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Evaluation the role of CD34 in patients with acute myeloid leukemia

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Abstract---Acute myeloid leukemia (AML) is the most common leukemia among the adult population and accounts for about 80% of all cases, and its characterized by clonal expansion of immature "blast cells" in the peripheral blood and bone marrow .The stem cell marker CD34 is expressed by leukemia blasts only for a subset of patients with acute myelogenous leukemia (AML).The aim of this study is to determine the role of CD34 in AML and its relationship with the parameters of complete blood (CBC). A total of (60) venous blood sample from patients and (30) control their ages ranged between 18 and 66 years, The samples was collected from Baghdad medical city hospital. Sera from all participants were tested to assessment CD34 level by ELISA.In the current study, CD34 in patients and control groups show a significant difference in relation with AML patients at p-value (< 0.001). The levels of CD34 in the patients were 0.55 ± 0.03, whereas it's significantly increased to 0.81 ± 0.05 in the control. also we found The majority of patients presented with subtype M3 38.3% of total patients, The results of present study showed a significant differences (P<0.05) between control and AML groups in blood parameters. The mean of WBC, Neutrophil, Lymphocytes, RBC, Hb, HCT and PLT in control group were (7.60 ± 0.37), (70.14 ± 1.56),(40.84 ± 3.97),(4.76 ± 0.12),(13.09 ± 0.21),(41.01 ± 0.75) and (264.67 ± 12.72) respectively , whereas these values in the patients group were significantly decreased to(5.67 ± 0.65),(17.35 ± 2.50),(30.19 ± 1.51),(2.92 ± 0.10),(8.12 ± 0.20),(24.51 ± 0.72) and (86.48 ± 11.45) respectively. Furthermore the mean of Blast and MCHC in the control group were (0.00 ± 0.00) and (32.06 ± 0.24) respectively, whereas

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these values in the patients group were significantly increased to (62.63 ± 2.99) and (32.81 ± 0.22) respectively. on the other hand, there were insignificant differences (P<0.05) between control and AML patients groups in other blood parameters. Also there was a significant differences between subtypes of AML according to Blast (%), Neutrophil (%) and Lymphocytes (%). While, there are insignificant differences (P<0.05) between subtypes of AML according to other parameter of complete blood picture, The correlation between CD34 with studied variables was found positive between CD34 and white blood cells count in patients with AML (P=0.005).

Keywords---acute myeloid leukemia, CD34, blood parameters.

Introduction

ML is a type of blood cancer that is caused by the clonal expansion of myeloid hematopoietic precursors in the bone marrow. As proliferating leukemia cells interfere with normal hematopoiesis, granulocytopenia, anemia, and thrombocytopenia are common in addition to circulating leukemia cells (also known as blasts) in the peripheral blood (Sekeres and Mitigating,2015). AML was classified according to the F-A-B classification system using morphology and immune-phenotype criteria to define eight major AML subtypes (FAB M0 to M7) (Bonnet and Dick ,1997). AML is a diverse illness caused by a number of genetic disorders, such as chromosomal rearrangements and mutations, which promote the proliferation and survival of immature myeloid cells (Ferrara and Schiffer,2013). CD34 is a transmembrane phosphoglycoprotein, first identified in 1984 on hematopoietic stem and progenitor cells. Studies on hematopoietic cells reveal that CD34 plays a role in cytoadhesion, regulation of cell differentiation, and proliferation despite the fact that its structure has received a great deal of attention. (Nielsen and McNagny ,2008). The stem cell marker CD34 is expressed by leukemia blasts only for a subset of patients with acute myelogenous leukemia (AML) (Anne et al.,2005). the aim of the current study was to investigate the role of CD34 in patients with acute myeloid leukemia as a new markers for establishing AML diagnosis and prognosis and its correlation with blood parameters.

