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CLEC4E as Novel Tumor Marker. A Biochemical Study for Prediction Acute Lymphocytic Leukemia at Iraqi Children

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Abstract

During the period between the beginning of February 2016 and the end of October 2017, 60 individuals were enrolled in the present work. In the Center of Oncology for Hematology of Al-Hussein Medical City in Karbala, Iraq, 30 sera samples from patients with ALL who had no family history of cancer were collected, before receiving chemotherapy. The statistical analysis using the student's *t*-test showed significant differences ($p = 0.000$ for lectin and $p = 0.005$ for MDA) when the two study groups (patients with ALL and healthy individuals) compared together. The study showed a statistically significant increase ($p = 0.005$) in male patients with ALL compared with their corresponding males in the control group. This finding was consistent with the results of comparing lectin levels in female patients with the control group ($p = 0.019$), while no significant differences were observed in CLEC4E levels when the intra-subgroups compared, separately. The highest concentration and activity (36.062 g / L and 168,660 U / L) of Ceruloplasmin Oxidase were recorded in the samples of females with ALL. The study Results showed that this type of lectin is naturally produced in humans, but its levels are elevated when the incidence of ALL, where the study recorded a rise in CLEC4E levels in 26 of the 30 resident samples (sensitivity 87%), in addition, It has been found that the levels of this protein increase with the progressive of cancer.The study showed decrease in the serum CLEC4E levels at 80% of patients with ALL after treated with chemotherapy. The sensitivity of the evaluated lectin in the sera samples of patients with ALL reached to 100% when the levels of lectin were combined to the levels of MDA.

Key words: CLEC4E, ALL, Chemotherapy, Oxidative Stress, MDA, Ceruloplasmin Oxidase

INTRODUCTION

The rate of infection with cancer has increased with a clear rise in the incidence of leukemia, which is the second most common cancer in 2017 [1] after it was ranked seventh in 1989 [2**],** and leukemia is the number one cancer in children [3]. The incidence of leukemia varies according to age, sex, race and geographical distribution globally. Ten of the 100,000 people develop leukemia, while acute lymphoblastic leukemia (ALL) accounts for half the number of patients [4], particularly leukemia males are more likely than females [5], the ratio of males to females is 2:3 in acute types of leukemia and 1:2 in lymphocytic leukemia, while 1.3:1 in leukemia. Acute leukemia in all age groups, while cases of acute myeloma infarction in adults are increasing with age, on the other hand, previous researches showed people between 40 and 60 years are most susceptible to leukemia, finally reach the highest rate of infection with ALL in children from 3 to 7 years [6].

Lectin (Agglutinin) was firstly isolated from plant in the last decade of the 19th century, diagnosed by the ability to attach to the red blood cells [7], then many types of lectin were characterized in numerous organs [8-11]. Lectin has many different functions in different cells ranging from its role in controlling the production of sugary proteins and increasing cell interrelationships to its contribution to stimulating and developing the immune system against diseases by stimulating the division of lymphocytes [12] At the end of the twentieth century began the first attempts to investigate the presence of the Lectins in the human body, after the isolation and diagnosis of several types of samples of tissue and human serum [13,14]. Generally, lectins classified in two large groups based on their mineral dependence. The first: accredited adhesives for the presence of calcium ion (Ca^{2+}) (C-Type Lectins) and the second group: the independent calcium group (S-Type Lectins) [15].

CLEC4E is one of the human C-Type Lectins family, it contains both water-soluble and complementary proteins in the cell membrane, and a sequential amino acid sequence of approximately 15% carbohydrate content [16]. Ca^{2+} ion is one of the essential requirements in the process of carbohydrate binding and the effectiveness of metabolism [17].

Cellular Oxidative Stress (COS) known as oxidants and antioxidants imbalance for the profit of oxidizing molecules [18,19]. OS occurrence associated with many natural conditions [20,21], as well as; with wide range of pathological conditions [22-26]. Finally, many studies have indicated that OS is associated with different types of cancers [27]. Several guides are used to look for levels of OS in the body, including malondialdehyde (MDA), which is produced by the oxidation of unsaturated fat, or one of the end products of oxidation of unsaturated fatty acids, lipid peroxidation [28]. Moreover; measurement of intracellular antioxidants (like ceruloplasmin oxidase, glutathione Reductase, and superoxide dismutase) presented as tools for evaluation of OS.

