

# Angiogenic Factor VEGF and Its Relationship with Biological Prognostic Markers in Chronic Lymphocytic Leukemia

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B-CLL is a heterogeneous disease, distinguishing between good and poor prognosis represents a challenge to hematologists. The present study aimed to assess the relative merit of VEGF as a prognostic factor in CLL and to correlate it with other prognostic factors as CD38 & sP53 in the different stages of the disease. The median values of CD38%, sP53 and VEGF were significantly higher in both high and intermediate risk subgroups of the modified Rai staging system when compared to low risk subgroup. There was a statistically significant positive correlation between CD38% and VEGF in all subgroups. However, positive correlation between VEGF and sP53 was only observed in the high risk subgroup. The strong correlation between CD38 and VEGF in CLL patients, suggests the possibility that angiogenic factors might contribute to the more aggressive clinical behavior of CD38+ CLL. This could provide a rationale for the use of antiangiogenic agents in CD38+ CLL.

**B**-cell chronic lymphocytic leukemia (B-CLL) is a heterogeneous disease (Del Principe *et al.*, 2004; Moreau *et al.*, 2008). Its clinical course can be quite variable. Many patients survive for prolonged periods without any therapy, whereas others succumb rapidly despite aggressive treatment. It has been a long-term challenge for hematologists to distinguish between CLL patients with either good or poor prognosis (Del Poeta *et al.*, 2001).

Classical staging systems by Rai (Rai *et al.*, 1975) and Binet (Binet., 1981), while readily available and useful for initial assessment of prognosis, are not able to determine individual patient's ongoing clinical course of CLL at the time of diagnosis, especially in early stages and did not take into account the new understanding about the molecular pathology of CLL (Moreno *et al.*, 2008; Montillo *et al.*, 2005; Liu *et al.*, 2005).

Several parameters such as lymphocyte doubling time (LDT), serum levels of  $\beta_2$ -

microglobulin, soluble CD23 (sCD23), serum thymidine kinase levels, and bone marrow histology, have been added to the current staging systems to differentiate prognostic subsets (Montserrat *et al.*, 2006). Mutational status of variable region of immunoglobulin heavy chain genes (IgVH), cytogenetic aberrations, and both intracellular ZAP-70 and surface CD38 expression are recognized as parameters with established prognostic value. Molecules regulating the process of angiogenesis are also considered as promising markers (Vroblová *et al.*, 2009).

The CD38 molecule is a type II transmembrane glycoprotein with ectoenzymatic activity (Ferrero *et al.*, 1999). It is formed of 300 amino acid residues and consists of three domains, a short intracellular domain, a single transmembrane helix domain and a large extracellular domain (Ferrero *et al.*, 1999; Shanafelt *et al.*, 2004; Stilgenbauer *et al.*, 2002). It is broadly expressed on the surface of many cells of both haematopoietic and non haematopoietic origin dependent on

either their differentiation or activation (Ferrero *et al.*, 1999). Binding of the agonistic anti-CD38 monoclonal antibody to mature B-cells is followed by the up regulation of Bcl-2 anti-apoptotic molecule thereby protecting malignant cells from apoptosis (Vroblová *et al.*, 2009). It has been involved in CLL as a prognostic marker, then as a key element in the pathogenetic network underlying the disease. There is a general consensus that CD38 expression is a reliable negative prognostic marker for CLL patients (Vroblová *et al.*, 2009).

The *P53* gene codes for the *P53* protein which is a transcription factor involved in the cell cycle arrest and induction of apoptosis in genetically damaged cells. After DNA damage, the half-life of *P53* is prolonged and the protein accumulates in the nucleus where it regulates the transcription of the pro-apoptotic protein Bax and the antiapoptotic protein Bcl-2 (Zenz *et al.*, 2008).