Materials and Methods

Subjects of the Study

The study subjects comprised of 115 AML patients(38male and 77female) ,and their ages ranged between 18 and 66 years .These patients were suffered from Acute myeloid leukemia and there samples were collected from the Baghdad medical city hospital during the period from (December) 2020 to (September) 2021 under the supervision of specialized hematopathologist , and according to the medical ethics of the hospital and consent form taken from all patients and volunteers group. Also, a questionnaire was taken from the patients and case sheets including: number, age, sex, subtypes of AML (M0. M1, M2, M3, M4, M5, M6 .M7) and duration of disease. Then, 60 patients were selected based on

quantity and natural color of their samples (28male and 32 female) , in addition to the 30 samples from healthy subjects as control group (11 male and 19 female) and this group matched with the patients group

Blood collection

Blood sample were collected by venipuncture from these groups (five millimeter of venous blood) were drawing by disposable syringe under aseptic technique 2 ml was placed into EDTA tubes and the remaining (3 ml) pushed slowly into disposable gel containing tubes. Blood in the EDTA tubes was used to determine CBC . while blood in the gel containing tubes was allowed to clot at room temperature for 15 minutes and then centrifuged at 2,000 x g for 10 minutes , after that serum was obtained (Barbara and Anna,2012) and stored eppendrof tube then stored at -20 to be used for CD34 ELISA assay .

Estimation the levels of CD34 by ELISA assay
Assay Principle

The kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human CD34 antibody. CD34 present in the sample is added and binds to antibodies coated on the wells , then biotinylated Human CD34 Antibody is added and binds to CD34 in the sample. After that , Streptavidin-HRP is added and binds to the Biotinylated CD34 antibody. The incubation unbound Streptavidin-HRP is washed away during a washing step , then the substrate solution is added and color develops in proportion to the amount of Human CD34. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

Reagent Preparation

- All reagents should be brought to room temperature before use.
- Standard reconstitute the 120µl of the standard (16ng/ml) with 120µl of standard diluent to generate a 8ng/ml standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (8ng/ml) 1:2 with standard diluent to produce 4 ng/ml, 2 ng/ml, 1 ng/ml and 0.5ng/ml solutions. Standard diluent serves as the zero standard(0 ng/ml) , and any remaining solution should be frozen at -20°C and used within one month.
- 3-Wash Buffer , diluent 20ml of Wash Buffer concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer , and if crystals have formed in the concentrate ,mix gently until the crystals have completely dissolved .

Assay Procedure

- Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.

- Determine the number of strips required for the assay. Insert the strips in the frames for use , and the unused strips should be stored at 2-8°C.
- Add 50µl standard to standard well. Note: Don't add biotinylated antibody to standard well because the standard solution contains biotinylated antibody.
- Add 40µl sample to sample wells and then add 10µl anti-CD34 antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.
- Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with 300ul wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate or decant each well and wash 5 times with wash buffer. Blot the plate onto paper towels or other absorbent material.
- Add 50µl substrate solution A to each well and then add 50µl substrate solution B to each well , then the incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.
- Add 50µl Stop Solution to each well, the blue color will change into yellow immediately.
- Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

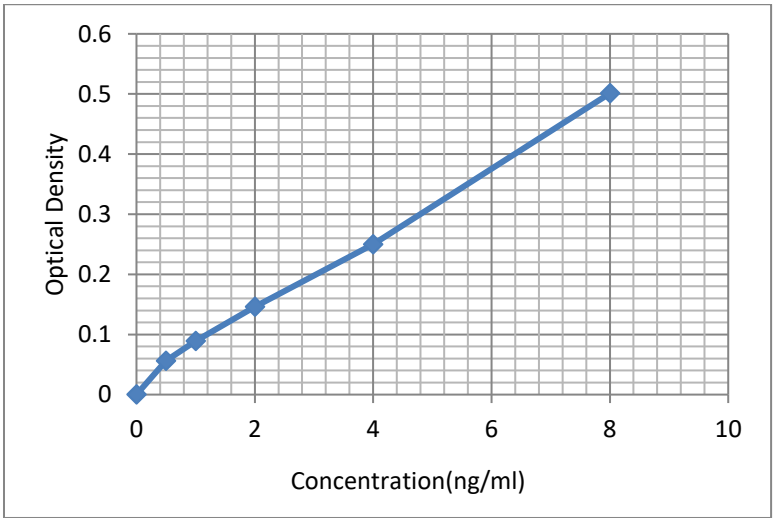

Figure 1. Standard curve of CD34 concentration

Calculation of Results

Construct a standard curve by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X)axis and draw a best fit curve through the points on the graph.These calculations can be best performed with computer-based curve-fitting software and the best fit line can be determined by regression analysis. The standard curve was done as shown in figure (1).