Aim of Study

Investigation of CLEC4E as a new tumor marker for prediction, detection of ALL in the samples of children who haven't familiar history with cancers, and follow up the response of patients for treatment chemotherapy

SAMPLES AND METHODS

Subjects: During the period between the beginning of February 2016 and the end of October 2017, 60 individuals were enrolled in the present work. In the Center of Oncology for Hematology of Al-Hussein Medical City in Karbala, Iraq, 30 sera samples from patients with Acute Lymphocytic Leukemia (ALL) ranging between 1-13 years old (6.80 ± 3.79) who had no family history of cancer were collected, before receiving chemotherapy. The initial diagnosis was performed by specialist physicians by clinical and laboratory tests. The full information about the current study patients was provided through oral interviews with patients' families, in cooperation with their supervisors and according to the questionnaire in the current work questionnaire prepared in advance, based on the opinion of the specialists. Healthy individuals were selected as a control group according to a set of criteria and determinants, total data about the study groups were summarized in **Table 1.**

Methods: Sandwich-ELISA technique was applied to estimate levels of lectin in the serological samples of the two current study groups using Human CLEC4E Kit that prepared by Elabscience Company, China. MDA concentrations were measured using thiobarbituric acid. Levels of activity and concentration of ceruloplasmin oxidase were evaluated using the Rice method [29].

The statistical analysis of the results obtained in the present study was carried out using the 20th edition of the Statistical Package for the Social Science (SPSS). The results were expressed in terms of Mean ± Standard Deviation (Mean ± S.D.) using the student's *t*- test the statistical comparison was conducted between the two main study groups, while the analysis of variance (ANOVA) was used to compare the results of the four subgroups included in the study based on gender differences. The results were statistically significant at 5% probability ($p<0.05$).

Table 1: Distribution of Study Samples According to Their Age and Gender

Subjects (n)	Gender (n)	Age (Year) Mean ± S.D.	Min-Max Age (Year)	p- value
Controls 30	Male 18	5.960 ±3.355	1 – 12	0.831 For 1vs2
	Female 12	6.700 ±3.773	2- 13	0.603 For 3vs4
Patients 30	Male 19	6.690 ± 3.941	2- 12	0.527 For 1vs3
	Female 11	7.000 ± 3.651	2-13	0.856 For 2vs4

1: Healthy Males, 2:Healthy Females, 3:Male Patients, and 4: Female Patients

RESULTS AND DISCUSSION

- ***Study Individuals and Methods***

The study showed that the incidence of ALL in males was greater than that of females. The selection of control group members was in line with the number of patients. The results were close to local and international statistics on the epidemiology of this type of cancer and [30], which confirmed the high incidence of ALL in males compared with females, with a male to female ratio of 1.3: 1. Previous studies have indicated that the difference in rates of ALL in males compared to females is due to the potential effect of hormones and sex-related factors [31].

The results obtained from the questionnaire designed in the current study showed that the effects of passive smoking, exposure to chemicals, oil derivatives, previous treatments taken by the patients, as well as a group of factors and uncontaminated contaminants contributed to the occurrence of leukemia. The results were consistent with a number of Studies that led to various environmental and chemical causes of the disease [32-34]. Other sources of causes of cancers other than type ALL, the current study found that exposure for ionizing radiation may be associated with ALL [35-37].

The statistical data on the epidemiology of cancer, which was based on the current study, showed that the number of cancer cases recorded in the holy province of Karbala over the past five years (from 2012 to 2017) has reached 3892 cases, and in a more recent statistic, 127 children were infected with ALL Karbala, the holy province of 2017, most of whom have a family history of cancer. The increase in the number of cancer cases, especially leukemia, is due to the exposure of the region to radioactive and chemical pollution, which led to an increase in the occurrence of mutations causing leukemogensis in the generation of leukemogensis and thus the disease [38].

