



# Clinical significance of interleukin-4 and interleukin-18 levels in aggressive non-Hodgkin's lymphoma patients

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Genet. Mol. Res. 15 (3): gmr.15038590

Received March 1, 2016

Accepted May 5, 2016

Published July 29, 2016

DOI <http://dx.doi.org/10.4238/gmr.15038590>

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**ABSTRACT.** Strong evidence indicates that tumor growth can be actively controlled by the immune system, and interleukins (ILs) are known to play an influential role in immune response regulation. Moreover, inflammatory cytokines are significantly involved in lymphoma pathogenesis. We aimed to investigate serum levels of IL-4 and IL-18 in aggressive non-Hodgkin's lymphoma (A-NHL) patients and their relationship with prognostic parameters and therapy outcome. These serum factors were measured by enzyme-linked immunosorbent assay in 46 patients with pathologically verified A-NHL before and after chemotherapy, and in 20 healthy controls. No significant difference in serum IL-4 ( $P=0.11$ ) and IL-18 ( $P=0.261$ ) levels was observed between the A-NHL and controls groups. None of the prognostic parameters analyzed significantly correlated with serum IL-4 concentration, while

only lactate dehydrogenase (LDH) measurements were associated with IL-18 values. Serum IL-18 was elevated in the patients with high LDH levels compared to those exhibiting normal values ( $P = 0.045$ ). In addition, no correlation was found between the concentrations of serum IL-4 and IL-18 in A-NHL patients ( $r = 0.188$ ,  $P = 0.187$ ). While IL-18 values did not change, serum IL-4 levels decreased following chemotherapy, independently from treatment response ( $P = 0.002$ ). Our study is the first to report the response of serum IL-4 levels to chemotherapy. In conclusion, although IL-4 serum concentration has no diagnostic role, it is sensitive to standard chemotherapy in A-NHL. However, serum IL-18 measurements have no diagnostic or prognostic role in this disease.

**Key-words:** IL-4; IL-18; Non-Hodgkin's lymphoma; Cytokines

## INTRODUCTION

Although the etiology of non-Hodgkin's lymphoma (NHL) remains unclear, subclinical immune dysfunction is the most consistently identified risk factor, and dysregulation of cytokines may mediate its progression (Vendrame and Martínez-Maza, 2011). A variety of NHL subtypes are recognized, but cases are generally classified into two major groups: B- and T-cell lymphomas. Of the former, diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) are the two most common subtypes. The exact causes of NHL remain largely unknown. However, immune dysregulation has been considered a hallmark of lymphoma (Zhang et al., 2015).

Aggressive NHLs (A-NHL) represent a heterogeneous group of malignancies that vary in biological pattern and outcome (Turner et al., 2010). The diagnosis and classification of NHL continue to be based on morphology and immunophenotype, and specific genetic changes are not taken into account in the definition of lymphoma subtypes. Certain recurrent molecular genetic abnormalities in NHL are associated with prognosis or represent potential therapeutic targets, thus there exists an emerging role for marker testing to help predict outcomes and guide therapy (Miles et al., 2016). Approximately half of A-NHL patients cannot be cured using standard treatment (Alici et al., 2003); therefore, the identification of new markers of treatment response and outcome is of some importance. Despite the description of a number of potential biomarkers, few have been integrated into clinical practice. The establishment of minimally invasive and easily accessible early diagnostic indicators is therefore necessary. Interleukins (ILs) play important roles in the pathogenesis of lymphomas and appear to be potential markers of tumor cell growth including that of NHL.

IL-4 is a cytokine produced by activated T lymphocytes (Paul, 1991). It exhibits pleiotropic activity, and can cause tumor regression via immunomodulation and/or direct anti-proliferative effects. The effects of IL-4 include: stimulating growth of B and T lymphocytes (Spits et al., 1987); enhancing lymphokine-activated killer cell and cytotoxic T lymphocyte activity, especially when combined with IL-2 (Treisman et al., 1990); promoting growth of tumor-infiltrating lymphocytes (Kawakami et al., 1988); and stimulating differentiation and proliferation of hematopoietic cells (Saito et al., 1988).