Statistical Analysis

Statistical analysis was carried out using SPSS version 27. Categorical variables were presented as frequencies and percentages. Continuous variables were presented as (Means ± SE). Student t-test was used to compare means between two groups. ANOVA test was used to compare means between three groups or more. A p-value of ≤ 0.05 was considered as significant.

Results

Acute Myeloid Leukemia patients and blood parameters
Distribution of patients with AML according to the complete blood count

The results in table (1) showed the mean differences of complete blood picture including , (WBC (*10^3/µl), Blast (%), Neutrophil (%), Lymphocyte (%),RBC (*10^6/µl), Hb (g/dl), HCT(%) , MCV (FL), MCH (pg), MCHC (g/dl) and PLT (*10^3/µl) according to study group including (acute myeloid leukaemia and control group). There were significant differences between means of WBC , Blast , Neutrophil, Lymphocyte, RBC, Hb, HCT,MCHC and PLT between patients and control groups.

Table 1
The means of complete blood count in patients and control groups

Complete blood picture	Study group	N	Mean ± SE	P-value
WBC (*10^3/µl)	Acute myeloid leukaemia	60	5.67 ± 0.65	0.011*
	Control group	30	7.60 ± 0.37	
Blast (%)	Acute myeloid leukaemia	60	62.63 ± 2.99	<0.001*
	Control group	30	0.00 ± 0.00	
Neutrophil (%)	Acute myeloid leukaemia	60	17.35 ± 2.50	<0.001*
	Control group	30	70.14 ± 1.56	
Lymphocyte (%)	Acute myeloid leukaemia	60	20.30 ± 1.47	<0.001*
	Control group	30	30.19 ± 1.51	
RBC (*10^6/µl)	Acute myeloid leukaemia	60	2.92 ± 0.10	<0.001*
	Control group	30	4.76 ± 0.12	
Hb (g/dl)	Acute myeloid leukaemia	60	8.12 ± 0.20	<0.001*
	Control group	30	13.09 ± 0.21	
HCT (%)	Acute myeloid leukaemia	60	24.51 ± 0.72	<0.001*
	Control group	30	41.01 ± 0.75	
MCV (FL)	Acute myeloid leukaemia	60	84.44 ± 1.22	0.156
	Control group	30	87.17 ± 1.13	
MCH (pg)	Acute myeloid leukaemia	60	28.09 ± 0.43	0.649
	Control group	30	27.85 ± 0.34	
MCHC (g/dl)	Acute myeloid leukaemia	60	32.81 ± 0.22	0.039*
	Control group	30	32.06 ± 0.24	
PLT (*10^3/µl)	Acute myeloid leukaemia	60	86.48 ± 11.45	<0.001*
	Control group	30	264.67 ± 12.72	

* Significant difference

The complete blood count was used as a tool to diagnosis the AML , in addition the results showed a significant differences in WBCs, Blast , Neutrophil, Lymphocyte, R.B.Cs, Hb, HCT, MCHC and PLT according to the statistical analysis, so that these parameters reflected their association with AML patients, that increasing with the severity of disease , therefore these results was agreed with Sreedhara *et al* .,(2013) who diagnosis AML depending on the complete blood film examination, gives definite and positive results in diagnosing of disease. AML is a heterogeneous disorder consisting of clonal expansion of myeloblasts, in addition , the blood parameters plays an important role in their pathogenicity , so the blood components (such as , lymphocytes, WBCs, Blast , PLT, Hb, etc.) are strongly associated with disease (Prasad *et al.*,2021). In the current study the mean values of W.B.Cs , Hb and platelets was 5.67 ± 0.65×10^3cell/µl , 8.12 ± 0.20 g/dl , and 86.48 ± 11.45 $\times10^3$cell/µl respectively, these results were similar to that reported by Weinberg *et al.,* (2009) who mention the same value in patients with AML.

Table (1) showed that the relation between complete blood count (CBC) and AML and their important in diagnosis. The researchers David(2014) and Hunger(2015) indicated that diagnosis of AML revolves around the complete blood count (CBC), given the clinical situations, in which the CBC test simply allows to diagnosis of disease in a timely manner. With regard to the correct interpretation of CBC, in addition a number of very important data should be considered including , W.B.C.s, Blast , Neutrophil , lymphocytes, R.B.Cs, PLT, Hb, MCV, MCH, and MCHC , also the researchers indicated four out of five patients with AML have anemia, so the diagnosis should not be excluded just because the patients does not have anemia. Meantime, one in five patients with AML has no thrombocytopenia so having a normal platelet count does not exclude important this test for diagnosis.

