Role of TNF-α as a Survival Prognostic Marker in Chronic Lymphocytic Leukemia Patients

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Pro-inflammatory cytokines play a central role in the pathogenesis of chronic leukemia. This pilot study assesses the potential value of measuring TNF-α, IL-6, IL-18 and CRP levels as prognostic markers for disease monitoring in CLL patient. These parameters were evaluated in 45 CLL patients and 25 healthy control subjects IL-6 on contraire to the other cytokines, was significantly higher (*P*<0.05) in patients compared to controls. At hematological remission, only CRP was significantly reduced (*P*<0.005). IL-6 is inversely correlated with Hb (*P*<0.05). In the meantime, IL-18 is correlated to splenomegaly, and CRP (*P*<0.05; for both). TNF- α level in non-survived patients was significantly higher than both survived patients (*P*<0.05), and controls (*P*<0.01). In conclusion, TNF- α can be potentially used as a survival prognostic factor in CLL.

Chronic lymphocytic leukemia (CLL) is a chronic lympho-proliferative malignancy characterized by progressive lymphocytosis caused by the clonal accumulation of $CD5^{+}CD19^{+}$B-cells in peripheral blood, bone marrow and lymphoid organs. Half of CLL patients progress more rapidly than initially anticipated, these patients might benefit from treatment before progression occurs, so it is necessary to identify more reliable prognostic markers, (Van Bockstaele *et al.*, 2009). Many pleiotropic proinflammatory cytokines, play a role in the activation, growth and apoptosis of leukemic B-cells, (Cordingley *et al.*, 1988), which are able to produce TNF, (Hoffbrand *et al.*, 1993), leading to B-CLL aggressiveness and disease progression, (Bojarska-Junak *et al.*, 2008). In the meantime, Interleukin-6 (IL-6) is a major participant in hematopoiesis, immune responses, (Heinrich *et al.*, 2003), and leukemic generation, (Nachbaur *et al.*, 1991), suggesting its involvement in leukemogenesis by modulating the other cytokines expression, (Thomas *et al.*, 1997), moreover it acts as a regulator for CRP release, (Kishimoto, 2005), which is a mediator of inflammation, (Haider *et al.*, 2006), and its pre-diagnostic levels correlate with the risks of cancer, (Kim *et al.*, 2009).

Tumor necrosis factor-alpha (TNF-α) was initially thought to be a product only of macrophages, T-cells, B-cells and monocytes. TNF-α has now been shown to be produced by a wide variety of tumor cells, including those of CML, B-Cell lymphoma, and many other kinds of tumors. It was first isolated as an anticancer cytokine, where TNF-α can mediate a wide variety of diseases, including cancer, (Aggarwal, 2003). TNF-α has been shown to be one of the major mediators of inflammation, induced by a wide range of pathogenic stimuli. It induces other inflammatory mediators and proteases that orchestrate inflammatory responses. It is also produced by tumors and can act as an endogenous tumor promoter. The role of TNF-α has been linked to all steps involved in tumorigenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis, (Balkwill, 2002 & 2009). Multifunctional cytokine, IL-18, interferes with both pro-cancerous and anti-cancer activities, (Samsami *et al.*, 2009), its level was elevated in leukemia patients sera, (Taniguchi

et al., 1997), suggesting that it is a tumor-associated protein in hematological malignancies, (Takubo *et al.*, 2000), representing its pivotal role in leukemic pathogenesis (Dinarello *et al.*, 1998). IL-18, initially defined as a potent inducer of IFN-gamma production, is a systemic, multifunctional cytokine with both pro-cancerous and anti-cancer activities, (Samsami *et al.*, 2009). It is a pleiotropic pro-inflammatory cytokine with stimulatory effects on both T helper-1 and T helper-2 cell responses, (Haas *et al.*, 2009). Elevated IL-18 levels were found in serum of some leukemia patients, especially those with chronic myeloid leukemia (CML), (Taniguchi *et al.*, 1997; Kiersnowska-Rogowska *et al.*, 2005), have shown that there is an increased level in TNF-α and IL-18 concentrations in serum of CML patients.