Several reports have proposed a rationale for using IL-4 as therapy for patients with lymphoid malignancies (Parkinson et al., 1993). However, few prospective studies have

evaluated pre-diagnostic cytokine levels and subsequent NHL risk in immunocomponent populations (Purdue et al., 2011). IL-4 also modulates expression of human leukocyte antigen (HLA) class I and II proteins and cell adhesion molecules. These factors are relevant to lymphoma immunosurveillance and tumorigenicity, and are of particular clinical pertinence to large-cell lymphoma, in which the absence of HLA-DR expression is associated with poorer patient survival following chemotherapy (Miller et al., 1988).

IL-18, a member of the IL-1 family, is synthesized as an inactive precursor before being converted to a bioactive form because of cleavage by an IL-1 $\beta$ -converting enzyme (Tas et al., 2015). IL-18 is a 18.3-kDa multifunctional cytokine synthesized by Kupffer cells, activated macrophages, keratinocytes, intestinal epithelial cells, osteoblasts, and adrenal cortex cells (Gillespie and Horwood, 1998). Human IL-18 stimulates cytotoxic natural killer (NK) cell activity and promotes the production of interferon- $\gamma$ , IL-2, and granulocyte/macrophage colony-stimulating factor by T cells (Ushio et al., 1996). Increased plasma IL-18 levels have been observed in acute lymphoblastic leukemia and chronic myelocytic leukemia patients (Taniguchi et al., 1997). Thus, IL-18 could be a useful and sensitive serum indicator of therapeutic effect and clinical course in haematologic malignancies (Taniguchi et al., 1997; Takubo et al., 2000a; Takubo et al., 2000b).

Knowledge of the serum levels of these cytokines in newly diagnosed A-NHL patients may help inform us of the possible prognosis and development of this disease, and decide on appropriate individualized therapeutic approaches.

This study was conducted to investigate IL-4 and IL-18 serum levels in A-NHL patients before and after chemotherapy and establish their relationship with prognostic parameters.

## MATERIAL AND METHODS

### Patients

Serum samples were obtained from 46 consecutively recruited A-NHL patients who had been referred to Istanbul University Institute of Oncology.

The clinical characteristics of these patients are summarized in Table 1. A number of clinical and pathological parameters were recorded, including age, tumor stage and histology, B-symptoms, extranodal lesions, performance status, bone marrow invasion, and serum lactate dehydrogenase (LDH) and  $\beta$ 2-microglobulin levels. Histopathological diagnosis was in accordance with the World Health Organization classification system (Turner et al., 2010).

The study population consisted of patients with newly diagnosed aggressive lymphoma, defined as DLBCL or grade III FL. As low-grade lymphomas are of lower priority in clinical and treatment terms, they were not included in the present study. Initial staging according to the Ann Arbor system was performed at diagnosis before initiation of treatment. Staging procedures included a computed tomography scan of the chest, abdomen and pelvis and unilateral bone marrow biopsy. Serum samples were obtained on admission and after completion of chemotherapy. All patients were treated with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone at 3-week intervals. None of the patients had received previous therapy, including immunomodulators, cytokines or steroids.

Pregnant individuals, or those with severe infections, known allergic diseases, or poor performance status were excluded. Written informed consent was obtained from all participants before the investigation began and our study was approved by the Istanbul University Istanbul

Faculty of Medicine Ethical Committee (No.: 772). Control subjects (N = 20) were recruited from among the Istanbul University Institute of Oncology personnel, and all were in excellent health at the time that the study was carried out.

**Table 1.** Patient Characteristics.

Parameter		Value	%
No. of patients		46	100
Gender	Male	28	60.9
	Female	18	39.1
Age (median, years)		52	
≤60		34	74
>60		12	26
Histology	Diffuse large B cell	40	86.9
	Follicular grade III	6	13.1
Stage	I	15	32.6
	II	15	32.6
	III	7	15.3
	IV	9	19.5
Response rate	Complete	39	84.7
	Partial	7	15.3
B-symptoms	A (Absent)	27	58.6
	B (Present)	19	41.4
Performance status	0	24	52.2
	1	14	30.4
	2	8	17.4
Bone marrow	Positive	2	4.3
	Negative	44	95.7
Extra nodal lesion	Yes	17	37
	No	29	63
Serum LDH level	≤1x normal	23	50
	>1x normal	23	50
Serum β2- microglobulin	≤1x normal	23	50
	>1x normal	16	34.88
	Absent	7	15.21

LDH = lactate dehydrogenase.