Mutations or deletions of the *P53* gene may facilitate the transmission of a genetic damage and the emergence of neoplastic clones with a survival advantage (Lane *et al.*, 1992). Over expression of *P53* protein was found to be correlated with *P53* gene mutations, progressive disease and short survival (Del Principe *et al.*, 2004). Also, *P53* dysregulation may occur by inactivation of its regulatory gene, the ATM gene. Low ATM protein levels due to ATM mutations are like TP53 mutations, associated with an adverse clinical outcome (Inamdar *et al.*, 2007). So, *P53* abnormalities in CLL are associated with advanced stage at presentation, resistance to fludarabine and alkylating agents-based therapies, high incidence of transformation, disease progression in 1 to 2 years, and worst clinical outcome (Schetelig *et al.*, 2008).

Angiogenesis which is a process of new blood vessels formation from already existing vasculature, appears to be an important player in biology of chronic lymphocytic leukemia

(CLL) (Smolej *et al.*, 2006; Maffei *et al.*, 2010). Several studies have also shown that in B-CLL there is increased vascularity in bone marrow (Kini *et al.*, 2000; Chen *et al.*, 2000) and angiogenic cytokines in peripheral blood (Aref *et al.*, 2005; Pepper *et al.*, 2007).

Among the variety of cytokines with angiogenic activating properties, the vascular endothelial growth factor (VEGF) is considered the most predominant, direct and selective (Chen *et al.*, 2000). Its signaling pathway has been characterized as one of the most important endothelial regulator in human angiogenesis (Molica *et al.*, 2002).

VEGF is a multifunctional protein that, on binding to its receptors on endothelial cells, affects vascular permeability, cell proliferation, migration, and survival. All of which are required for angiogenesis. Although data tend to support the idea that angiogenic cytokines may play a role in the leukaemogenic process of CLL, the relative merit of VEGF in predicting the outcome of disease has not been assessed thus far (Smolej *et al.*, 2006).

The aim of the present study was to assess the prognostic value of serum vascular endothelial growth factor as angiogenic marker alone and in combination with other prognostic parameters, such as CD38 antigen expression, and soluble *P53* (sP53) serum levels in B-CLL and the relation between these parameters in different stages of the disease.

## Patients and Methods

### Patients

The present study was conducted on 2 groups:

- Group I

The control group which included 15 apparently healthy individuals, age and sex matched with the patient group.

- Group II

The patient group which included 35 newly diagnosed B-CLL patients of both sexes admitted to hematology

oncology department of Alexandria Main University Hospital during the period February 2009 to January 2010.

Informed written consent was obtained from patients and controls, according to requirements of the medical ethics committee of Alexandria Faculty of Medicine.

Diagnosis of CLL was based on the clinical features and the National Cancer Institute-Working Group (NCI-WG) diagnostic criteria (Bruce *et al.*, 1996). Then patients were sub-classified according to the modified Rai staging system (Rai *et al.*, 1987) into 3 subgroups: Low risk patients: 13 patients (37.1%), Intermediate risk patients: 9 patients (25.8%) and High risk patients: 13 patients (37.1%)

### Methods

**Blood Samples:** A volume of 6 ml of venous blood were obtained from each of the patients and controls; 3 ml freshly collected on K2 EDTA, used for complete blood count (CBC), CLL immunophenotyping and CD38% expression by flowcytometry. Sera were separated from the other 3 ml. Serum samples were stored at -20 till used for measuring VEGF and sP53.

All patients were subjected to full history taking, complete clinical examination, CBC performed using a Bayer ADVIA120 cell counter (Lewis *et al.*, 2006). Immunophenotyping of peripheral blood lymphocytes was performed according to the method of Braylan *et al.*, 2001, using a panel of antibodies to diagnose CLL. Expression of CD38 on CLL cells was done by flowcytometry. sP53 and VEGF in serum samples were assessed by ELISA.

- Immunophenotyping of lymphoid cells:

The direct immunofluorescence technique was used for typing viable leukemic cell suspensions that were analyzed using Becton Dickinson, FACS Caliber Flow Cytometer equipped with cell quest software. To diagnose CLL, we used a panel of monoclonal antibodies (DAKO, Denmark); CD5/CD19 (Dual color reagent Cat # FR882); CD22 (Cat # R7062); CD23 (Cat # F7063); FMC7 (Cat # F7110), Anti human kappa light chain/Anti human lambda light chain (Dual color reagent Cat # FR481).