Distribution of patients with AML according to the CD34 concentration

Table (2), and figure (2) showed the level of CD34 concentration (pg/µl) in patients and control groups . Where it shows a significant difference in relation with AML at p-value (< 0.001).

Table 2
The mean levels of study biomarkers in patients and control groups

Biomarkers	Groups	N	Mean ± SE	P-value
CD34 (pg/µl)	Patients	60	0.55 ± 0.03	<0.001*
	Control	30	0.81 ± 0.05	

* Significant difference

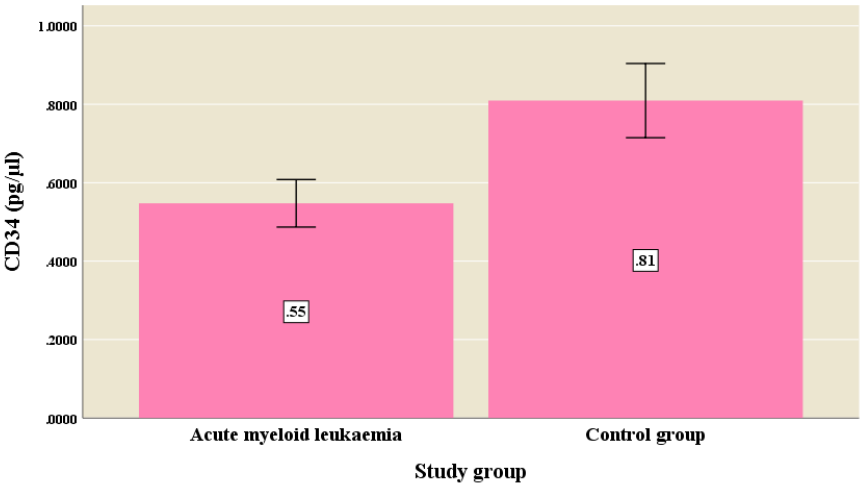

Figure 2. The mean levels of CD34 (pg/µl) in patients and control groups(N.90)

In the current study there was a highly significant differences between AML patients and healthy subjects groups in the biomarker CD34 at p-value (< 0.001), these highly correlation was clarified by several studies on AML patients and the role of CD34 proteins in disease events, which indicated that such biomarker in AML syndromes play a crucial role in the behavior of the disease because it potentially contains the neoplastic precursors with clonogenic capability, in addition their role in the expression of apoptosis- and MDR-related proteins (Parker and Mufti,2001; Pecci *et al.*,2003). The statistical analysis for the results on patients showed that those with positive CD34 had significantly higher severity of disease compared to CD34 other types of receptor , these results are consistent with data of other studies that mentioned CD34 have important role in pathogenicity and mortality in patients with AML (Amer *et al.*,2019).

The Acute myeloid leukemia subtypes with blood an virulence parameters
Distribution of Acute myeloid leukemia subtypes according to the age

The data in table (3) and figure (3) shows the mean differences of age (years) according to subtypes of AML including M1 and M2, M3, M4 and M5. The results revealed no significant differences between means of age according to subtypes of AML .

Table 3
The mean differences of age according to the subtypes of AML (N=57)

Study variable	Subtypes of AML	N	Mean ± SE	P-value
Age (years)	M1 and M2	13	40.69 ± 3.76	0.214
	M3	23	33.83 ± 3.20	
	M4	12	37.42 ± 2.48	
	M5	9	44.44 ± 4.95	

