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Study of serum interleukin 35 level in patients with acute myeloid leukemia

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Abstract
Background: Acute myeloid leukaemia (AML) is a dangerous cancer of the blood-forming stem cells, which leads to a rise in the number of myeloid cells in the bone marrow. This may cause severe infections, bleeding, or the invasion of organs, ultimately leading in death. This research aimed to evaluate the significance of serum interleukin IL-35 levels in individuals with AML.
Methods: serum IL-35 was measured using enzyme-linked immunosorbent assays and evaluated as biomarkers for the diagnosis and prognosis.
Results: There was positive significant correlation between serum IL-35 level and Age, WBC, peripheral blood blasts, BM blasts, lactate dehydrogenase (LDH), cluster of differentiation (CD) 45, myeloperoxidase (MPO), human leukocyte antigen - DR isotype (HLA-DR) and CD34 (P <0.001). There was significant negative correlation between serum IL35 level and platelets, CD13 and CD 117 (p <0.001, p=0.001, p=0.001 respectively). While there was no significant correlation between serum IL35 level and haemoglobin (Hb), erythrocyte sedimentation rate (ESR) 1st hr, CD14, CD33 and CD64. ROC curve analysis showed that serum IL-35 had diagnostic value for AML with 92% sensitivity, 90% specificity.
Conclusions: Serum IL 35 level may have a role in the diagnosis of AML. Serum IL 35 correlates positively with prognostic markers as age, complete blood count (CBC) parameters, peripheral blood blasts, BM blasts, serum LDH, HLA-DR and CD 34, so it may be a useful prognostic marker.

Keywords: Serum Interleukin 35 level, AML, BM blast

Introduction
Acute myeloid leukaemia (AML) is a dangerous cancer of the blood-forming stem cells. It is defined by an abnormal increase of myeloid cells in the bone marrow and a halt in their development, leading to life-threatening infections, bleeding, or the invasion of organs [1]. AML may develop in people with a pre-existing haematological problem, known as secondary AML, or as a primary disorder in persons without any previous conditions (de novo AML) [2].
The exact cause of AML is not completely understood, although it is widely acknowledged that aberrations in the immune system have a role in its development. It is generally accepted that immune cells, such as regulatory T cells (Treg), effector T cells, and B cells, have an impact on cancer growth [3].
Treg are a kind of CD4+ cells that are distinguished by their constant production of elevated amounts of the interleukin (IL)-2 receptor α chain (CD25). Treg cells play a crucial role in regulating the immune system and are responsible for limiting autoimmune reactions, promoting acceptance of transplanted organs, and inhibiting the body's immunological response to tumors [4].
IL-35 is a cytokine produced by Treg cells that consists of two subunits, p35 and EBi3, and belongs to the IL-12 family. Within the tumor microenvironment, the secretion of IL-35 by Treg cells stimulates the activation of many inhibitory receptors and genes linked with T cell fatigue in CD8 T cells that have infiltrated the tumor. As a result, this process hinders the ability of the immune system to effectively combat the tumor [5].
The study revealed that IL35 had a stimulatory impact on the growth of AML blasts and also prevented their programmed cell death. The results indicated that IL35 plays a role in the immunological escape mechanism in AML [6].

The present rates of survival and recurrence in AML are disheartening, highlighting the need for more efficacious treatment options. Interleukin immunotherapy has promise as a powerful cancer treatment [7].

This research aimed to evaluate the significance of serum IL35 levels in individuals with AML.

Patients and Methods

Study design

This research was conducted prospectively and included a cohort of 50 patients who were attending the hematology and oncology units of Tanta University hospitals between December 2021 and May 2023. All patients had been diagnosed with AML (AML was diagnosed based on the presence of 20% blast cells in the BM film, as recommended by WHO, as well as MPO positivity and immunophenotyping results consistent with AML (positive CD13, CD33, and CD117) [8]. Exclusion criteria were AML patients in relapse and who start chemotherapy. The control group consisted of 50 individuals who were in good health, matched in terms of age and sex, and had no history of chronic or acute illness.