The aim of this study is to determine the effect of chemotherapy on some pro inflammatory cytokines (TNF-α, IL-6 and IL-18) and CRP, and its viability to be used as prognostic markers for monitoring therapy or indicators of disease severity in CLL patiens and to elucidate the role of these cytokines in the pathogenesis of the disease.

Subjects and Methods

Patient selection

The study group composed of 55 subjects (age from 25 – 78) divided into 2 groups. The first group consists of 45 de novo CLL patients (33 male & 12 females) and the second group consists of 10 apparently normal healthy controls (5 Male, 5 female). All CLL patients were chosen such that they showed clinical signs of leukocytosis and relative & absolute lymphocytosis in peripheral blood samples. Their examined bone marrow aspirate showed relative lymphocytosis in addition to CLL scoring system by immunophenotyping analysis. All patients were subjected to treatment with accredited therapy in NCI which are Fludarabin or Vincristine or CHOP. The patients who suffer from other tumors, viral infection, diabetes, or chronic diseases were rejected from the study.

Sample collection

Venous blood samples of patients were obtained after signing the clinical referral and ethical forms approved by the ethics committee at the Egyptian National Cancer Institute. Blood samples were drawn in a sterile vaccutainer tubes from de novo CLL cases before therapy. Another blood samples were obtained after accomplishing the first partial and/or complete hematological remission which is ranged in duration from 3 to 8 months for all patients.

Laboratory Diagnosis

Clinical routine tests such as complete blood count (CBC), liver function tests (LFT) and kidney function tests (KFT) were performed to evaluate the general status for the patients, patients with hepatic or renal complications were excluded from this study. Patients diagnosis was achieved according to the diagnostic criteria of World Health Organization (WHO), which was done through 2 main steps:

1- Morphological examination of bone marrow aspirate (BMA) and peripheral blood (PB): patients with relative and absolute lymphocytosis in PB and excessive lymphoid series cells in BMA (i.e.: Lymphocytes are encroaching other bone marrow elements) were initially diagnosed as subgroup of Non-hodgkin lymphocytic leukemia / Lymphoma (NHL).

2- Another mandatory test to verify the differential diagnosis of NHL which is done by immunophenotyping (IPT) and the establishment of CLL scoring system; scoring of 5/5 is considered as typical CLL while scoring of 4/5 is atypical CLL.

Cytokine analysis and CRP measurement

- Determination of IL-6 & IL-18

Cytokines levels were determined in sera via a multiarray, sandwich immunoassay kit provided by Human IL-6 BMS213/2 (Bender Medsystems GmbH, USA) for IL-6 & Human IL-18 BMS267/2 (Bender Medsystems GmbH, USA) for IL-18. Procedures were performed according to the manufacturer instructions. Briefly, 50 µl of diluted biotin-conjugate in addition to 100 µl of standard for calibration or diluted sample (1:1) or buffer (as blank) were pipetted per well, the plate was incubated at room temperature (18º to 25ºC) for 2 hours while shaking. Then the wells were washed, and 100 µl of Streptavidin-HRP was added to each well. The plate was incubated at room temperature for 1 hour while shaking. After incubation, the wells were washed and 100 µl of TMB Substrate were pipetted to each well, then the plate was incubated at room temperature for about 10 minutes. The enzyme reaction was stopped by quickly pipetting 100 µl of stop

solution into each well and the absorbance was read at 450 nm.

Determination of TNF-α

TNF-α concentration was measured in serum via a multiarray, sandwich immunoassay kit Immunotech REF: IM1121 (Beckman Coulter Co. France). Procedures were performed according to the manufacturer instructions. Briefly, 100 µl of conjugate and 100 µl of standard or sample, was added per well, the plate was incubated for 2 h. at room temperature (25ºC) while shaking. The wells were washed and 200 µl of substrate was added to each well, then the plate was incubated for 45 min at room temperature in dark, while shaking. Then, 50 µl of stop solution was added to each well and the absorbance was read at 405 nm.

