*Original Research Article*

# Study of CD 47 Expression in Patients with Acute MyeloidLeukemia


## Abstract

**Background:**Acute Myeloid Leukemia (AML)is the most common acute leukemia affecting adults, and its frequency increases with age.CD47may not only be valuable biomarker to recognize leukemic stem cells (LSCs) but also may denote a clinically relevant pathogenetic factor of disease.The aim of this work was to study the expression of CD47 in patients with acute myeloid leukemia and to identify its role as a prognostic marker.

**Methods:**The present study was carried out on 40 newly diagnosed untreated AML patients. AML patients were divided into two groups according to status of expression: Negative Group including patients expressing CD47 on less than 20% of their blast cells (28 patients) and positive Group including patients expressing CD47 on 20 % or more of their blast cells (12 patients). All patients were subjected tolaboratory investigation for detection of CD47 expression m bone marrow aspirate and venous blood samples of AML patients by flowcytometry.

**Results:** Complete remission was foundin 2 patients (16.67%), 6 patients relapsed (50%) and 4 patients died (33.33%) of 12 cases that had exhibitedCD47 positive expression. Kaplan-Meier Survival curve shows significant higher overall survival and disease-free survival in negative CD47 expression group than in positive CD47 expression group.

**Conclusions:**Positive CD47 expression levels are associated with a poor outcome in AML patients and its expression can be easily determined in routine flow cytometric analysis. Therefore, it should be regularly investigated as a bad prognostic factor for theassessment of AML patients.

**Keywords:**CD 47, Expression, Acute Myeloid Leukemia.

## Introduction:

“Acute myeloid leukemia (AML) is a cancer of the myeloid line of blood cells, which ischaracterized by the rapid growth of abnormal white blood cells that accumulate in the bone marrow and interfere with the production of normal blood cells. AML is the most common acute leukemia affecting adults, and its frequency increases with age”[1].

“AML has many subtypes; treatment and prognosis differ among subtypes. AML is cured in 35-40% of people under 60 years old and 5-15% over 60 years old. Older people who are not able to withstand intensive chemotherapy have an average survival of 5-10 months”[2].

CD47 is a transmembrane glycoprotein, which is widely expressed in several human tissues and functions as a ligand for many receptors, including signal regulatory protein alpha (SIRPa).

“CD47- SIRPa signaling on macrophages or dendritic cells results in inhibition of phagocytosis by immunoreceptor tyrosine- based inhibition motif (ITIM) mediated recruitment of protein tyrosine phosphatase Src homology region 2 damain-containing phosphatase 1\2 (SHP-1\2)”[3].

“CD47 inhibits nitric oxide (NO) \cyclic guanine monophosphate (cGMP) signaling pathway and restricts NO-mediated vasodilatation and decrease the inhibition of platelet aggregation”[4].

“Upregulation of CD47 expression is a physiological mechanism: administration of cyclophosphamide granulocyte colony stimulating factor (G-CSF) and lipopolysaccharide, respectively stimulate mobilization of hematopoietic stem cells (HSCs), resulting m extensively elevated CD47 expression on circulating HSCs in comparison to their bone marrow (BM)-resident counterparts. This CD47 up regulation apparently protects mobilized HSC fromsubsequent macrophage mediated phagocytosis”[5].

“Evasion of macrophage mediated phagocytosis by CD47-SIRPasignaling resulted in decreased blast clearance by the innate immunesystem and conferreda survival advantage to the leukemic stem cells(LSCs) as compared with the normal HSC counterparts”[6].

“The residual leukemic stem cells fraction of cells followingconventional chemotherapies can lead to relapse of the disease. CD47has been demonstrated to be differentially expressed on AML LSCcompared with normal HSC”[7].

“So, CD47may not only be avaluable biomarker to recognize LSCsbut also itmay denote a clinically relevant pathogenetic factor ofdisease”[6].

The aim of this work wasto study the expression of CD47 in patients with acute myeloid leukemia and to identify its role as a prognostic marker.

## Patients and Methods:

The present study was carried out on 40 newly diagnosed untreated AML patients, 26 male and 14 female, ages 29-71 years at hematology /oncology units, Internal Medicine Department, Tanta University Hospital.