The results obtained from the current study questionnaire indicate that rural areas and parties have reported the most cases of infection among members of the affected group, that may be explained in several ways: **(1)** the infection is caused by BLV virus, which is one of the main causes of the disease Acute leukemia in people who deal with cattle with reactive virus. The disease was studied in 571 cases of acute leukemia in Asian countries [39]. **(2)** the study also examined the most important cellular genetic changes that may be experienced by workers in the manufacture and use of pesticides [40-42], which gives an indication of the impact of chemicals and pesticides in raising cases**. (3)** finally, rural areas and the outskirts of the holy Karbala governorate were exposed to radiation through the use of weapons containing uranium during The Gulf War and its maturation of battles and this element is radioactive activity, where the atoms dissolve at an absolute slow energy in the form of radiation, and the age of half of the disappearance of 4.9×10^9 years, which makes these areas contaminated for a very long time, and known according to many previous studies, this element causes many of the mutations leading to cancer [43-45].

- ***Evaluation of CLEC4E Levels in Patients and Controls Groups***

The statistical analysis using the student's *t*-test showed significant differences ($p = 0.000$) when the two study groups (patients with ALL and healthy individuals) compared together, as shown in **Table 2**.

Table 2: Levels of CLEC4E (pg / ml) in Sera of Patients with ALL and Healthy Individuals

Subjects (n)	CLEC4E Concentration (pg / ml) Mean ± S.D.	Min-Max CLEC4E (pg / ml)	p-value
Controls 30	0.222 ± 0.129	0.060 - 0.480	0.000
Patients 30	0.429 ± 0.265	0.030 - 1.070	

In order to investigate the effect of gender on serum CLEC4E levels, the analysis of variance (ANOVA) was applied to compare the recorded results in the subgroups. The study showed a statistically significant increase ($p = 0.005$) in male patients with ALL compared with their corresponding males in the control group. This finding was consistent with the results of comparing lectin levels in female patients with the control group ($p = 0.019$), while no significant differences were observed in CLEC4E levels when the intra-subgroups compared, separately **(Table 3).**

Although the lowest Lectin levels (0.03) was recorded in a sample of a 12 year-old male who was in the earlier malignant stage, moreover, the highest levels of the estimated lectin (1.07) were also noted at male with 5 years-old, this finding can indicate that the patient age has a role in the susceptibility to cancer, which explains the high recovery rates in children older than 7 years compared to their peers at younger ages.

Table 3: CLEC4E (pg / ml) levels in Serum Samples of Males and Females with ALL and Healthy Individuals

Subjects (n)	Gender (n)	CLEC4E Concentration (pg / ml) Mean ± S.D.	Min-Max CLEC4E '(pg / ml)	p - value
Controls 30	Male 18	0.228 ±0.130	0.062 - 0.480	0.865 For 1vs2
	Female 12	0.210 ±0.131	0.060 - 0.460	0.832 For 3vs4
Patients 30	Male 19	0.424 ± 0.269	0.030 -1.070	0.005 For 1vs3
	Female 11	0.438 ± 0.271	0.100 -0.810	0.019 For 2vs4

1: Healthy Males, 2:Healthy Females, 3:Male Patients, and 4: Female Patients

In order to investigate the effect of chemotherapy in production of serum lectin levels were followed in patients after receiving at least two consecutive doses of chemotherapy. **Figure 1** shows decrease in the serum CLEC4E levels at 80% of patients with ALL (24 of 30 patients) after treated with chemotherapy.

Yet, there are no literatures indicated to CLEC4E levels in healthy individuals or samples of ALL. The present study is an attempt to present complete evaluation of CLEC4E levels in this type of leukemia as well as in the healthy individuals, on the other hand, this study was designed as an attempt to provide this type of lectin

as a new tumor marker. The study Results showed that this type of lectin is naturally produced in humans, but its levels are elevated when the incidence of ALL, where the study recorded a rise in CLEC4E levels in 26 of the 30 resident samples (sensitivity 87%), in addition, It has been found that the levels of this protein increase with the progressive of cancer.