Measuring of CRP

CRP was measured semi-quantitatively by kits provided by (Avitex CRP). Procedures were performed according to the manufacturer instructions. Briefly, isotonic saline is used for serial dilutions of the patients' sera, 50 µl of each serum dilution was transferred to the test circle on the slide, shaking the latex reagent, and one drop of suspension was added to the test circle. The drops were mixed using a disposable stirrer and rotated gently for 2 minutes while examining the test slide for agglutination.

Statistical Analysis

The comparison of the measured parameters between CLL & controls was carried out by One-way ANOVA; meanwhile the comparison between dead *&* survived patients for cytokines and CRP was performed by Independent t-test and Mann-Whitney test respectively. The significance of the effect of chemotherapy can be detected on cytokines & CRP levels by Paired-t-test & Friedman test respectively. All data in the tables and graphs are expressed as mean value ± standard error unless otherwise indicated. The SPSS statistical package software v.17 (IBM Corporation) was used. A *P* value < 0.05 was considered significant.

Results

Cytokines and CRP concentrations in serum before chemotherapeutic administration

Significant elevation of IL-6 in serum concentration of CLL patient group compared to controls were noted ($P<0.05$) (Table 1), while no statistically significant difference was observed for TNF-α and IL-18. CRP was elevated ($P<0.005$) in CLL patients indicating the presence of inflammation caused by cancer. Also, there was no significant difference in cytokines levels according to stages (Table 2). The time between the administration of chemotherapy and the first partial or complete hematological remission was 3-8 months. A distinctive result indicated that the TNF-α serum level is increased in patients having CLL who's no longer surviving and that there is a correlation between the TNF-α plasma level and overall survival (Table 3).

Cytokines and CRP concentrations in serum after therapy

Post-treatment results, attained after partial remission revealed that no important changes in TNF-α levels, Meanwhile a significant decrease in CRP levels ($P<0.005$) (Table 4), IL-6 and IL-18, table 1.

ROC Curve and Cross Tabulation of IL-6 & IL-18

The normalization of IL-6 and IL-18 by treatment was measured by calculating the cut-off value, which separates normal from abnormal levels. From the ROC-curve analysis, the optimum cut-off values were 6.05 pg/ml for IL-6 and 95.7 pg/ml for IL-18. Cross tabulations using the optimum IL-6 cut-off value of 6.05 pg/ml for all subjects, revealed that 100% of normal controls had IL-6 < 6.05 pg/ml, while 67% of pre-treated CLL had IL-6 > 6.05 pg/ml. Chemotherapeutic treatment caused normalization of IL-6 in 55%, while only 45% had abnormal elevated values. While the optimum IL-18 cut-off value of 95.7 pg/ml for all subjects, revealed that all controls had IL-18 < 95.7 pg/ml, and all pre-treatment CLL patients had IL-18 > 95.7 pg/ml. After treatment, IL-18 levels revealed that only (18%) of CLL patients have normalized levels of IL-18 and (82%) is still elevated, Figure 1.