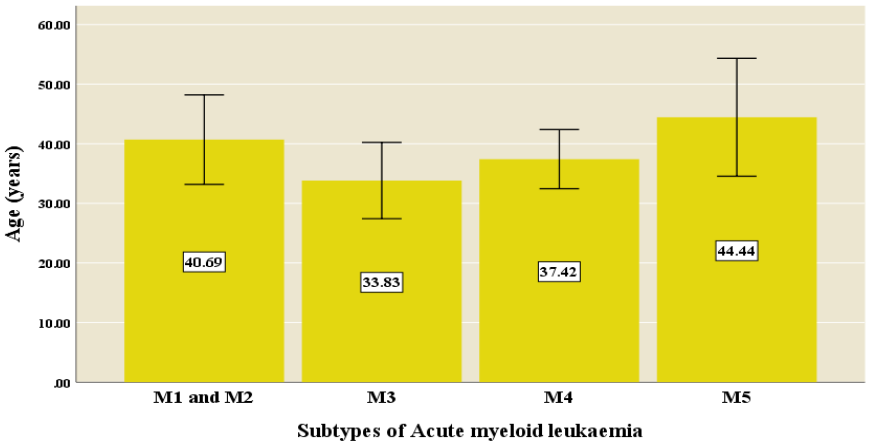

Figure 3. The mean differences of age (years) according to subtypes of AML (N=57)

In the current study it was found a highly number of patients in M3 subtype and at mean value 33.83 ± 3.20, while the high mean at 44.44 ± 4.95 was shown in M5 subtype with low number of patients (10) , in addition, the highest age of patients with AML was at 20-40 years , so that these correlation between age > 30 years and AML subtypes or abnormal mutation and other parameters such as cytogenetics, could be depending on it as prognostic parameter for diagnosis (Haferlach *et al.*,2003) .The age of AML patients presentation in current results showed increase between 20-40 years especially with the M3 subtype , and these result was consistent with Estey (2014) that showing age and AML subtypes mainly affected in severity and prognostic of disease.

AML is closely related to age, as most researchers has focused on the relationship between age and disease due to the concept of age-related clonal hematopoiesis . Most of the patients were found in ages equal to 30 years due to the increase of receptors in the cells that get the disease and the AML increases with increasing age until the age of 60 years (Jaiswal *et al.*,2014) ,these finding was consistent with present results where the highest incidence of patients was between the age group 20- 40 years. There are clinical differences according to age and gender in patients with AML and it would like to clarify that the focus of the disease among this age periods referred to previously may be because the ages are less than 20 years They may undergo less diagnostic procedures, such as morphological sub-classification and genetic evaluation , this explanation was consistent with the results Sorror *et al.*, (2014).

Distribution of AML subtypes according to the blood picture

Table (4) showed the mean differences of complete blood picture including WBC ($\times 10^3$cell/µl), Lymphocytes (%),RBC ($\times 10^6$cell/µl), Hb (g/dl), HCT(%) , MCV (FL), MCH (pg), MCHC (g/dl) and PLT ($\times 10^3$cell/µl) according to subtypes of AML (M1 and M2, M3, M4 and M5). The lymphocytes showed that significant differences at value (0.003), while the other parameters don't shows any significant differences.

Table 4
The mean of complete blood picture according to subtypes of AML (N=57)

Complete blood picture	Subtypes of AML	N	Mean ± SE	P-value
WBC (×10^{3}cell/µl)	M1 and M2	13	4.75 ± 1.37	0.268
	M3	23	6.85 ± 0.90	
	M4	12	3.89 ± 1.50	
	M5	9	7.25 ± 2.17	
Lymphocytes (%)	M1 and M2	13	43.96 ± 9.57	0.003*
	M3	23	22.37 ± 3.97	
	M4	12	56.83 ± 6.93	
	M5	9	44.48 ± 10.14	
RBC (×10^{6}cell/µl)	M1 and M2	13	3.15 ± 0.37	0.215
	M3	23	3.06 ± 0.13	
	M4	12	2.53 ± 0.13	
	M5	9	2.86 ± 0.19	
Hb (g/dl)	M1 and M2	13	7.97 ± 0.58	0.062
	M3	23	8.73 ± 0.27	
	M4	12	7.24 ± 0.32	
	M5	9	7.98 ± 0.50	
HCT (%)	M1 and M2	13	25.38 ± 2.17	0.066
	M3	23	26.47 ± 1.06	
	M4	12	21.93 ± 1.06	
	M5	9	22.18 ± 1.28	
MCV (FL)	M1 and M2	13	81.27 ± 1.50	0.513
	M3	23	83.94 ± 2.54	
	M4	12	87.13 ± 1.55	
	M5	9	84.36 ± 3.69	
MCH (pg)	M1 and M2	13	25.88 ± 0.73	0.073
	M3	23	28.39 ± 0.84	
	M4	12	29.00 ± 0.59	
	M5	9	28.37 ± 0.85	
MCHC (g/dl)	M1 and M2	13	31.66 ± 0.36	0.056
	M3	23	33.15 ± 0.44	
	M4	12	33.28 ± 0.44	
	M5	9	32.61 ± 0.33	
PLT (×10^3/µl)	M1 and M2	13	80.46 ± 27.89	0.068
	M3	23	126.55 ± 21.11	
	M4	12	59.59 ± 15.70	
	M5	9	52.13 ± 13.59	