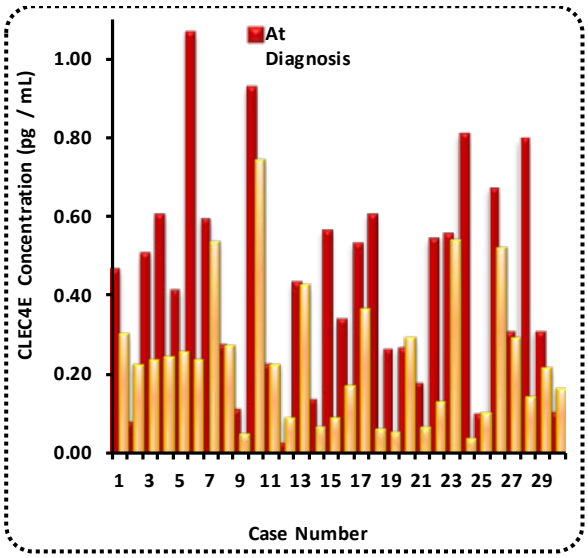

Figure 1: Serum CLEC4E Levels in Patients with ALL at Diagnosis and After Chemotherapy

The study showed that there were no differences in the levels of CLEC4E between the genders within the group, whether for ALL patients or healthy individuals, while the study showed that the lower and higher levels of this protein was recorded in males samples.

The observed increase in CLEC4E levels at patients with ALL can be explained by the association of induced synthesis by the transformation of cancer cells. Results of the study agreed with other studies that evaluated different types of lectin in samples of individuals infected with different types of cancers [46-48].

reduction in CLEC4E levels after chemotherapy, suggesting that this protein can use as monitor to the patient's health and response to treatment, and the decrease in the lectin to be similar to its level in the control group refer to the body's response to curative treatment, more than; an inhibition of cancer cells proliferation, and that could lead to decrease in the production of many proteins, and decrease in the level of lectin CLEC4E in response to the process of annihilation in healthy and infected cells after treatment with chemotherapy, and the four samples that did not register a reduction of the level of lectin may be due to the progress of the infection stages or due to the resistance of the applied treatment. The obtained findings are consistent with a number of studies [49-51], which were based on studying the levels of crude Lectins in patients with cancer, these studies attributed the increase in the level of lectin as one of the types of proteins produced and separated due to diseases, especially cancerous diseases.

- ***Assessment of Cellular Oxidative Stress in the Sera of the Two Study Groups***

The level of probable cellular oxidative stress was assessed as a response to cancer in patients with ALL compared with healthy individuals in the control group by measuring the level of Malondialdehyde (MDA) as a indicator for over cellular oxidation. Activity and concentration of ceruloplasmin oxidase were evaluated as indicator for intracellular antioxidant system.

The results of the evaluated MDA levels in the current study showed a significant increase ($p < 0.05$) for this parameter in patients with ALL compared to the control group, as shown in **Table 4.**

Table 4: MDA (mM) Levels in Patients with ALL and Healthy Individuals

Subjects (n)	*MDA Concentration (mM) Mean ± S.D.*	*Min-Max MDA (mM)*	*p - value*
Controls 30	*0.126 ±0.111*	*0.000 - 0.390*	*0.001*
Patients 30	*0.433 ±0.481*	*0.010 - 1.860*	

When the comparison was carried out between the male and female subgroups, results showed absence of statistical differences at comparing of males to females done in each group, while the study illustrated a statistically significant difference ($p = 0.010$) for MDA levels when comparing females with their peers in control group, with the same manner, the study demonstrated a significant difference ($p = 0.043$) for MDA levels when healthy and patient male subgroups were compared together, as shown in **Table 5.**

T able 5: MDA (mM) Levels in Patients Samples of Males and Females with ALL and Healthy Individuals

Subjects (n)	*Gender (n)*	*MDA Concentration (mM) Mean ± S.D.*	*Min-Max MDA (mM)*	*p - value*
Controls 30	*Male 19*	*0.127 ±0.116*	*0.000 - 0.390*	*0.848 For 1vs2*
	Female 11	*0.123 ±0.107*	*0.010 - 0.290*	*0.977 For 3v4*
Patients 30	*Male 18*	*0.424 ± 0.423*	*0.010 - 1.410*	*0.010 For 1v3*
	Female 12	*0.451 ± 0.605*	*0.010 -1.860*	*0.043 For 2v4*