Exclusion criteria were secondary, mixed or pediatric AML, other malignancies or treated AML.

AML patients were divided into two groups according to status of expression: Negative Group including patients expressing CD47 on less than 20% of their blast cells (28 patients) and positive Group including patients expressing CD47 on 20 % or more of their blast cells (12 patients).

All patients were subjected to: Detailed history, careful clinical examination to presence and extent of leukemia involvement including pallor, purpuric eruptions, size of liver and spleen, lymphadenopathy and CNS involvement. Also, routine laboratory investigationsincluding complete blood count (CBC), erythrocyte sedimentation rate (ESR), serum lactate

dehydrogenase (LDH), liver function tests, bone marrow aspiration, immunophenotyping of blast cell in BM aspirate samples using Becton Dickinson(BD FACS calibre) flow cytometer. The panel for acute leukemia was fluorescent isothiocyanate (FITC/phycoerythrin PE), Conjugated monoclonal antibodies (MoABs) which were used to diagnose AML and include common progenitor marker (CD34, HLA-DR), myeloid markers (CD13, CD33, myeloperoxidase (MPO)), Monocyticmarker (CD14, CD64), Erythroidmarker (Glycophorin A), megakaryoblastic markers (CD61, CD41), lymphoidmarkers (B cell markers: CD10, CD 19 and T cell markers: CD2, CD7). Specific laboratory investigations were performed for detection of CD47 expression m bone marrow aspirate and venous blood samples of AML patients by flowcytometry.

After being fully investigated, all patient received chemotherapy and were observed for 12 months as regard clinical and laboratory findings of remission and relapse taking care to estimate the date of first complete remission (CR), date of first relapse and date of death or last seen alive. A patient was relapsed when the bone marrow blasts >5%, reappearance of blasts in the blood, or development of extramedullary disease[8].

The patient was followed up for one year then the time at which the patient achieved remission, relapsed, died or last seen alive detected for calculation of overall survival (OS) and the disease free survival (DFS)[8].

TWO values were used in evaluating prognosis of patients: overall survival (OS) which is measured from the date of diagnosis of the patient to the date of death or date last seen and disease-free survival alive (DFS) which is measured from the date of occurrence of disease-free state to disease recurrence or death[8].

Peripheral blood and bone marrow aspiration samples were taken. Bone marrow aspiration was performed under complete aseptic technique and samples were collected in sterile covered EDTA tube and labelled for immunophenotyping and detection of CD47 expression.

Smears were stained with Leishman's stain and cytochemical SBB stain for morphological diagnosis.

**Flowcytometric immunophenotyping:**Immunophenotyping is the term applied to the identification of cells using antibodies to antigens expressed by these cells. Flowcytometry is a process of passing cells singly in a fluid stream (isotonic sheath fluid) through a beam of light, the light source is a laser.Fluorescent dyes may bind or intercalate with different cellular components such as DNA or RNA. Additionally, antibodies conjugated to fluorescent dyes can bind specific proteins on cell membranes or inside cells. When labelled cells are passed by a light source, the fluorescent molecules are excited to a higher energy state. Upon returning to their resting states, the fluorochromes emit light energy at higher wavelengths.

The use of multiple fluorochromes each with similar excitation wavelengths and different emission wavelengths (or "colours) allows several cell properties to be measured simultaneously. Commonly used dyes include propodeum iodide, phycoerythrin, and fluorescein, although many other dyes are available. Tandem dyes with internal fluorescence resonance energy transfer can create even longer wavelengths and more colors[9]. Detection of CD47 expression was done by clone B6H12 (CD47 monoclonal antibody)

**Flowcytometric analysis:**

FACS calibre flowcytometry from Becton Dickinson (BD) was used, where 10.000events (cells) at least were acquired. Analysis was done using automated CELL QUEST Pro software, the percentage positive CD47blasts were calculated by gating on blast population. The instrument setting was set by using calibrated beads provided the manufacture. Isotopic quality control was used to exclude non-specific binding and auto- fluorescence.Light scatter histogram, forward light scatter versus logside scatter, was used to delineate cell populations of interest by bitmap drawing (gating). Gating fluorescence histogram is evaluated for positive cells by using cursor position from histograms for isotopic controls, sothat98% of positive are defined. In routine diagnostic, the universal accepted cutoff for positivity is 20%

in AML. As regard CD47, a case with positive expression was defined if 20% of the gated cell or more expressed the marker.