* Significant difference

The results of current study(table 4) revealed a significant differences between lymphocytes and AML subtypes at (0.003) , this is may be the lymphocytes plays an important role in tumor induction pathogenesis , while another blood

parameters are less related to AML (Sidney *et al.*,2014). The mean of WBCs (7.25 ± 2.17 × 10^3 cell/µl) was strongly correlated with AML subtypes , so the lower WBCs count was, the lower severity of AML . This results agrees with study of Greenwood *et al.*, (2006) and Oliveira *et al.*, (2010). The higher mean value of Hb and platelets was closely correlated with higher relapse rates of AML subtypes in comparison to the lower mean value of it. The increase in the incidence of AML subtypes and its relationship to these parameters, especially Hb, platelets, MCV, MCH and MCHC, indicates that this disease causes many blood disorders such as decrease the amount and content of Haemoglobin that decrease the oxygen level in the blood , formation of clots inside the capillaries, and a higher normal red cell volume indicates that the red cells are larger than normal (macrocytosis), These pathological changes in the blood parameters can be used in diagnosing the disease, monitoring the progression of the disease, and thus controlling the exacerbation of the disease (Jahic *et al.*,2017). In present study, most patients showed highly mean value with M3 subtype in contrast to another AML subtypes , in addition to the mean value of total leukocyte count (TLC), Hemoglobin, platelets count, MCV, and MCHC , which is indicates that there was a significant correlation between AML-subtypes expression and these parameters , and this was matching with the results certain studies (Schuurhuis *et al.*, 2010; Zhu *et al.*, 2013).

The relationship between AML subtypes and CD34 concentration

Table (5), and figure (4) showed that the mean levels of according to AML subtypes (M1 and M2, M3, M4 and M5). There were insignificant differences according to the subtypes of AML.

Table 5
The mean levels of biomarkers according to the acute myeloid leukemia subtypes (N=57)

Study variables	subtypes of AML	N	Mean ± SE	F	P-value
CD34 (pg/µl)	M1 and M2	13	0.54 ± 0.02	0.753	0.525
	M3	23	0.56 ± 0.06		
	M4	12	0.49 ± 0.04		
	M5	9	0.65 ± 0.13		

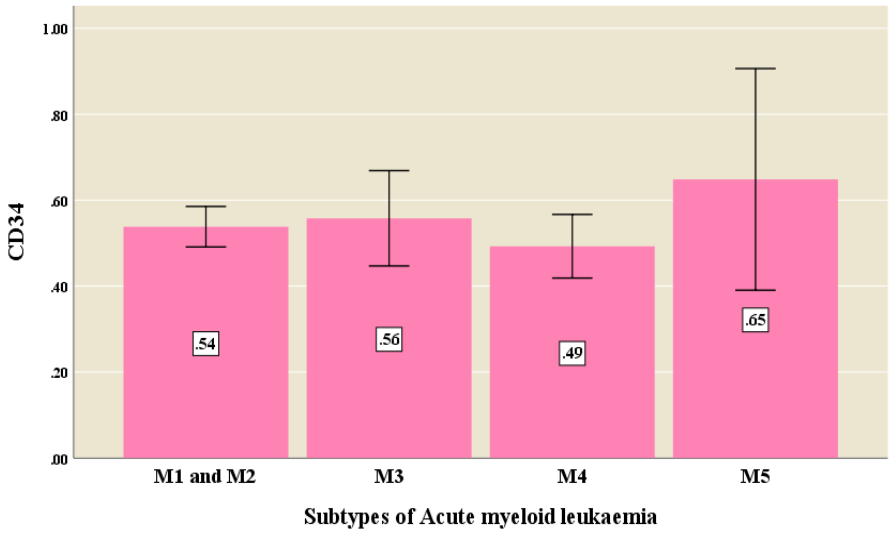

Figure 4. The mean levels of CD34 (pg/µl) according to subtypes of AML (N=57)