Ethical approval

The current investigation received approval from the Scientific Research Ethics Committee of Tanta University. Prior to participation in the trial, all subjects gave written informed permission.

Data Collection

Every patient had a comprehensive assessment that included: gathering medical history, conducting a physical examination, and doing laboratory tests.

Laboratory Investigations

Sample collection

Under very sterile conditions, 8 ml of peripheral blood was collected into one serum separator tube (SST), 2 tubes containing ethylenediaminetetraacetic acid (EDTA), and 1 tube containing sodium citrate. The serum separator tube was left undisturbed for a period of 10-20 minutes to allow for coagulation, after which it was subjected to centrifugation at a speed of 2000 revolutions per minute for a duration of 20 minutes. The serum was divided into two portions: one ml was immediately analyzed for LDH using the automated INDIKO plus analyzer, while two ml was kept at -20 °C for later calculation of serum IL-35 using ELISA. One tube containing EDTA was used for conducting a comprehensive blood count analysis using an electronic automated analyzer manufactured by ERMA Inc., Poland. Additionally, Giemsa-stained smears were prepared from this tube. The other tube was employed for flow cytometric immunophenotyping. A citrated tube was used for the determination of ESR.

Measurement of serum IL-35

Serum IL35 was estimated by Enzyme-linked immune-sorbent assay (ELISA) kit by SunRed Company made in China supplied by Biokit Egypt catalogue No. 201-12-0043.

Principle of the Assay

The kit employs a double-antibody sandwich ELISA to measure the concentration of Human IL-35 in samples. Apply IL-35 to the well coated with Human IL-35 monoclonal antibody, followed by incubation. Next, introduce biotin-labeled IL-35 antibodies and combine them with Streptavidin-HRP to create an immune complex. Subsequently, incubate and wash the mixture to eliminate any unbound enzyme. Next, introduce Chromogen Solution A and B. As a result, the liquid undergoes a transformation and becomes blue. Finally, when exposed to acid, the color changes to yellow. There was a positive correlation between the color's chroma and the content of Human Substance IL-35 in the sample.

Statistical Analysis

The statistical analysis was performed using the SPSS v26 program (IBM Inc., Chicago, IL, USA). The normality of the data distribution was evaluated by doing the Shapiro-Wilks test and examining histograms. The average and variability (standard deviation, SD) of the quantitative parametric variables were documented and compared between the two groups using an unpaired Student's t-test. The quantitative non-parametric data were presented as the median and interquartile range (IQR) and were analyzed using the Mann Whitney-test. The qualitative variables were represented as frequency and percentage (%) and were assessed using either the Chi-square test or Fisher's exact test, depending on the circumstances. ROC analysis was conducted to determine the overall predictive ability of a parameter and identify the optimal cut-off value. Sensitivity and specificity were assessed at this cut-off value. The Pearson correlation coefficient (r) was calculated to evaluate the strength and direction of the association between two continuous numerical variables, with at least one of them following a normal distribution. A two-tailed P value below 0.05 was considered to have statistical significance.

Results

There were no significant differences in age or sex between the two groups tested Table 1.

Table 1: Comparison between the two studied groups according to demographic data

		Cases (n = 50)	Control (n = 50)	p
Age (years)		51.30 ± 12.0	47.18 ± 17.24	0.169
Sex	Male	34(68.0%)	30(60.0%)	0.405
	Female	16(32.0%)	20(40.0%)	

Data are presented as mean ± SD or frequency (%). *Significant p value<0.05.