**Statistical analysis**

Statistical analysis was done by SPSS v26 (IBM Inc., Chicago, IL, USA). Quantitative variables were presented as mean and standard deviation (SD) and compared between the two groups utilizing unpaired Student's t- test. Qualitative variables were presented as frequency and percentage (%) and were analysed utilizing the Chi-square test or Fisher's exact test when appropriate. A two tailed P value $\leq 0.05$ was considered statistically significant.

## Results:

Table 1 shows gender data, hepatosplenomegaly, lymphadenopathy, pallor, laboratory data, FAB classification and distribution of the studied AML cases according to CD 47 expression % of studied AML cases.**Table 1**

**Table 1: Gender data, hepatosplenomegaly, lymphadenopathy, pallor, laboratory data, FAB classification, distribution of the studied AML cases according to CD 47 expression % and Outcomein studied AML cases (n=40)**

| | | |
|---|---|---|
| **Sex** | **Male** | 26 (65 %) |
| | **Female** | 14 (35%) |
| **HSM** | | 18 (45%) |
| **LN** | | 14 (35%) |
| **Pallor** | | 29 (72.5%) |
| **Laboratory data** | **Hb (gm/dl)** | 7.953 ± 1.421 |
| | **WBC (xl0$^9$/L)** | 43.093 ± 26.088 |
| | **Platelets (xl09/L)** | 74.800 ± 22.758 |
| | **Blasts (%)** | 27.600 ± 12.993 |
| | **ESR (1$^{st}$ hr.)** | 75.350 ± 20.252 |
| | **LDH (IU/L)** | 933.850 ± 349.519 |
| | **BM blast (%)** | 62.900 ± 16.086 |
| **FAB subtypes** | **M1** | 4 (10%) |
| | **M2** | 11 (27.5%) |
| | **M3** | 10 (25%) |
| | **M4** | 7 (17.5%) |
| | **M5** | 8 (20%) |
| **CD47 (%)** | | 12 (30%) |
| **Outcome** | **Complete remission** | 25 (62.5%) |
| | **Relapse** | 9 (22.5%) |
| | **Death** | 6 (15 %) |

HSM: Hepatosplenomegaly, LN: Lymphadenopathy, Hb: hemoglobin, WBC: White blood cells, ESR: Erythrocyte sedimentation rate, BM. Bone marrow,M1: Acute myeloblastic leukemia with minimal maturation,

M2: Acute myeloblastic leukemia with maturation, M3: Acute promyelocytic leukemia, M4: Acute myelomonocytic leukemia, M5: Acute monocytic leukemia, CD47: Cluster of differentiation 47.

There was no significant relation between Sex, HSM, LN, laboratory data at diagnosis, FAB classification and CD47 expression.**Table 2**

**Table 2: Relation between Sex, HSM, LN, laboratory data at diagnosis and FAB classification according toCD47expression**