Table 1. Baseline characteristic of the studied group *(mean ± S.E.)*

	Controls (n=10)	CLL – Pre-treatment (n=45)	CLL – Post-treatment (n=33)
Age	43 ± 4.0	62.5 ± 2.8	
Total Leucocyte Count (x 10^9/L)	5.76 ± 0.53	105.3 ± 31.3	8.1 ± 1.06
Hemoglobin (g/dl)	13.3 ± 0.3	10.9 ± 1.0	11.9 ± 0.7
Platelets (x 10^9/L)	283.2 ± 15.9	122.3 ± 23.9	192.6 ± 24.6
Relative neutrophil (%)	49.0 ± 2.2	12.4 ± 3.7	46.3 ± 5.5
Relative lymphocyte (%)	34.0 ± 2.4	85.2 ± 4.6	40.6 ± 3.9
Absolute lymphocyte (x 10^9/L)	1.9 ± 0.15	99.0 ± 31.0	3.2 ± 0.5
Urea (mg/dl)	24.3 ± 1.7	35.8 ± 7.6	27.2 ± 2.0
Creatinine (mg/dl)	0.88 ± 0.03	1.1 ± 0.09	0.86 ± 0.05
AST (IU/L)	21.1 ± 1.9	29.1 ± 3.1	27.6 ± 4.4
ALT (IU/L)	15.5 ± 1.7	18.3 ± 2.5	18.8 ± 2.8
TNF (pg/ml)	186.6 ± 4.7	217.5 ± 23.4	216.0 ± 64.4
IL-6 (pg/ml)	2.6 ± 0.3	11.6 ± 2.3	7.4 ± 1.6
IL-18 (pg/ml)	67.2 ± 4.0	346.2 ± 81.7	193.0 ± 28.3

Table 2: Cytokines concentration among CLL stages (mean ± S.E.)

	Controls (n=10)	Stage II (n=27)	Stage III-IV (n=18)	Total (n=45)
TNF-α (pg/ml)	186.6 ± 4.7	179.8 ± 25.7	238.5 ± 97.0	217.5 ± 23.4
IL-6 (pg/ml)	2.6 ± 0.3	16.0 ± 4.7	8.7 ± 1.8	11.6 ± 2.3
IL-18 (pg/ml)	67.2 ± 4.0	324.1 ± 99.4	361.9 ± 123.6	346.2 ± 81.7

Table 3 Cytokines concentration in survived and non-survived patients (median).

	Statistics	Control	CLL	
			Dead (n=21)	Alive (n=21)
TNF-α (pg/ml)	Median	186.3	340.7	160.8
	Minimum	169.4	159.3	109.6
	Maximum	217.2	1739.2	278.9
	P (*vs.* Control)	---	0.01	NS
	P (Dead vs. Alive) = 0.025			
IL-6 (pg/ml)	Median	2.4	7.4	10.0
	Minimum	1.62	4.5	3.8
	Maximum	4.55	20.6	35.8
	p (*vs.* Control)	---	NS	0.0355
	P (dead *vs*. alive) = NS			
IL-18 (pg/ml)	Median	72.5	269.5	312.3
	Minimum	45.3	95.7	151.9
	Maximum	80.6	428.2	1302.3
	P (*vs.* Control)	---	NS	0.0298
	P (dead *vs*. alive) = NS			

*P<0.*05 is significant., NS= not significant

Table 4. Statistical analysis for CRP level before and after therapy in both groups.

		Friedman Test				
		N	Mean±SD	Range		*P* value
				Min.	Max.	
CLL	Pre-therapy	33	3.18±1.78	1	6	0.005
	Post-therapy	33	1.64±0.81	1	3	