- CD38% Expression by Flowcytometry :

Peripheral mononuclear cells were analyzed for surface expression of CD19/CD38 using monoclonal antibodies (Cat #R0808/F701) according to the method of Deaglio *et al.*, 2001.

The cut-off point of positivity for all monoclonal antibodies was considered when more than 20% of the cells stained with a particular antibody in excess of the background fluorescence in the negative controls.

- Soluble P53 Measurement

Soluble P53 was measured using a commercial kit of Biosource international, California (Cat. # KHO0151) according to manufacturer instructions and recommended by Hirao *et al.*, 2000. The system is a solid phase sandwich enzyme linked immunosorbant assay. A monoclonal antibody specific for P53 has been coated onto the wells of the microtiter strips. Absorbance was read at 450nm. The color intensity is directly proportional to the concentration of P53 present in the original specimen. SP53 concentrations for unknown samples and controls were read from the curve constructed from the supplied standards.

- VEGF Measurement

VEGF was measured using a commercial kit (Quantikine Human VEGF Immunoassay from R& D Systems, Cat No DVED00) according to the manufacturer protocols and recommended by Aguayo *et al.*, 2000.

The assay is based on a sandwich enzyme linked immunoassay technique. A monoclonal antibody specific for VEGF has been pre-coated onto a microplate. Standards and samples were placed into the wells and any VEGF present was bound to the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody conjugated to horseradish peroxidase-specific for VEGF was added to the wells. Following a wash to remove any unbound reagent, a substrate solution was added to the wells and color developed in proportion to the amount of VEGF bound in the initial step. The color development was stopped and the intensity of the color measured at 450nm. A standard curve was constructed based on supplied standards. Concentrations for unknown samples and controls were read from the plotted curve.

### Statistical Methods

Data were fed to the computer and analyzed using the Statistical analysis System software SPSS (Version 17, Chicago, IL, USA). The distribution of VEGF was tested for normality using Kolmogorov-Smirnov test which revealed abnormal distribution. Thus, non-parametric statistics were considered. Quantitative data were described using median, minimum (Min) and maximum (Max) values. Qualitative data were described using number and percent. Kruskal-Wallis and Mann-Whitney were used to test quantitative

independent variables. Significance of the obtained results was judged at the 5% confidence level. However, for Mann-Whitney tests applied after Kruskal-Wallis test, Bonferroni correction of the alpha value for multiple comparisons was carried out. Spearman's rho correlation test was used for correlation between 2 quantitative variables.

## Results

This study included 35 patients and 15 controls of matched age and sex. The mean

age of the patient group was 58.94±10.27 years, while that of the control group was 56.73±10.33 years.

Table (1) demonstrates CD38% on CLL cells, *sP53* and VEGF serum levels in the patient and control groups. A statistically significant difference was found between patients and controls in the 3 parameters.

Table 1. Comparison between the two studied groups regarding serum levels of VEGF, *sP53* expression and CD38% expression.