## Measurement of IL-4 and IL-18 levels

Circulating IL-4 and IL-18 levels were evaluated by solid-phase enzyme-linked immunosorbent assay (ELISA; BioSource International, Inc., Camarillo, CA, USA) using 96-well microplates following the manufacturer's protocol.

This assay comprises microtiter well strips coated with a monoclonal antibody specific for IL-4. Samples, including standards of known IL-4 content, control specimens, and unknowns, are then pipetted into the wells, followed by addition of a secondary biotinylated monoclonal antibody. During the first incubation, the IL-4 antigen binds to the immobilized (capture) antibody at one site and to the fluid-phase biotinylated antibody at a second site. After removal of excess secondary antibody, peroxidase-conjugated streptavidin is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to completely remove unbound enzymes, a substrate solution is added, which is acted upon by the bound enzyme to produce color. Color development is arrested with Stop Solution and its intensity measured at 450 nm using an ELISA plate reader (ChroMate 4300; Awareness Technology

Inc., Palm City, FL, USA).

Similarly, for detection of IL-18, microtiter strips coated with a specific IL-18 monoclonal antibody were used. Samples, including standards of known IL-18 content, control specimens, and unknowns, were pipetted into these wells, and incubated for 1 h. During this first incubation, IL-18 is captured and immobilized on the plate. After washing, a horseradish peroxidase (HRP)-conjugated anti-IL-18 antibody was added and the reaction incubated for 1 h. The binding of this HRP conjugated antibody to the wells completes a three-member solid-phase sandwich. After a second wash step, HRP activity was detected by addition of 3,3',5,5'-Tetramethylbenzidine Substrate Reagent which produces color in proportion to the quantity of IL-18 contained in each sample.

At the end of the incubation period, Stop Solution was added to terminate the HRP-catalyzed reaction and stabilizes the color formed. The absorbance of each well at 450 nm was then measured using a microtiter plate reader (ChroMate™ 4300; Awareness Technology Inc., Palm City, FL, USA). IL-4 and IL-18 levels are expressed in picograms per milliliter.

### Statistical analysis

Data analyses were performed using SPSS 16 (SPSS Inc., Chicago, IL, USA). The Wilcoxon signed-rank test was used to identify the statistical significance. Correlations were assessed with Spearman's correlation test, and P values < 0.05 were considered significant.

## RESULTS

There were no significant differences in serum IL-18 (P = 0.261) and IL-4 (P = 0.11) levels between the patient and controls groups. Table 2 shows IL-18 and IL-4 levels in the A-NHL patients and control subjects.

**Table 2.** Serum IL-18 and IL-4 values in non-Hodgkin's lymphoma patients and healthy controls.

	Patients (N = 46)		Controls (N = 20)		P
	Median	Range	Median	Range	
IL-18 (pg/mL)	307.1	(29.6-1965.8)	241.2	(104.5-1734.2)	0.261
IL-4 (pg/mL)	16	(9-27)	14	(9-19)	0.11

None of the prognostic parameters analyzed significantly correlated with serum IL-4 concentration. This was also true for serum IL-18 values, with the exception of LDH. Serum IL-18 levels were elevated in patients with increased LDH compared to those with normal readings (P = 0.045). In addition, we found no correlation between IL-18 and IL-4 measurements among A-NHL patients (r = 0.188, P = 0.187). Table 3 shows the relationships between serum IL-18 and IL-4 levels and clinicopathological factors.

When we analyzed the effect of chemotherapy on serum parameters, we found that IL-4 levels had decreased following treatment, independently of response to from therapy (P = 0.002). However, serum IL-18 values were unchanged (P = 0.528; Table 4).

