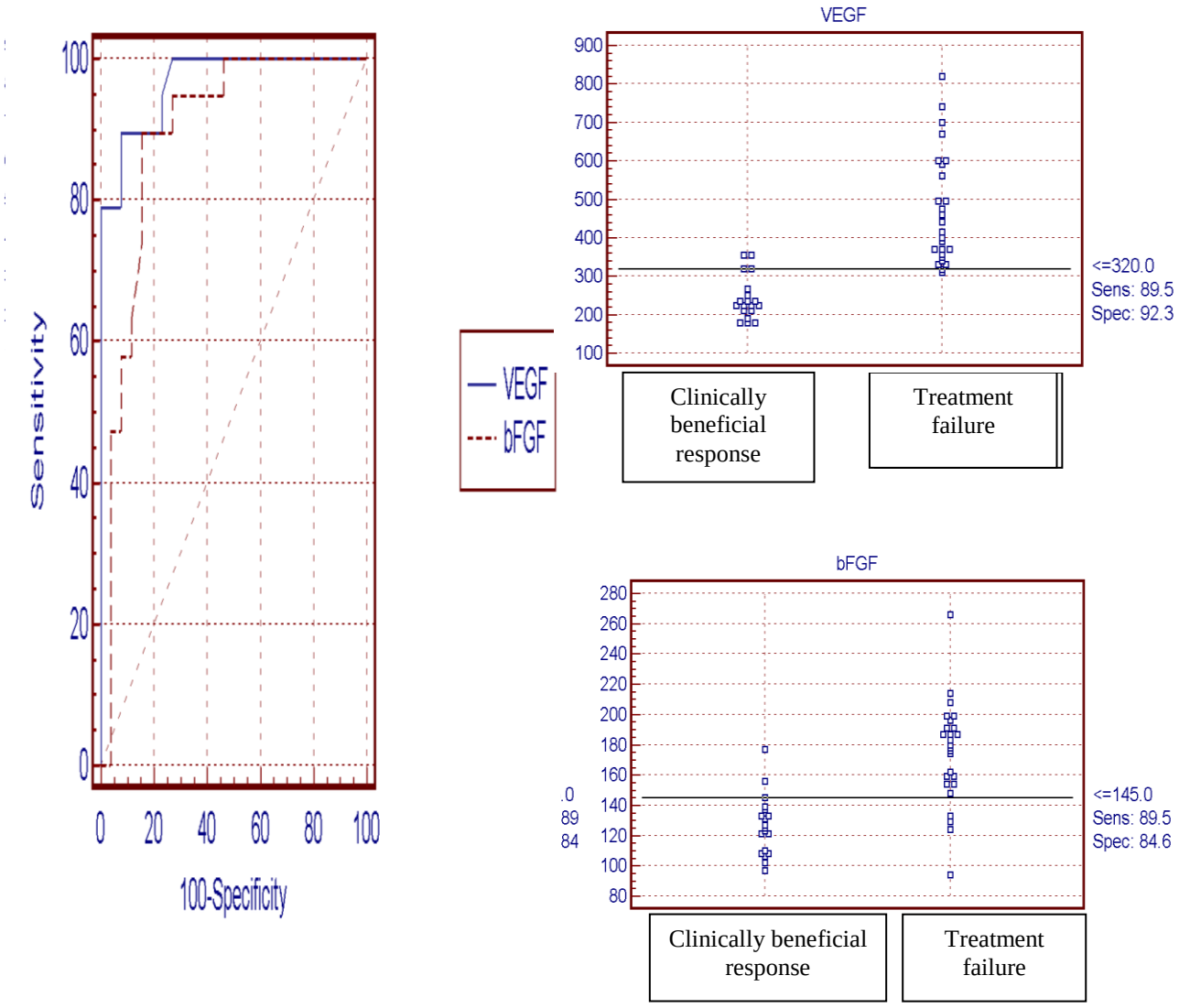


**Figure (2):** Best cutoff value for VEGF and bFGF for prediction of poor prognosis (treatment failure) in CLL patients

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