1: Healthy Males, 2:Healthy Females, 3:Male Patients, and 4: Female Patients

After at least two weeks from the second dose of chemotherapy (as a minimum dose in the treatment protocol) the results showed a statistically significant decrease in 18 patients with ALL, while the study showed an increase in MDA in 10 infected samples, while the levels of this parameter did not change in 2 of the evaluated samples. The fluctuation in MDA levels obtained can be explained by several hypotheses:

First: the moderate decreases in MDA levels after receiving two doses of chemotherapy can be explained by the fact that the number of doses taken (two doses) was insufficient to produce the preferred effect in reducing the level of cellular damage caused by oxidation, the aggressive decrease in the levels of MDA in each of the 6, 9, 16, 18, 19, 29 and 30 cases which received more than two doses (3-6 doses) of chemotherapy, this finding accentuate the prime assume, so the levels of excessive cellular oxidation are directly decreased with the progression of treatment plan is, which gives this parameter importance in tracking the success of the treatment protocol. **Second**: the increase in recorded MDA levels may be due to cell resistance to chemotherapy, that may be caused increase in the amount of oxidative stress as a reflect to the chemical compound which contained in the chemotherapy. **Third:** The convergent MDA levels at the same samples (indicated in archeries in **Figure 2**) during diagnosis and after chemotherapy can explained due to inappropriate treatment or progression of cancer, ineffectively of represented treatment in inhibiting carcinogenesis or preventing tumor progression.

The transforming process of natural cell into a cancerous cell synchronize with many alterations in many metabolic events, leading to generation of numerous molecules at higher levels. Intermediates and products of oxidation reactions are the most prominent of these abnormal cellular products [52].

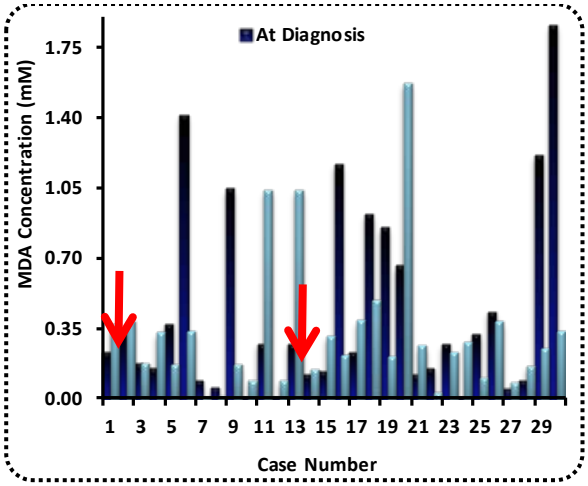

Figure 2: MDA levels in Patients with ALL at Diagnosis and After Chemotherapy

Naturally, there is a strict controlled system for the entrance and exit of substances into and out of the normal cell, and the flow system (Efflux System) into the normal cell prevents the entry or accumulation of foreign compounds into the cell. In cancer cells, however, this system is completely or partially disabled allowing in the presence of these compounds into the cell and thus adversely affect them although the real imbalance of the system of permeability of the walls of cancer cells is not yet known [53].

MDA is an organic compound with low molecular weight produced by the lipid peroxidation that reacts with the amine group of basal amino acid (arginine, lysine and histamine). This molecule also interacts with both ketones and aldehydes from different sources such as sugars or sugary products associated with proteins or fats and nucleic acids. The MDA molecule is found in two forms, the first one is free and the second is associated with amine and thiol groups of nucleic acids, protein and lipoproteins [54]. The results of the present study are consistent with Demir study, which found that the MDA molecule is able to interact directly with nucleic acid, causes genetic mutations by stimulating tumor suppressor genes and thus exacerbating the complications of cancerous symptoms [55].

Results of the study were agreed with a number of previous studies that focused on the follow-up levels of oxidative stress caused by various cancer infections by evaluating different chemical parameters [56-60]. Results of the present study were agreed with a number of studies that investigated levels of oxidative stress before and after receiving non-surgical treatments in many cancers [61-62].