*P<0.*05 is significant

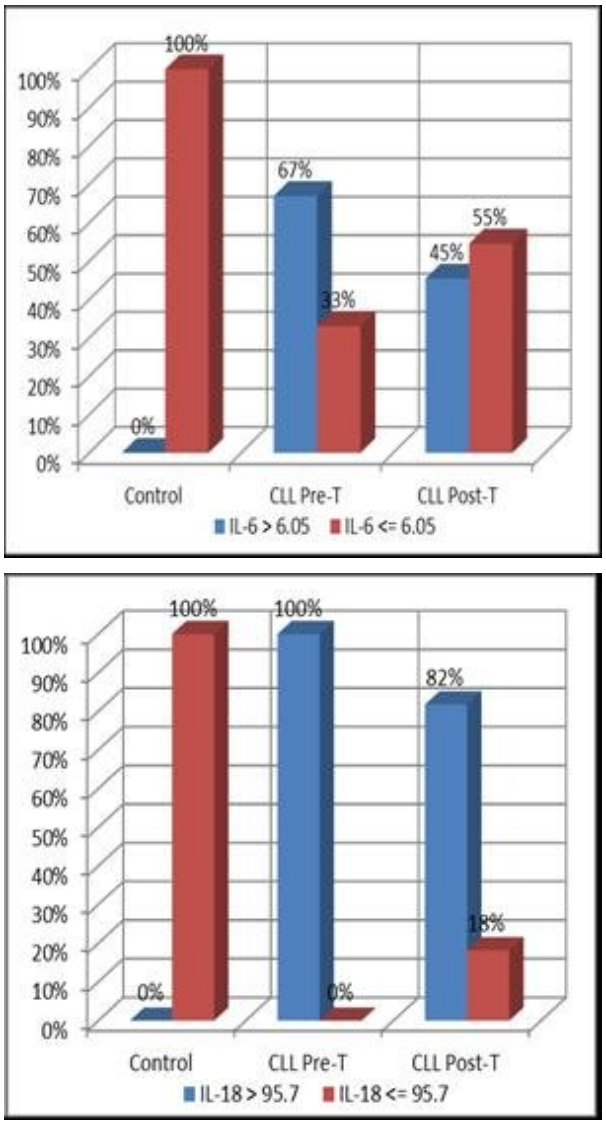

Figure 1. Showing percent of patients with normal or abnormal IL-6 and IL-18, (Using cut-off value= 6.05 pg/ml for IL-6 and 95.7 pg/ml for IL-18).

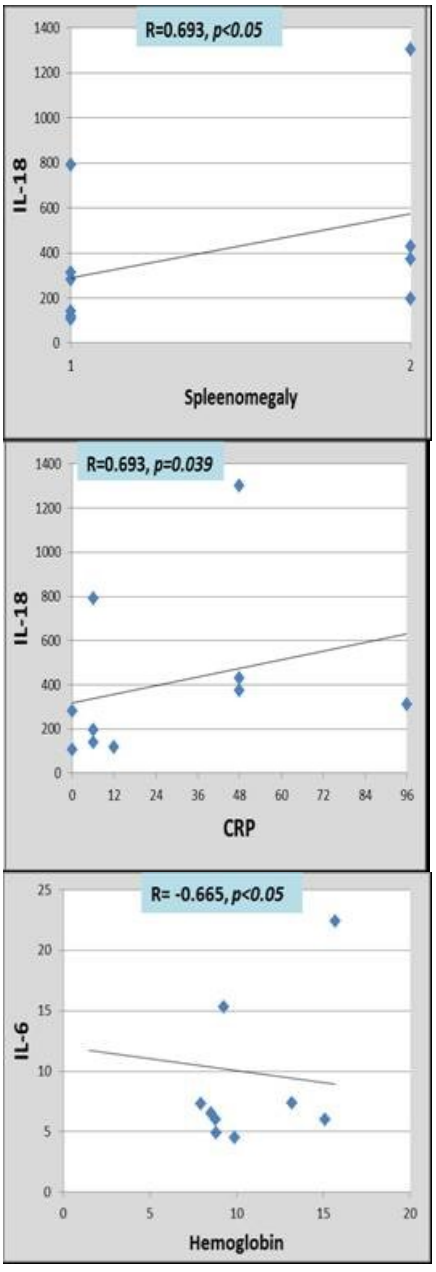

Figure 2. Showing various correlations between IL-18, splenomegaly and CRP.

Cytokines correlation with other parameters

Correlation analyses of our data revealed that IL-6 level was directly correlated to AST *(P<0.004)* and inversely correlated to Hemoglobin, *(P*<0.05). IL-18 correlated positively with the presence of splenomegaly *(P*<0.039) and CRP level *(P*<0.039). It is obvious that the normalization of immunological makers is expected to correlate with hematological and chemical in hematological remission.

Discussion

A substantial proportion of CLL patients are diagnosed prior to the age of 60, and that 70% of CLL patients will die either directly or indirectly by their disease, (Tam & Khouri, 2009). There is a need to the establishment of prognostic markers in chronic lymphocytic leukemia for good prognosis and thereby approve the therapeutic decision, (Hamblin, 2006).