Out of the 50 AML patients, 41 (82.0%) patients were presented with fever, 38 (76.0%) with bleeding, 27 (54.0%) with hepatosplenomegaly, 16 (32.0%) with lymphadenopathy. The mean of peripheral blood blast was 27.90 ± 28. 32. The mean of BM blast was 51.28 ± 24.99. M5 was the most prevalent 13(26%), followed by M4 12(24%), M0 10(20%), M1 4(14%), M2 (612%) and M3 2(4%). Table 2

Table 2: Descriptive analysis of the studied cases according to clinical findings, peripheral blood blast and BM blast and FAB classification of AML group

	N=50
Clinical findings	
Fever	41 (82.0%)
Bleeding	38 (76.0%)
HSM	27 (54.0%)
LN	16 (32.0%)
Peripheral blood blast	10.0 (6.0-60.0)
BM blast	40.0 (30.0-77.0)
FAB classification	
M0	10.0 (20.0%)
M1	7.0 (14.0%)
M2	6.0 (12.0%)
M3	2.0 (4.0%)
M4	12.0 (24.0%)
M5	13.0 (26.0%)

Data are presented as frequency (%) or median (IQR), AML: Acute myeloid leukemia, HSM: Hepatosplenomegaly, LN: lymph node, BM: Bone marrow, FAB: fragment antigen-binding region.

There were significant differences between patient and control group regarding CBC parameters (Haemoglobin, WBCs and platelets), LDH, ESR and IL 35 Table 3

Table 3: Comparison between the two studied groups according to CBC, LDH, ESR and serum IL35 level findings

	Cases (n = 50)	Control (n = 50)	p
Hb	9.10 (8.30 – 10.0)	14.05 (13.50 – 15.10)	<0.001*
PLT	62.0 (30.0 – 85.0)	271.50 (202.0 – 330.0)	<0.001*
WBCs	21.0 (13.0 –44.20)	7.55 (6.50 – 8.70)	<0.001*
LDH	1197.40 ± 619.19	179.66 ± 31.42	<0.001*
ESR 1hr	92.80 ± 18.16	13.30 ± 5.94	<0.001*
IL35	140.21 ± 47.02	58.50 ± 13.17	<0.001*

Data are presented as mean ± SD or median (IQR), *significant p value<0.05, AML: Acute myeloid leukemia, Hb: Hemoglobin, PLT: platelets WBCs: White blood cell count, LDH: lactate dehydrogenase, ESR: erythrocyte sedimentation rate, IL35: Interleukin-35.

The results of flowcytometric immunophenotyping revealed that out of the 50 patients, 13 (26%) were positive for CD14, 6 (46.2%) with high expression and 7 (53.8%) with low expression, and 37 (74%) were negative for CD14. All AML patients in the study were positive for CD45 (100%), 23 (46%) with high expression and 27 (54%) with low expression. Out of the 50 patients, 48 (96%) were positive for CD13, 24 (50%) with high expression and 24 (50%) with low expression, and 2 (4%) were negative for CD13. Out of the 50 patients, 30 (60%) were positive for MPO, 14 (46.7%) with high expression and 16 (53.3%) with low expression, and 20 (40%) were negative for MPO. All AML patients in the study were positive for CD33 (100%), 25 (50%) with high expression and 25 (50%) with low expression. Out of the 50 patients, 48 (96%) were positive for HLA-DR, 24 (50%) with high expression and 24 (50%) with low expression, and 2 (4%) were negative for HLA-DR. Out of the 50 patients, 30 (60%) were positive for CD34, 15 (50%) with high expression and 15 (50%) with low expression, and 20 (40%) were negative for CD34. Out of the 50 patients, 48 (96%) were positive for CD117, 24 (50%) with high expression and 24 (50%) with low expression, and 2 (4%) were negative for CD117. Out of the 50 patients, 13 (26%) were positive for CD64, 6 (46.2%) with high expression and 7 (53.8%) with low expression, and 37 (74%) were negative for CD64 Table 4.