| | | CD47 (%) | | P-value |
|---|---|---|---|---|
| | | Negative | Positive | |
| **Sex** | **Male** | 17 (60.71%) | 9 (75%) | 0.385 |
| | **Female** | 11 (39.29%) | 3 (25%) | |
| **HSM** | **Present** | 13 (46.43%) | 5 (41.67%) | 0.781 |
| | **Absent** | 15 (53.57%) | 7 (58.33%) | |
| **LN** | **Present** | 9 (32.14%) | 5 (41.67%) | 0.563 |
| | **Absent** | 19 (67.86%) | 7 (58.33%) | |
| **Laboratory data at diagnosis** | **Hb (gm/dl)** | 8.004 ± 1.380 | 7.833 ± 1.569 | 0.733 |
| | **WBC (x $10^9$/L)** | 43.254 ± 27.009 | 42.717 ± 24.944 | 0.953 |
| | **Platelets (x $10^9$/L)** | 76.929 ± 22.264 | 69.833 ± 24.105 | 0.373 |
| | **Blasts (%)** | 27.500 ± 13.585 | 27.833 ± 12.059 | 0.942 |
| | **ESR ($1^{st}$ hr.)** | 76.429 ± 19.101 | 72.833 ± 23.424 | 0.613 |
| | **LDH (IU/L)** | 951.929 ± 353.904 | 891.667 ± 350.606 | 0.624 |
| | **BM blast (%)** | 62.500 ± 15.999 | 63.833 ± 16.964 | 0.814 |
| **FAB subtypes** | **M1** | 1 (3.57%) | 3 (25%) | 0.259 |
| | **M2** | 9 (32.14%) | 2 (16.67%) | |
| | **M3** | 8 (28.57%) | 2 (16.67%) | |
| | **M4** | 5 (17.86%) | 2 (16.67%) | |
| | **M5** | 5 (17.86%) | 3 (25%) | |

HSM: Hepatosplenomegaly, LN: Lymphadenopathy, Hb: hemoglobin, WBC: White blood cells, ESR: Erythrocyte sedimentation rate, BM: Bone marrow,M1: Acute myeloblastic leukemia with minimal maturation, M2: Acute myeloblastic leukemia with maturation, M3: Acute promyelocytic leukemia, M4: Acute myelomonocytic leukemia, M5: Acute monocytic leukemia, CD47: Cluster of differentiation 47.

Complete remission was in 2 patients (16.67%), 6 patients relapsed (50%) and 4 patients died (33.33%) of 12 cases CD47 positive expression. There was a significant value between two groups regarding overall survival and disease-free survival (Pvalue<0.001). **Table 3**

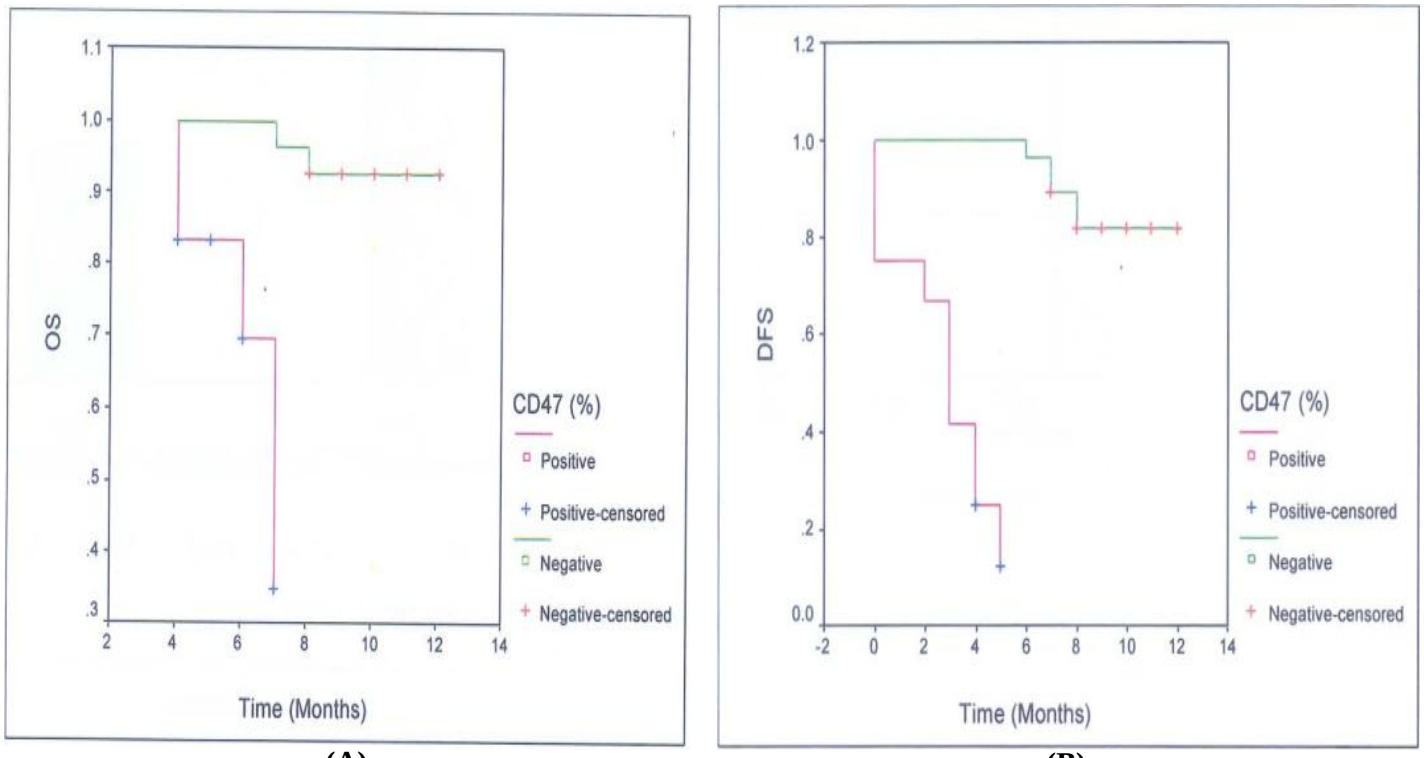
**Table 3: Relation between outcome and CD47 expression and comparison between overall survival and disease-free survival with CD47 expression positive and negative groups.**