The differences in the AML subtypes with the biomarkers under study in total sample, does not reflect the subtypes of AML patients in the another region , because the study did not include larger number of patients. The results confirmed that the correlation between AML subtypes and biomarker (CD34) as tool for monitoring the severity of disease and shorter overall survival, which is consistent with the results of Appelbaumm *et al.*, (2006) . The biomarker CD34 is expressed on the surface of immature hematopoietic progenitor cells and is involved in cellular adhesion and mediates resistance to apoptosis. differential CD34 expression in the blasts of acute leukemia patients has been reported to be a significant prognostic indicator. High percentages of CD34 in some types and low in another types of AML have been shown to correlate with good prognosis in AML patients in several studies (Rahul *et al.*,2014) , and these results are consistent with current data that found the expression of CD34 with M3 subtype are more expression from others. In present study, it was found that all patients with AML expression, were CD34 high (>50% level) ,these meaning that the important of this biomarker in detection the severity of AML . These results were similar to a study of AML patients, as the value of CD34 was higher in some subtypes of AML . The differences between these subtypes were statistically significant (P<0.001) when analysed by a two-tailed (T) test according to Mean ± SE (Oyan *et al.*,2005).

Correlation between CD34 with study parameters

The correlation between CD34 and study variables were shown in Table (6), it was found significant positive correlation between CD34 and WBC count (r=0.359 ,p=0.005) patients as shown in Figure (5), Furthermore, no correlation between CD34 and other studied parameters was found.

Table 6
Show the correlation between CD34 (%) and study variables among patients with acute myeloid leukaemia (N=60)

Study variables	CD34 (%)		
	N	r	P-value
WBC (*10^3/µl)	60	0.359	0.005*
Blast (%)	60	0.071	0.59
Neutrophil (%)	60	-0.126	0.336
Lymphocyte (%)	60	0.043	0.746
RBC (*10^6/µl)	60	-0.028	0.832
Hb (g/dl)	60	-0.105	0.426
HCT (%)	60	-0.171	0.191
MCV (FL)	60	-0.129	0.327
MCH (pg)	60	-0.067	0.611
MCHC (g/dl)	60	0.078	0.552
PLT (*10^3/µl)	60	-0.066	0.617

* Significant difference

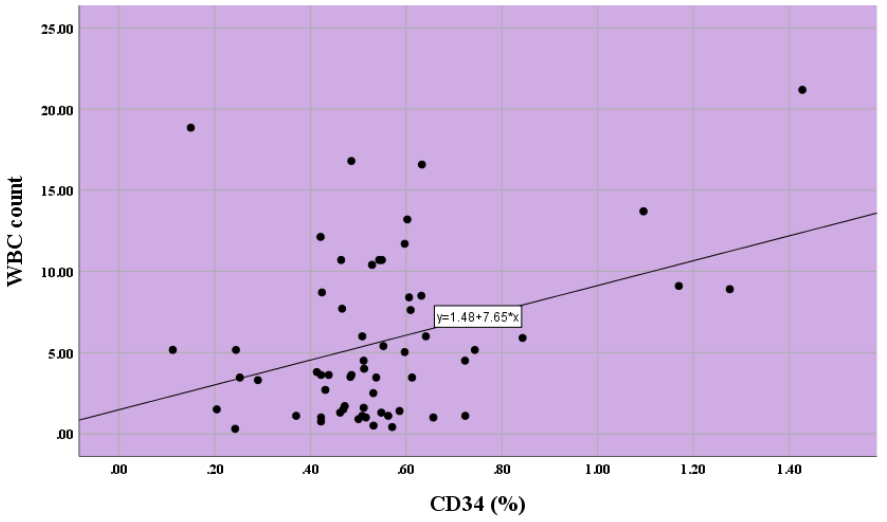

Figure 5. The correlation between CD34 (%)and WBC count (*10^3/µl) among patients with acute myeloid leukaemia (N=60, r=0.359, P=0.005*)

Conclusions

CD34 as a prognostic marker might be easily adopted in clinical practice to rapidly identify patients with AML. Furthermore, Complete blood picture (WBC, Blast , Neutrophil , Lymphocyte, RBCs, Hb, HCT, MCHC, and PLT) can also be used in prognosis of AML patients.

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