The imbalance between pro-inflammatory (TNF-α) and anti-inflammatory cytokines may favor the induction of chronic inflammation and the blockage of pro-inflammatory cytokines like TNF-α or IL-6 can prevent disease progression in some autoimmune disease, (Balague *et al.*, 2009)**.**

TNF-α is a crucial pro-inflammatory cytokine in the establishment and maintenance of inflammation in multiple autoimmune and non-autoimmune disorders. It was suggested that TNF-α neutralization seems to be a useful target in a variety of disorders, (Anand *et al.*, 1998a)**.** Similar results were reported that TNF-α level was non-significantly different from normal levels and was elevated only in secondary complications, (Anand *et al.*, 1998a) and (Aref *et al.*, 2007), even though others found its level was higher than normal controls in some leukemia, (Kiersnowska-Rogowska *et al.*, 2005). This increase can be explained due to high expression of enzymes that block the TNF-α surface receptors, (Kiersnowska-Rogowska *et al.*, 2005). This data were in ordinance with (Han *et al.*, 2007), that the change in TNF-α before and after chemotherapy in leukemic patients was previously explained due to changes in the immunological response. They have implied that the release of some pro-inflammatory cytokines may promote tumor growth and hence influence survival (Han *et al.*, 2007)**.**

Proteins of (TNF) family are implicated in the regulation of essential cell processes such as proliferation, differentiation, survival and cell death, (Planelles *et al.*, 2008). It may be involved in more complex interactions between a leukemic clone and normal bone marrow cells which provide a favorable environment for leukemic cells to survive, (Bojarska-Junak *et al.*, 2008)**.**

A remarkable notification by, (Lichtman, 2007), have stated that patients with CLL are prone to developing systemic autoimmune disease, explaining that the autoantibody might be produced by the neoplastic B cell clone, or mostly by bystander non-neoplastic B cells, reflecting a disease-associated dysregulation in humoral immune tolerance to self-antigen, (Lichtman, 2007). Consequently patients with CLL who generally do not appear to have an increased incidence of pathologic autoimmunity other than that directed against hematopoietic cells might develop pure red blood cell aplasia or neutropenia secondary to the development of autoantibodies against marrow hematopoietic progenitor cells. Altered expression of TNF which is often associated with pathological conditions can play a role in developing autoimmune disease, (Planelles *et al.*, 2008).

A warning report by FDA that Anti-TNF-α inhibitors have been largely administered to patients affected by autoimmune diseases, considering the pivotal role of the pro-

inflammatory cytokine TNF-α in the pathogenesis of these disorders, (Caramaschi *et al.*, 2009).

In the present study, plasma TNF-α level was determined in CLL patients in different stages, before and after treatment, and its level were compared to that of normal controls for both genders, also between its initial levels found in survivors versus non-survivals.

Results revealed that TNF-α level of CLL patients before treatment was non-significantly higher from that of healthy controls. However, by comparing its initial level in alive patients versus those who died, we found that the non-survivor CLL patients had significantly higher TNF-α levels as compared to either healthy controls (83 %; $P<0.01$) or the alive ones (112%, $P< 0.025$).

Studies on CLL by (Anand *et al.*, 1998a; Anand *et al.*, 1998b) showed that TNF-α was not significantly different from those of healthy controls and was elevated only in secondary complications, which agree with the present results. They inferred that TNF-α induced B-CLL growth is decreased by IL-6 secretion, and that TNF-α is a known autocrine growth factor for lymphocytes in CLL. In the meantime, (Ferrajoli *et al.*, 2002; Bojarska-Junak *et al.*, 2008), found that patients with malignant tumors including CLL have high plasma levels of TNF-α. Similarly, (Adami *et al.*, 1994), found that increased values of TNF-α were detected in B-CLL patients, compared to normal age-matched controls, and was observed in all stages with a progressive increase in relation to the stage of the disease.