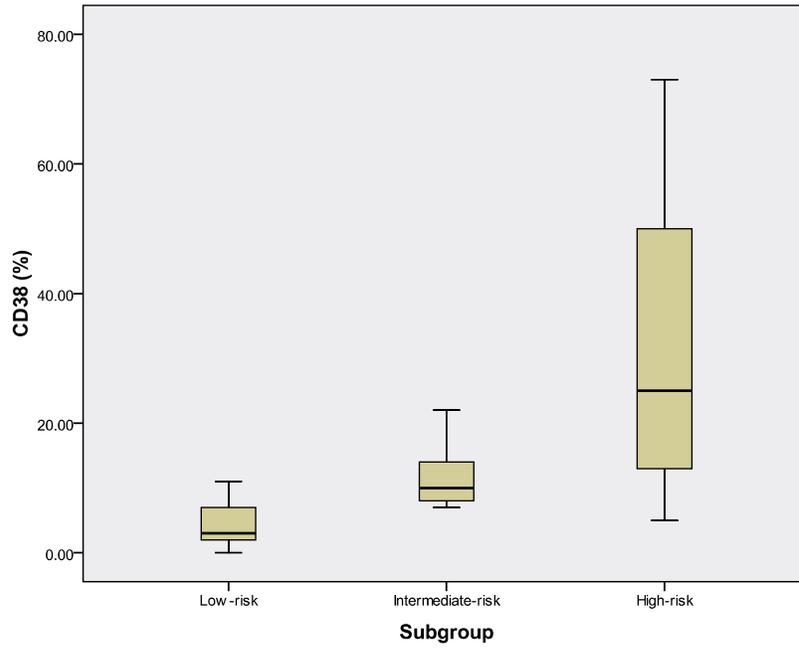
	Control	Patient	<i>P</i> value*
VEGF(pg/ml)			
Min-Max	23.19-53.50	22.10-732.00	<0.001
Median	33.45	230.00	
CD38%			
Min-Max	1.00-10.00	0.00-73.00	<0.001
Median	2.00	11.00	
<i>sP53</i> (pg/dl)			
Min-Max	0.00-60.00	0.00-720.00	<0.001
Median	0.00	120.00	

\*Statistically significant at  $P<0.05$

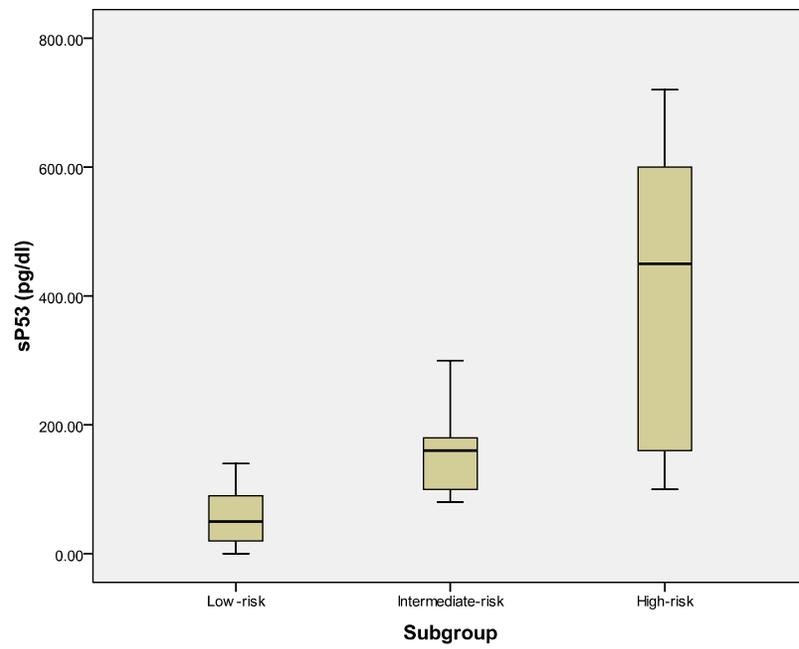
The patients were classified according to the modified Rai staging system into 3 subgroups: low risk patients included 13 patients (37.1%), intermediate risk patients included 9 patients (25.8%) and high risk patients included 13 patients (37.1%).