Table 4: Flowcytometric immunophenotyping results in AML patients

	N=50
CD 14	69.15 ± 15.27
Negative	37 (74.0%)
Positive	13 (26.0%)
Low expression	7 (53.8%)
High expression	6 (46.2%)
CD 45	54.36 ± 21.79
Negative	0 (0.0%)
Positive	50 (100.0%)
Low expression	27 (54.0%)
High expression	23 (46.0%)
CD 13	61.79 ± 21.78
Negative	2 (4.0%)
Positive	48 (96.0%)
Low expression	24 (50.0%)
High expression	24 (50.0%)

MPO	56.47 ± 20.58
Negative	20 (40.0%)
Positive	30 (60.0%)
Low expression	16 (53.3%)
High expression	14 (46.7%)
CD 33	70.88 ± 13.39
Negative	0 (0.0%)
Positive	50 (100.0%)
Low expression	25 (50.0%)
High expression	25 (50.0%)
HLA-DR	54.10 ± 27.02
Negative	2 (4.0%)
Positive	48 (96.0%)
Low expression	24 (50.0%)
High expression	24 (50.0%)
CD 34	59.93 ± 28.68
Negative	20 (40.0%)
Positive	30 (60.0%)
Low expression	15 (50.0%)
High expression	15 (50.0%)
CD 117	61.79 ± 21.78
Negative	2 (4.0%)
Positive	48 (96.0%)
Low expression	24 (50.0%)
High expression	24 (50.0%)
CD 64	69.15 ± 15.27
Negative	37 (74.0%)
Positive	13 (26.0%)
Low expression	7 (53.8%)
High expression	6 (46.2%)

Data are presented as mean ± SD or frequency (%). AML: Acute myeloid leukemia, CD: cluster of differentiation, MPO: Myeloperoxidase, HLA-DR: Human Leukocyte Antigen – DR isotype.

A strong positive connection was seen between the serum IL35 level and Age, WBC, peripheral blood blasts, BM blasts, LDH, CD45, MPO, HLA-DR, and CD34 (P value <0.001). A strong inverse relationship was seen between the blood IL35 level and platelets, CD13, and CD117 (*p* <0.001, p=0.001, p=0.001 correspondingly). There was no notable association seen between the serum IL35 level and Hb, ESR 1st hr, CD14, CD33, and CD64 (P value=0.766, 0.155, 0.515, 0.508, 0.515 correspondingly).Table 5

Table 5: Correlation between serum IL35 level and prognostic factors in AML group

	IL35	
	r_s	**p**
Age (years)	0.542	<0.001*
Hb	0.043	0.766
PLT	-0.599	<0.001*
WBCs	0.635	<0.001*
bl. blast	0.795	<0.001*
bm. blast	0.779	<0.001*
LDH	0.671	<0.001*
ESR 1hr	0.204	0.155
CD 14	-0.199	0.515
CD 45	0.753	<0.001*
CD 13	-0.454	0.001*
MPO	0.537	0.002*
CD 33	0.096	0.508
HLA-DR	0.782	<0.001*
CD 34	0.723	<0.001*
CD 64	-0.199	0.515
CD 117	-0.454	0.001*

r_s: Spearman coefficient, *: significant t $p < 0.05$, AML: Acute myeloid leukemia, Hb: Hemoglobin, PLT: platelets WBCs: White blood cell count, LDH: lactate dehydrogenase, ESR: erythrocyte sedimentation rate, CD: cluster of differentiation, MPO: Myeloperoxidase, HLA-DR: Human Leukocyte Antigen – DR isotype.

ROC curve analysis demonstrated that serum IL35 had diagnostic value for AML with 92% sensitivity, 90% specificity, 90.2%, positive predictive value (PPV) and 91.8% negative predictive value (NPV) Fig 1.

The relationship between IL35 and both overall survival and disease-free survival was evaluated by using Kaplan-Meier

survival curves. Those with blood IL 35 levels below 127 demonstrated extended overall life and disease-free survival compared to those with serum IL 35 levels over 127 Fig 2.