**Table 3.** Relationships between serum interleukins and various demographic and disease characteristics.

Parameter	Comparison	IL-18	IL-4
Age	(≤60 vs >60)	ns	ns
Gender	(Male vs female)	ns	ns
Histology	(Diffuse large cell vs others)	ns	ns
Stage	(I,II vs III,IV)	ns	ns
Performance status	(0.1 vs 2)	ns	ns
Response Rate	(Complete vs partial)	ns	ns
B-symptoms	(Absent vs present)	ns	ns
LDH	(≤1x normal vs >1x normal)	0.045	ns
Serum β2- microglobulin	(≤1x normal vs >1x normal)	ns	ns
Bone marrow involvement	(Positive vs negative)	ns	ns
Extra nodal lesion	(Yes vs no)	ns	ns
Progression	(Absent vs present)	ns	ns

LDH = lactate dehydrogenase, ns = not significant.

**Table 4.** Distribution of serum IL-18 and IL-4 values in patients with non-Hodgkin's lymphoma before and after chemotherapy.

	Before chemotherapy		After chemotherapy		P
	Median	Range	Median	Range	
IL-18 (pg/mL)	307.1	(29.6 - 1965.8)	301.3	(72.8 - 1910.7)	0.528
IL-4 (pg/mL)	16	(9 - 27)	14.3	(9.3 - 19.2)	0.002

## DISCUSSION

The communication between malignant cells is considered as important as the genetic or epigenetic changes acquired by tumor cells. The bone marrow microenvironment contains hematolymphoid and stromal compartments, each, composed of a large number of cell types, including hematopoietic stem, blood, endothelial, and stromal cells. These secrete a wide array of soluble factors, orchestrating the survival and proliferation of transformed cells (Yu et al., 2016).

Modern techniques have demonstrated that cytokines exert broad and intriguing cell-regulatory effects on a heterogeneous group of cell, forming a “cytokine network” (Fritsch et al., 1980). One current area of cytokine research concerns their role in the proliferation of neoplasms. Among human lymphoid malignancies, B-cell neoplasms have received the most attention in this respect (Cordingley et al., 1988; Biondi et al., 1989).

There exists strong evidence that tumor growth can be actively controlled by the host immune system and ILs are known to play a significant role in immune response regulation (Vendrame and Martínez-Maza, 2011). Previous studies have shown that; polymorphisms in a gene encoding an inflammatory mediator affect the biological activity of the corresponding protein, and may be associated with oncogenesis (Chen et al., 2015).

Recent reports have also revealed relationships between several ILs and nasopharyngeal, prostate, cervical, pancreatic, gastric, breast, and esophageal cancers, in addition to NHL (Guney et al., 2009; Chen et al., 2015; Li et al., 2015; Sun et al., 2015; Tas et al., 2015; Yu et al., 2015; Karabulut et al., 2016).

Inflammatory cytokines have an important influence on lymphoma pathogenesis and may reflect underlying biological processes, such as tumor-host interactions, providing prognostic information not afforded by conventional clinical parameters. Cytokines secreted



by tumor or host cells function as key mediators and/or regulators of tumor-host interactions causing the progression of malignancies (Turner et al., 2010).

IL-18 is a multifunctional cytokine of 18.3 kDa synthesized by Kupffer cells, activated macrophages, keratinocytes, intestinal epithelial cells, osteoblasts, adrenal cortex cells, and the murine diencephalon (Gillespie and Horwood, 1998). In addition to multiple activities relating to its immunity-stimulating capacity, it exerts antitumor effects mediated by enhancement of NK cell activity, reduction of tumorigenesis, induction of apoptosis, and inhibition of angiogenesis in tumor cells. Recent data suggest that abnormal IL-18 production contributes to the pathogenesis of malignancies and may influence clinical outcome (Tas et al., 2015). Increased IL-18 levels have been detected in hematologic malignancies, for which this cytokine may serve as a sensitive serum marker of therapeutic effect and clinical course (Taniguchi et al., 1997; Takubo et al., 2000b).

IL-4 is produced by a sub-set of CD4<sup>+</sup> T cells, basophiles and mast cells. It affects various steps in the human B-cell antigen-dependent maturation process and increases the expression of several functionally important surface antigens. IL-4 also regulates the differentiation of antigen-stimulated naïve T-cells which go on to produce IL-4 and a series of other cytokines including IL-10. Moreover, IL-4 is a key factor in tissue adhesion and the stimulation of B-cell growth (Nelms et al., 1999).