Comparison between the 3 subgroups revealed significantly higher median values of CD38%, *sP53* and VEGF serum level in the

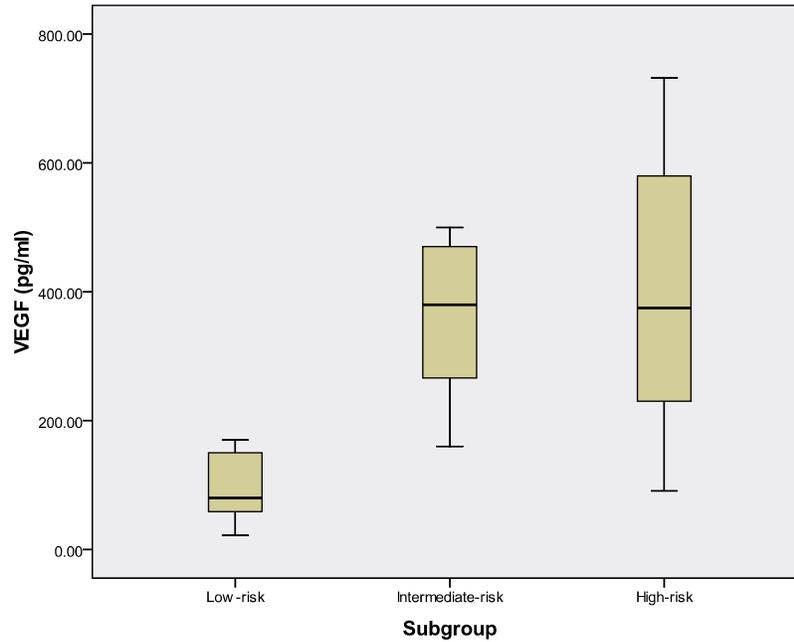
high risk ( $P=0.07$ ,  $P<0.001$ ,  $P<0.001$ , respectively) and intermediate risk sub groups ( $P=0.007$ ,  $P<0.001$ ,  $P<0.001$ , respectively) when compared to low risk subgroup as shown in figures (1, 2, 3). However, the median values of CD38%, *sP53* and VEGF serum levels did not differ significantly between the intermediate risk and high risk subgroups.



**Figure 1.** Comparison between the three subgroups as regards CD38%.



**Figure 2.** Comparison between the three subgroups as regards sP53 serum level.



**Figure 3.** Comparison between the three subgroups as regards VEGF serum level.

The relation between CD38%, *sP53* and VEGF serum levels and clinical parameters of patients is illustrated in table (2). There were significantly higher levels of CD38%, *sP53* and VEGF serum levels in patients with splenomegaly than patients without splenomegaly ( $P<0.001$ ,  $P=0.001$ ,  $P<0.001$ , respectively) and the same apply for patients with hepatomegaly ( $P=0.008$ ,  $P=0.001$ ,  $P<0.01$ , respectively).

However, as regards lymphadenopathy only serum VEGF level showed a

significantly higher level in patients with lymphadenopathy than patients without lymphadenopathy ( $P<0.001$ ).

For laboratory parameters, a statistically significant negative correlation was observed between both CD38% and *sP53* and hemoglobin concentration ( $P=0.020$ ,  $P=0.017$ , respectively). Otherwise, no significant correlations were elicited between any other parameters.

Table 2. Relation between CD38%, sP53, VEGF serum levels and clinical parameters of the patients.

	Splenomegaly		<i>P</i> value*	Hepatomegaly		<i>P</i> value*	Lymphadenopathy		<i>P</i> value*
	Present (19)	Absent (16)		Present (15)	Absent (20)		Present (22)	Absent (13)	
VEGF(pg/ml)									
Min-Max	91.00- 732.00	22.10- 296.00	<0.001	91.0- 732.0	22.10- 490.00	0.04	61.50- 732.00	22.10- 155.00	<0.01
Median	385.00	92.00		390.00	152.50		300.00	69.00	
CD38%									
Min-Max	5.00- 73.00	0.00- 22.00	<0.001	5.00- 73.00	0.00- 70.00	0.008	0.00- 73.00	1.00- 70.00	NS
Median	16.00	5.50		16.00	7.50		11.00	10.50	
sP53(pg/dl)									
Min-Max	20.00- 720.00	0.00 - 270.0	<0.001	100.00- 700.00	0.00- 720.00	0.001	0.00- 700.00	20.00- 720.00	NS
Median	160.00	80.00		240.00	85.00		140.00	75.00	

\*Statistically significant at  $P < 0.05$ . NS= Not significant.