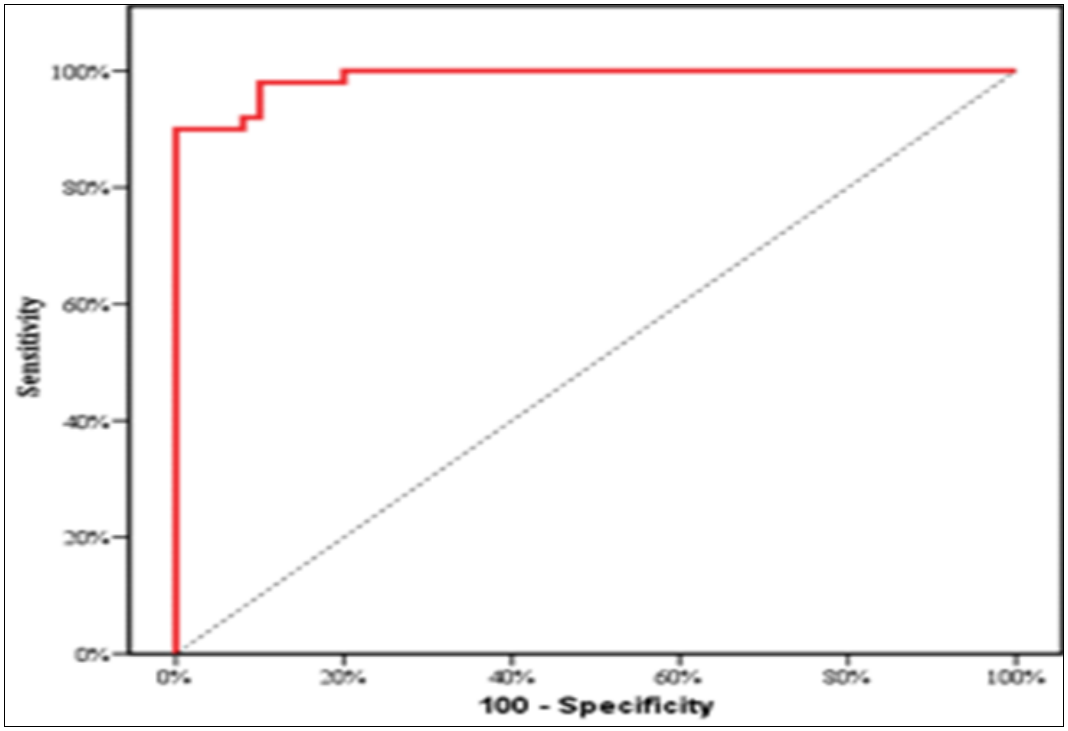
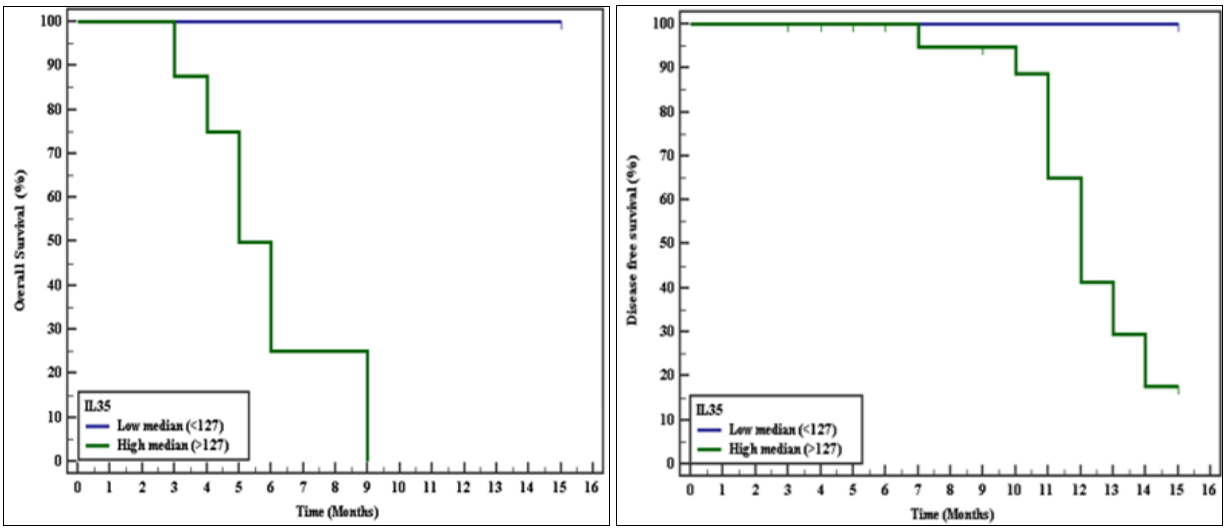