| | | CD47 (%) | | P-value |
|---|---|---|---|---|
| | | Negative | Positive | |
| **Outcome** | **Complete remission** | 23 (82.14 %) | 2 (16.67 %) | <0.001 * |
| | **Relapse** | 3 (10.71 %) | 6 (50 %) | |
| | **Death** | 2 (7.14 %) | 4 (33.33%) | |

| | | | |
|---|---|---|---|
| **OS (Months)** | 9.929 ± 1.464 | 5.417 ± 1.084 | <0.001 * |
| **DFS (Months)** | 9.214 ± 1.729 | 2.750 ± 1.865 | <0.001 * |

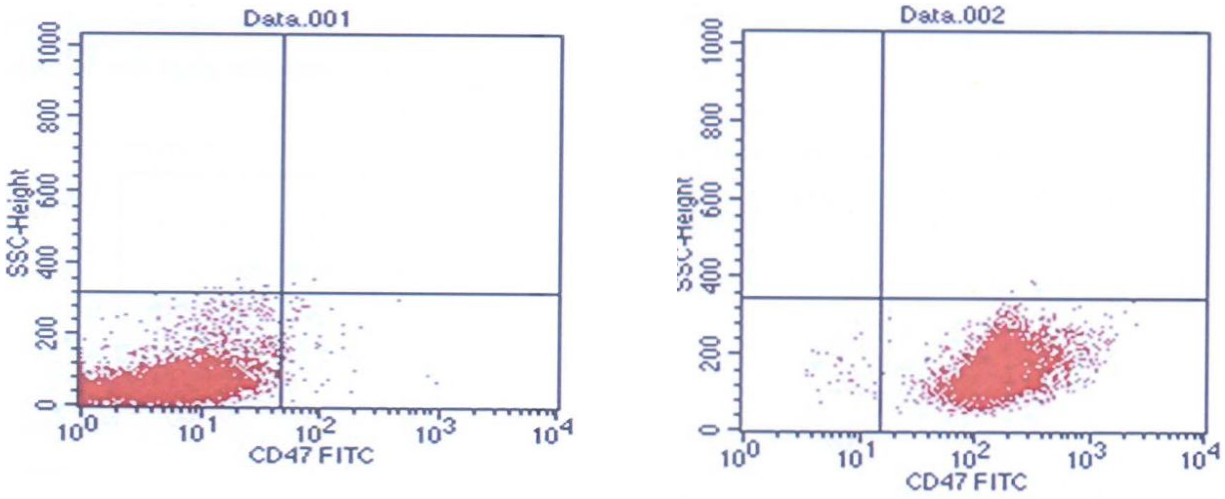
* Significant as P value ≤ 0.05. HSM: Overall survival, DFS: Disease-free survival, CD47: Cluster of differentiation 47.