### Correlation between VEGF and other prognostic factors

In the whole patient group a significant positive correlation was detected between serum VEGF and both CD38% and sP53 ( $P < 0.001$ ) as illustrated in figures (4, 5)

In the different patients subgroups: A significant positive correlation was found

between VEGF level and CD38% in the low risk, intermediate risk and high risk subgroups ( $r = 0.860$   $P < 0.001$ ,  $r = 0.895$   $P = 0.001$ ,  $r = 0.895$   $P < 0.001$ , respectively). However, the correlation between VEGF and sP53 revealed a significant positive correlation only in the high risk subgroup ( $r = 0.804$   $P = 0.001$ ).

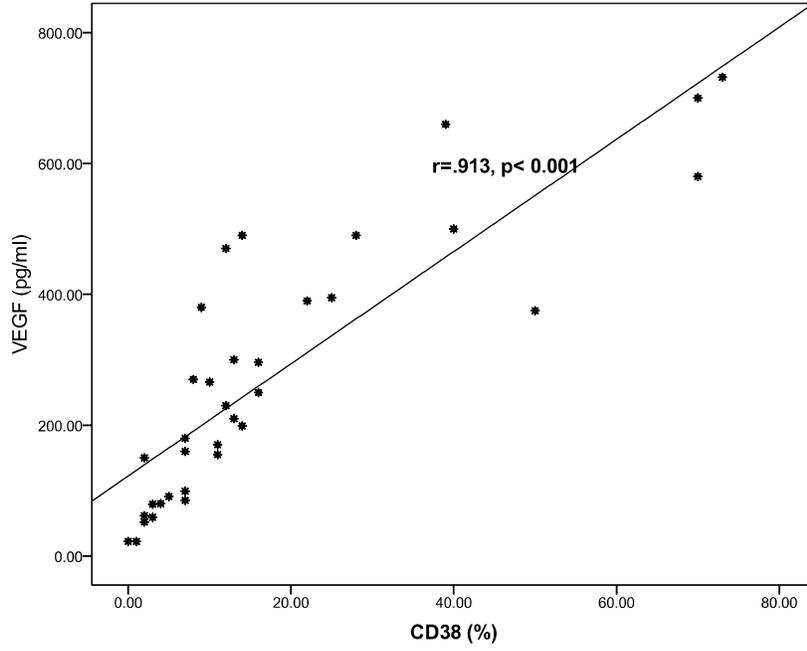


Figure 4. Correlation between VEGF serum level and CD38%.