- ***Evaluation of Concentration and Activity of Ceruloplasmin Oxidase Levels in Patients with ALL and Control Groups***

The results of the present study showed a statistically significant difference (p = 0.005) for the concentration of Ceruloplasmin Oxidase in the samples of the study groups as shown in **Tables 6.**

Table 6: Ceruloplasmin Oxidase (g / L) Concentration Levels in Patients with ALL and Healthy Individuals

Subjects (n)	***Ceruloplasmin Oxidase Concentration (g / L) Mean ± S.D.***	***Min-Max Ceruloplasmin Oxidase Concentration (g / L)***	***p - value***
Controls 30	*19.630± 25.240*	*0.960 - 76.740*	*0.005*
Patients 30	*35.660 ± 29.670*	*0.440 - 142.800*	

Comparison of Ceruloplasmin Oxidase activity in the two study groups contrasted with the results of enzyme concentration levels. There were no significant differences (p > 0.05) in the activity of this enzyme between the two study groups (**Table 7**).

Table 7: Ceruloplasmin Oxidase Activity (U / L) in Patients with ALL and Healthy Individuals

Subjects (n)	***Ceruloplasmin Oxidase Activity (U/ L) Mean ± S.D.***	***Min-Max Ceruloplasmin Oxidase Activity (U/ L)***	***p - value***
Controls 30	*110.500 ± 89.190*	*8.380 - 316.230*	*0.096*
Patients 30	*111.720 ± 146.740*	*0.700 - 602.440*	

From the above results it can be concluded that this enzyme creates high levels in the group of patients with ALL as a result of the disease (according to the definition of cancer) exposed to the body and here appears its role as one of the Acute Phase Proteins, but this enzyme was unable to show its function, that may be due to the combination of transitions that occur during the transition from the normal cell to the cancer cell and the associated loss of many cellular components and products to their vital functions.

When the ANOVA test was used to compare the implicit total males and females in the current study, the results of the statistical analysis failed to find significant differences in the concentration of the Ceruloplasmin Oxidase when comparing the males and females in the healthy individuals group while differences were observed between the male and female patients (as shown in **Table 8**).

Table 8: Ceruloplasmin Oxidase Concentration (g / L) Levels in Sera Samples of Males and Females of ALL and Healthy Individuals

Subjects (n)	***Gender (n)***	***Ceruloplasmin Oxidase Concentration (g / L) Mean ± S.D.***	***Min-Max Ceruloplasmin Oxidase Concentration (g / L)***	***p - value***
Controls 30	*Male 19*	*27.221 ±16.09*	*6.56 - 58.28*	*0.378 For 1vs2*
	Female 11	*21.282 ±25.870*	*0.96 - 76.74*	*0.048 For 3vs4*
Patients 30	*Male 18*	*26.470 ± 24.021*	*0.44 - 83.13*	*0.093 For 1vs3*
	Female 12	*36.062 ± 53.020*	*2.63 - 142.80*	*0.006 For 2vs4*

1: Healthy Males, 2:Healthy Females, 3:Male Patients, and 4: Female Patients

At the same time, no significant differences were observed when males with ALL compared with their counterparts in the control group, while there were high differences when infected females compared to those in healthy group (**Tables 8**).

The results of Ceruloplasmin Oxidase activity in the implicit subgroups showed a significant difference between males and females in the total number of people with ALL, while no such results were recorded when comparing the two genders in the control group (**Table 9**). The results of the study showed that there was a high difference between the males in the two subgroups of study (p = 0.023).

The highest concentration and activity (36.062 g / L and 168,660 U / L) of Ceruloplasmin Oxidase were recorded in the samples of females with ALL. In normal cases, Ceruloplasmin Oxidase was higher in healthy male samples than in female samples. Results of the present work were similar to previous studies that focused on the evaluation of Ceruloplasmin Oxidase levels in different cancers [65,19].