The aim of our study was to investigate serum IL-4 and IL-18 levels in A-NHL patients. The results were evaluated in relation to tumors prognostic parameters and chemotherapy.

No significant difference was established between the patient and control groups in terms of serum IL-4 and IL-18 levels. It is well known that serum IL-2, IL-6, and IL-10 concentrations are significantly higher in A-NHL patients than in healthy blood donors (Stasi et al., 1995; Fabre-Guillevin et al., 2006; Yu et al., 2015). Fabre-Guillevin et al. (2006) tested a large panel of cytokines using samples from A-NHL patients. They demonstrated that only IL-2 and IL-6 serum levels are elevated in high-grade NHL, while IL-4 concentration does not appear to be predictive of clinical development.

Contrary to our findings, Amo et al. (2001) found that serum IL-18 values are increased in cutaneous NK/T-cell lymphoma patients. Similarly, Taniguchi et al. (1997) reported that IL-18 levels in two groups of patients with acute lymphocytic and chronic myelocytic leukemia were significantly increased compared to the control group. In two studies carried out by Takubo et al. (a,c) IL-18 was also elevated in individuals with various hematologic disorders and 27 patients with NHL. The authors suggested that IL-18 may be produced in excess by lymphoma cells as a tumor-associated protein in hematological malignancies. Takada et al. (1999) found increased serum levels of this cytokine in patients with hemophagocytic lymphohistiocytosis, including those with anaplastic large-cell lymphoma or Epstein-Barr virus (EBV) infection. It has been suggested that tumor necrosis factor- $\alpha$  produced by T-cell lymphomas or EBV-infected T cells stimulates macrophages to produce IL-18 (Takada et al., 1999).

A second objective of our study was to investigate the relationship between serum prognostic factors and IL-4 and IL-18 levels. None of the prognostic parameters analyzed significantly correlated with serum IL-4 concentration. However, we determined a statistically significant relationship between IL-18 and LDH values.

The International Prognostic Index (IPI), taking into account A-NHL patient age, stage, LDH level, performance status, and number of extranodal sites, was devised by the International Non-Hodgkin's Lymphoma Prognostic Factor Project (1993). These criteria are used to determine aggressive lymphoma treatment plans and prognoses. There are several

examples in the literature, suggesting that high serum cytokine levels are important prognostic factors in A-NHL (Aydin et al., 2002; Guler et al., 2014; Uskudar Teke et al., 2014).

In an investigation by Fabre-Guillevin et al., (2006) serum IL-2, IL-4, IL-6, and IL-10 concentrations were compared against only two components of the IPI: B symptoms and Ann Arbor stage. In agreement with our results, no correlation established between IL-4 levels and prognostic factors. Further, we found a relationship between raised IL-18 and LDH measurements. To our knowledge, this is the first report to demonstrate such an association.

Also of note in the present study is the serum IL-4 level reduction that occurred because of chemotherapy, regardless of response to such treatment. We understand this to be the first study in which administration of chemotherapy triggered significant decrease in serum IL-4 levels.

This is the first report to measure the concentration of IL-18 and IL-4 by ELISA using serum samples from healthy individuals and A-NHL patients having received chemotherapy. However, two limitations to our study should be considered. First, owing to the rarity of A-NHL, the patient sample size in our study was relatively small and this may have limited our ability to identify statistically significant differences between group. Second, the development of A-NHL may be influenced by many other factors besides changes in IL activity.

In conclusion, it is not possible to determine the value of serum levels of these important cytokines in A-NHL patients based on this small study. The measurement of ILs in serum samples may be a cost effective, convenient and minimally invasive method of utilizing these easily accessible potential biomarkers of treatment response and outcome. Larger-scale research is needed in this field, and exciting new finding will ultimately ensue. In addition, considering the critical role that they play in the differentiation and proliferation of normal and neoplastic B-cells, further investigation of the biological impact of high levels of cytokines on lymphoma pathogenesis and progression is warranted.

### Conflicts of interest

The authors declare no conflict of interest.

### ACKNOWLEDGMENTS

Research supported by the Istanbul University Research Fund (#1825).

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