Changes in TNF-α before and after chemotherapy in leukemic patients were previously explained to be due to changes in the immunological response, it had been implied that the release of some pro-inflammatory cytokines may promote tumor growth and hence influence survival. Correspondingly, (Balague *et al.*, 2009), reported that, the imbalance between pro-inflammatory (TNF-α) and anti-inflammatory cytokines may favor the induction of autoimmunity, chronic inflammation and tissue damage. In addition, the blockage of pro-inflammatory cytokines like TNF-α or IL-6 can prevent disease progression in some autoimmune disease like rheumatoid arthritis.

Ferrajoli and others (Ferrajoli *et al.,* 2002) and (Tsopra et al., 2009), had found that serum TNF- α level was increased in anemic CLL patients, thus directly inhibit the erythroid development in early stages of erythropoiesis. They concluded that CLL-related anemia was not due to intrinsic defects of erythroid precursors or erythropoietin, but due to the direct suppressive effect of TNF- α on erythroid and thrombopoietic lineages causing extensive bone marrow replacement by neoplastic B-cells which leads to anemia and thrombocytopenia in CLL patients.

A highly conserved acute phase reactant, CRP, with pluripotent properties has an important constituent of the innate immune system, playing pro-inflammatory as well as anti-inflammatory effects, where raised levels occur mainly to inflammation, (Anand *et al.*, 1998a). In this study, the elevated CRP level before therapy in the leukemic patients which is attributed to chronic inflammation, which agrees with (Fang *et al.*, 2004) and (Takamura *et al.*, 1983), and its level is significantly declined after therapy ($P<0.005$).

Interleukin-6 (IL-6) a pleiotropic cytokine of biological activities in the immune regulation, hematopoiesis, inflammation, and oncogenesis, (Tadamitsu, 2003), has the capacity to orchestrate transition from innate to acquired immunity, (Youinou & Jamin, 2009).

Unexpectedly, IL-6 level was found to be non-significantly higher in CLL patients than controls; it starts to decline after chemotherapy. The most significant correlation was found between IL-6 levels and

AST reflecting the influence of IL-6 on liver function, suggesting that IL-6 was associated with a high burden of the disease reflecting the secondary immune system activation, (Anand *et al.*, 1998a) and (Panteli *et al.*, 2005).

As an IFN-γ inducer, IL-18-mediates immune responses, it overcomes pathogenic, viral, bacterial and fungal infection by activated immune cells, (Dinarello and Fantuzzi, 2003) and (Vidal-Vanaclocha *et al.*, 2006). Nevertheless, IL-18 itself may play a potentially harmful role in the establishment of auto-reactivity due to impairment of the non inflammatory clearance of apoptotic cells and the increased rate of apoptosis, (Wittmann *et al.*, 2009), nevertheless it can also play a damaging role in some diseases like arterial damage which implies a contribution of IL-18 to the pathophysiology of cardiovascular disease, (Vlachopoulos *et al.*, 2010).

IL-18 was non-significant higher than controls. Correspondingly, cancerous lungs and bone metastases cells markedly produced IL-18 suggesting that elevated serum IL-18 levels may be associated with IL-18 producing cancer cells, (Okamoto *et al.*, 2009). Similarly, (Zhang *et al.*, 2003), have reported that plasma IL-18 level in leukemia patients and in normal control was similar. Their study estimated the value of IL-18 level in leukemic patients was significantly lowered than those of normal level after receiving chemotherapy for 2 weeks. They resolved that the decrease in IL-18 may be due to immunologic hypo-function, (Zhang, *et al.*, 2003). However, in the present study post-treatment levels were determined at first hematological remission.

Accordingly, it is observed although IL-6 and IL-18 play a major role in immune modulation, they are not yet valid for disease and therapy monitoring. TNF-α needs to be further studied on large scale population for its role in autoimmune development and the overall survival in CLL patients.

Thus, in conclusion, TNF-α is obligatory to be studied on large scale population to confirm our data, TNF-α blocker therapy may be a good adjuvant therapy for evading the clinical outcomes of the disease and autoimmune complications.

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