Table 9: Levels of Ceruloplasmin Oxidase Activity (U / L) in Sera Samples of Males and Females of ALL and Healthy Individuals

Subjects (n)	*Gender (n)*	*Ceruloplasmin Oxidase Activity (U / L) Mean ± S.D.*	*Min-Max Ceruloplasmin Oxidase Activity (U / L)*	*p - value*
Controls 30	*Male 19*	*128.870 ± 78.320*	*33.860 - 269.810*	*0.061 For 1vs2*
	Female 11	*73.740 ± 102.140*	*8.380 - 316.230*	*0.005 For 3vs4*
Patients30	*Male 18*	*83.100 ± 82.710*	*0.700 - 261.780*	*0.023 For1vs3*
	Female12	*168.960 ± 222.430*	*14.660 - 602.440*	*0.004 For 2vs4*

1: Healthy Males, 2:Healthy Females, 3:Male Patients, and 4: Female Patients

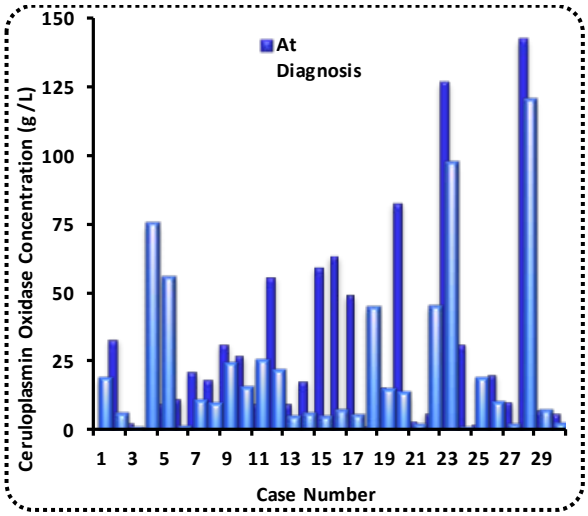

Figure 3: Concentration of Ceruloplasmin Oxidase (g / L) in Sera of Patients with ALL at Diagnosis and After Chemotherapy

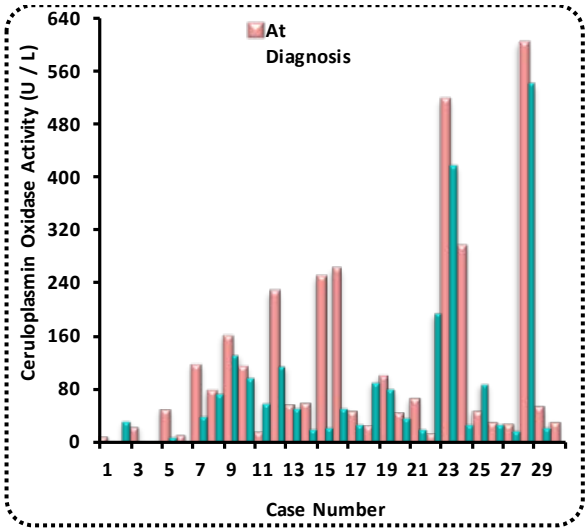

Figure 4: Ceruloplasmin Oxidase Activity (U / L) in Sera of Patients with ALL at Diagnosis and After Chemotherapy

Tracking the production levels of Ceruloplasmin Oxidase indicates a decrease in the concentration of the enzyme in 23 patients (about 77% of the included cases) after receiving at least two doses of chemotherapy (**Figure 3**), this result can confirm the role of this enzyme within the system of protection against any case of disease may be exposed to the body and low levels of the start of response to treatment and decline the division and proliferation of cancer cells and the destruction of a number of cells in the body before the start of treatment.

Great and significant reduction in the levels of Ceruloplasmin Oxidase activity in 25 cases (83%) of patients with ALL following chemotherapy was indicated to the action of the antioxidant system in the body cells decreased after receiving chemotherapy. The system of antioxidant enzymes in the response to the flood of cellular oxidation products resulting from the treatment of chemotherapy and support the possibility that this hypothesis is a reality that recorded in the continued production of cells to high rates of oxidants after receiving two doses of chemotherapy, in addition, the lowest levels of enzymatic activity were observed in samples of patients in advanced stages of infection.

The sensitivity of the evaluated lectin in the sera samples of patients with ALL reached to 100% when the levels of lectin were combined to the levels of MDA.

CONCLUSION

This observation made the measurement of these two criteria together an excellent diagnostic tool for the diagnosis of acute lymphocytic leukemia in its early stages. It is also possible to use these parameters to follow up the patient's health status and assess their response to chemotherapy.

RECOMMENDATIONS

Evaluation of *CLEC4E* levels at patients with ALL who had familiar history with cancers, moreover other cancers cases, and evaluated the specificity of this parameter in different benign tumors.

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