Fig 1: ROC curve for interleukin-35 to discriminate cases from control



A) **B)**

Fig 2: The Kaplan-Meier survival curve depicts the (A) overall survival rate when IL35 is administered, and (B) the disease-free survival rate when IL35 is administered

Discussion

AML is the most common type of acute leukaemia in adults. Without treatment, the disease is fatal within a few months [9].

IL-35, a cytokine mainly produced by regulatory T cells (Treg cells), was lately recognized as heterodimeric inhibitory cytokine and contributes to their immune suppressive activity by limitation of antitumor effect and contribution in dysfunction of T-cell [1].

Our study revealed that of the studied 50 cases, AML M5 was the most prevalent subtype (26%), followed by AML M4 (24%), AML M0 (20%), AML M1 (14%), AML M2 (12%) and AML M3 subtypes (4%). Elnagar MG *et al.* [10] stated that AML M4 and AML M5 represented the most prevalent FAB subtype accounted for 51.7% followed by AML M2 (23%) subtype. While Mahmood EF & Ahmed AA, 2020 [11] stated that AML M3 was the most frequent (23.3%) subtype followed by AML M1 (20%) and AML M2 subtypes (20%).

In the present study, Hb level showed significant decrease compared to control group and this agreed with Mahmood EF and Ahmed AA. [11] and Nagar V *et al.* [12].

We found that PLT count showed significant decrease compared to control group and this agreed with Haque S *et al.* [13] and Ahmed HA *et al.* [2]. This significant decrease may be due to the infiltrative effect of leukemic cells in BM [14]. Gao A, *et al.* [14] observed inhibitory effects of IL -4 throughout the process of megakaryopoiesis *in vivo*.

In this study WBC count showed significant increase compared to control group and this agreed with Dohner K *et al*. [15] and Ahmed HA *et al*. [2]. Our results as regard to peripheral blood blasts mean was 27.90±28.32 and this was in agreement with Nasreldin E *et al*. [16] with a mean value of 29.8±26.3 but disagreed with Wu H *et al*. [1] and Ahmed HA *et al*. [2] who showed higher peripheral blast count with a mean value of 56.52±27.36 and 69.5±21.68 respectively and this may be due to the difference in sample size and AML subtypes.

We also found that BM blasts ranged from 5.0-92.0 with a mean value of 51.28±24.99 this was in agreement with Wang J, *et al*. [17] with a mean value of 60.42±23.76, Nasreldin E *et al*. [16] with a mean value of 40.9±25.9 but disagreed with Wu H *et al*. [1] and Ahmed HA *et al*. [2] who showed higher BM blast count with a mean value of 72.48 and 81.42±12.29 respectively and this may be due to the difference in sample size and AML subtypes.

The results revealed that LDH level in AML group showed statistically significant increase as compared to the control group. This agreed with Jumaah HM *et al*. [18] who found significant increase in LDH levels at presentation.

In this study, first hour ESR in AML patients showed statistically significant increase as compared to the control group. This agreed with Padhan P *et al*. [19] who stated that ESR is elevated in solid and haematological malignancies.