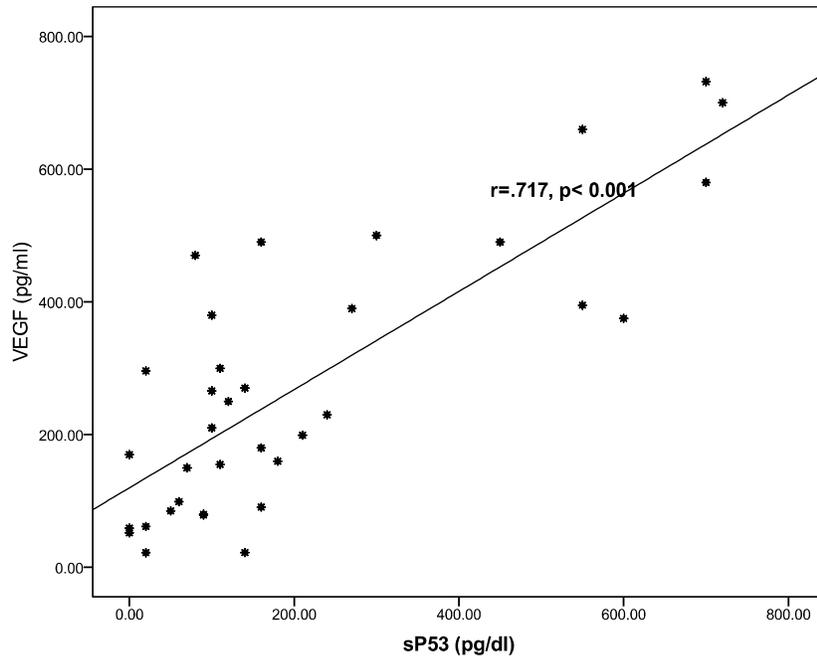


Figure 5. Correlation between VEGF and sP53 serum levels

## Discussion

Novel prognostic factors are being sought in order to identify high-risk CLL patients at the time of diagnosis and to optimize their treatment, VEGF is one of these new prognostic markers. The aim of the present study was to assess the prognostic value of serum vascular endothelial growth factor as angiogenic marker, alone and in combination with other prognostic parameters, and the relation between these parameters in different stages of the disease.

The study comprised 35 B-CLL patients and 15 healthy persons as a control group. The patients were classified into three subgroups according to the modified Rai staging system (Rai *et al.*, 1987) into low (n=13), intermediate (n=9) and high risk patients (n=13). CD38 antigen expression (%), sP53 and VEGF serum levels were the studied markers in this work.

In the present study, sP53 serum level was significantly higher in advanced Rai stages ( $P<0.001$ ) and had a positive correlation with CD38 % which is another negative prognostic marker in B-CLL. Similar results were obtained by Del Principe and others (2004) who found significant differences ( $P<0.001$ ) in sP53 levels among the low, intermediate and high modified Rai stages, and that high levels of sP53 were associated with the advanced Rai stage. They also found a significant positive correlation between the plasma levels of sP53 and the percentages of CD38+ cells ( $r=0.27$ ,  $P=0.0001$ ).

Del Poeta and coworkers (2001) found that high sP53 levels identified patients in advanced clinical stages and predicted poor prognosis. They also studied the clinical outcome and the survival data of the patients and they found that sP53 protein represented a significant additional biological parameter

that identified a "high risk" B- CLL subset candidate for novel therapeutic approaches.

Also, Ripolles and colleagues (2006) found that patients with advanced stages (Rai III-IV) had more frequent ATM deletion. And that P53 dysfunction due to mutation of the genes encoding either P53 or ATM was characterized by increased baseline levels of sP53, which could be determined by a simple immunoenzymatic technique.

On the other hand, Xu and others (2008) did not find correlation between ATM or TP53 deletion and Binet stage. This contradiction may be attributed to the difference in the number or characteristics of patients, different techniques used and different staging system as they used the Binet staging system while other studies used the modified Rai staging system.

In the present study there was significantly higher levels of CD38% in patients with splenomegaly than patients without splenomegaly ( $P<0.001$ ) and the same regarding hepatomegaly ( $P=0.008$ ). A significant negative correlation was found between both CD38% and hemoglobin concentration ( $P=0.020$ ). Moreover, the CD38 antigen expression significantly differed among the different modified Rai stages ( $P<0.001$ ).

Similarly, Ibrahim and others (2001) found that CD38+ patients had significantly lower hemoglobin levels ( $P=0.001$ ) and there was a trend towards more palpable hepatomegaly in the CD38+ patients ( $P=0.05$ ). But unlike the present study, they found a significant association between the number of nodal sites involved with the disease and CD38 expression. Also, they did not find any significant difference in terms of splenomegaly and Rai or Binet staging systems between CD 38+ and CD38- CLL patients.

Interestingly, Ibrahim *et al.*, (2001) found that CD38 positivity identified a subgroup of CLL patients with aggressive disease staged by Rai as indolent and intermediate (Rai stage 0-II) and negative CD38 expression also distinguished a subgroup of patients with less aggressive disease whose Rai stage indicated aggressive disease (Rai III-IV). Similar observations were found in the present study as 2 patients of the intermediate modified Rai stage had positive CD38 antigen expression and also 6 patients of the high risk group showed CD38 negativity. Thus, the measurement of CD38 expression allows the identification of a subset of patients with better prognosis within a presumably poor prognostic group (high modified Rai stage) and the identification of patients with poorer prognosis among what is considered intermediate prognostic group (intermediate modified Rai stage). This is particularly important in managing patients in the early stages of the disease which includes a subgroup whose disease will progress quickly.