In our study, all AML cases were positive for CD 45 and CD 33, and this agreed with Haque S, *et al*. [20] who stated that CD 45 was positive in all their cases while CD 33 was positive in 95% of their cases.

We also found that CD 14 was expressed in 26% of cases, CD 13 was positive in 96% of cases, MPO was positive in 60% of cases, HLA-DR was positive in 96% of cases, CD 64 was expressed in 26% of cases, CD117 was expressed in 96% of cases and CD 34 was positive in 60% of cases. This agreed with Haque S *et al*. [20] who found that among their cases CD 13 was positive in 89% of cases, MPO was positive in 80% of cases, HLA-DR was positive in 80% of cases, CD 117 was positive in 74% of cases and CD 34 was positive in 62% of cases. Shetty *et al*. [21] found that majority of AML cases show reactivity for CD 13, CD 15, CD33, CD 64 and CD 117.

The findings of the present investigation demonstrated a statistically significant elevation in serum IL 35 levels among newly diagnosed AML patients as compared to the control group ($p<0.001$). A study conducted by Ahmed HA *et al*. [2] revealed that the levels of IL 35 in individuals who were recently diagnosed with AML were notably elevated in comparison to the control group. In addition, a study conducted by yang PX *et al*. [22] revealed a substantial increase in the concentration of IL 35 in patients with AML compared to the control group. Mahmood EF and Ahmed AA [11] found that IL35 was higher in complete remission than non-remission but doesn't reach the level of significance.

To check for the effectiveness of IL35 as a diagnostic marker in newly diagnosed AML, we use ROC curve analysis. Our results demonstrated that serum IL35 had diagnostic value for AML with 92% sensitivity, 90% specificity, 90.2% PPV, 91.8% NPV. Ahmed HA *et al*. [2] demonstrated that IL35 optimum cutoff is >27.8 was 88% sensitivity and 100% specificity.

To investigate the use of IL35 as a prognostic marker in AML, we looked at its connection with other prognostic markers in the study participants such as age, CBC parameters, peripheral blood blasts, BM blasts, serum LDH, HLA-DR and CD 34.

In the current study there was a positive significant correlation between serum IL35 and age, WBCs, peripheral blood blasts, BM blasts, LDH, CD 45, MPO, HLA-DR, and CD 34.

There was a significant negative correlation between serum IL35 and platelets and CD13. While there was no significant correlation between serum IL35 and Hb, ESR 1st hr., CD14 and CD33. Ahmed HA *et al*. [2] reported that there is significant positive correlation of IL35 with age, WBCs, CD45, HLA-DR and CD34 and significant negative correlation with platelets. While they found no correlation between IL35 and Hb, peripheral blood blasts, BM blasts, CD14, CD13, MPO and CD33. Wang J *et al*., 2015 stated that IL35 was markedly elevated in BM of adult AML patients and this increase was correlated significantly with the advanced stages of malignancy.

In order to examine the correlation between IL35 and both overall survival and disease-free survival in patients with AML, we used the KAPLAN MEIER curve. The findings of our study indicate that patients with blood IL 35 levels below 127 had a greater overall survival and disease-free survival compared to patients with serum IL 35 levels above 127. Sariani and colleagues [23] as well as Li and colleagues [6] posit that IL-35 plays a role in the development of AML by promoting cell survival and proliferation in AML blasts. This suggests that IL-35 is significantly involved in the immune evasion mechanism in AML. Consequently, IL-35 is now being assessed as a possible target for modifying Treg activity in therapeutic applications. Hence, the inhibition of IL-35 by the use of monoclonal antibodies may be a compelling immunotherapeutic strategy.

Conclusions

Serum IL 35 level may have a role in the diagnosis of AML. Serum IL 35 correlates positively with prognostic markers as age, complete blood count (CBC) parameters, peripheral blood blasts, BM blasts, serum LDH, HLA-DR and CD 34, so it may be a useful prognostic marker.

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Conflict of Interest: Nil

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