Hamblin and coworkers (2002) reported a significant association between CD38 expression and clinical stage according to Binet staging system. Similar results were obtained by Del Principe and others (2004), as they also found a significant association between CD38 % and the modified Rai stages ( $P=0.002$ ). They suggested that percentage of CD38+ B cells is likely to be an accurate predictor of clinical outcome and therefore could be used to indicate when novel chemotherapeutic approaches are needed.

Angiogenesis proved to be involved in the pathogenesis of B cell-CLL, and high micro vascular density has been found to be associated with a poor prognosis in CLL (Wolowiec, 2004). It has already been fully appreciated that higher levels of VEGF are significantly correlated to the size of tumor in patients suffering from various solid tumors. Much less is known about the role of

abnormal angiogenesis in malignancies of hematopoietic origin (Vroblová, 2009).

In the present study, VEGF showed a significant higher median serum level in the patient group as compared to the control group ( $P<0.001$ ). Similar results were reported by other studies (Smolej, 2006; Aguayo, 2006; Molica, 1999).

A significantly higher VEGF serum level was found in patients with splenomegaly, hepatomegaly and lymphadenopathy compared to those without ( $P<0.001$ ,  $P=0.04$ ,  $P<0.001$ , respectively). Also, serum level of VEGF was significantly higher in both high and intermediate risk groups when compared to low risk group according to modified Rai staging system ( $P<0.001$ ). On the contrary, Smolej and colleagues (2006) observed no difference in VEGF plasma levels between patients in the different modified Rai stages.

There were significant positive correlations between serum VEGF level and CD38% in the three subgroups; low, intermediate and high risk modified Rai groups ( $P<0.001$ ,  $P=0.001$ ,  $P<0.001$ , respectively). Similarly, other studies (Molica *et al.*, 2007; McCabe *et al.*, 2004) reported that circulating levels of VEGF correlated positively with CD38 expression ( $P=0.03$ ) and by measuring surface expression of CD38 and VEGF on peripheral blood circulating CD19/CD5 CLL cells, a correlation was found between percentage of CLL cells expressing CD38 and percentage of CLL cells expressing VEGF ( $P = 0.001$ ). Also, Pepper and coworkers (2005) demonstrated 2-3 fold increased levels of VEGF in CD38+ cells.

These results suggest that the poor prognosis and more aggressive behavior of CLL patients with increased CD38 expression may, in part, be related to increased expression of angiogenic factor (VEGF) by CLL cells. VEGF may contribute to the poor prognosis associated with increased CD38 expression not only through increased bone

marrow angiogenesis, but other possible mechanisms include paracrine and autocrine interactions to promote CLL cell growth and survival (McCabe *et al.*, 2004).

Also, VEGF has been shown to inhibit the maturation of dendritic cells into antigen presenting cells, which might help tumor cells to evade an effective immune response (McCabe *et al.*, 2004). Molica and others (2004) suggested that the latter possibilities may be more important in explaining the strong correlation between CD38 expression and VEGF expression on CLL cells as they found lack of correlation between CD38 expression and extent of bone marrow angiogenesis in CLL.

On the contrary, a study (Shanafelt *et al.*, 2005) failed to show association of CD38 expression with serum levels of VEGF.

In conclusion, these results point out the need for larger prospective studies in order to further elucidate the significance of angiogenesis in CLL. The demonstration of more conclusive evidence of increased angiogenesis could provide a rationale for the use of antiangiogenic agents in CD38+ CLL. The combination of CD38, *P53* and VEGF, is likely to provide more accurate prognostic information in future studies. This will assist in identifying Stage I patients who may benefit from early and/or more intensive treatment, as well as Stage II and III patients who may require alternative treatment strategies at the outset.

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