Kaplan-Meier Survival curve shows significant higher overall survival and disease-free survival in negative CD47 expression group than in positive CD47 expression group.**Figure 1**




**(A)** **(B)**

**Figure 1: Kaplan - Meier survival curve (A) overall survival with CD47 Expression positive and negative groups (B) disease free survival with CD47 expression positive and negative groups**




**(A)** **(B)**

**Figure 2: Flowcytometric analysis represents: (A) dot plot showing negative control for CD47 expression (B) dot plot showing positive CD 47 expression**

## Discussion

"Acute myelogenous leukemia (AML) is a clonal, malignant disease of hematopoietic tissues that is characterized by accumulation of abnormal (leukemic) blast cells, principally in the marrow, and impaired production of normal blood cells. Thus, the leukemic cell infiltration in marrow is accompanied, nearly invariably, by anemia and thrombocytopenia. The absolute neutrophil count may be low or normal, depending on the total white cell count"[2].

"CD47 has been shown to participate in cellular processes such as apoptosis, proliferation, adhesion, and migration. In particular, CD47 functions as a marker of "self' by inhibiting phagocytosis of autologous cells through interaction with SIRPa expressed by professional phagocytes, such as macrophages"[10].

This Study stated that AML can occur in any age, but in general the incidence of AML increases with age also, Deschler and Lubbert [11]stated that "incidence of AML increases through adulthood period; during with 70% to 80% of acute leukemias are AML, with marked rise in incidence in elderly. Much of this increase is attributed to AML with a

myelodysplastic related change, which become more with age, while the incidence of de novo AML remains approximately constant across all adult age groups".

In the present Study, it was found that 26 patients (65%) were males, and 14 patients (35%) were females. This in concordance with a study done by Jemal et al. [12]who reported that, the incidence of AML is higher in male than in females.

As regards the hepatosplenomegaly it was observed in 18/40 (45%) of the studied Patients. In contrast Lichtman and Lieveld[13]who reported that hepatosplenomegaly occurs in one about one third ofAML patients.

Lymphadenopathy was absent in 26 patients (65%). This is concordance with Lichtman and Lieveld[13]who reported thatLymphadenopathy is uncommon feature of AML.

As regards pallor, Out of the studied patients 29 patients (72.5%)showed pallor and 11 patients (27.5%) absent pallor This is concordancewith Mathur et al. [14]who reported that Pallor was seen as apresenting symptom in 100% ofthe patients.

As regards FAB classification and its distribution among

"patients'groups with CD47 Expression (12 patients).Ml was in 3 patients (25%), M2 was in 2 patients (16.67%), M3 was in 2patients (16.67%), M4 was in 2 (16.67%) and M5 was in 3 patients (25%) ofl2 cases CD47 expressed. So, the present study showed no significant difference in CD47 expression in relation to FAB subgroups. This is in concordance with" kassemet al[15].It was also in agreement with previously studies Majeti[7].

"CD47 Expression in relation to gender, Hb, TLC, platelets count, blast count, ESR, LDH, showed no statistically significant difference between the negative and positive groups. These findings were in Concordance with" kassemet al[15].

"IN this study by comparing between groups, it was found that patients with positive CD47 expression had a significant lower CR rate and a significant higher relapse and death rate.

Thus, there was a significant association between CD47 expression and poor outcome of patients($p<0.001$). These findings were in concordance with" Majeti et al[7].

"Overall survival (OS) and disease-free survival (DFS) were evaluated in negative CD47 expression group using Kaplan-Meier Survival curve. A Significant decrease in both OS ($p< 0.001$) and DFS ($p<0.001$) was found in positive CD47 expression group. This is in accordance with" Majeti et al[7]. Also, kassem et al[15] observed that "there was inverse correlation between CD47 expression and Overall survival where increased expressionwas associated with worse overall survival".

Acute myelogenous leukemia (AML) 1s organized as a cellular hierarchy initiated and maintained by a subset of self-renewing leukemic stem cells (LSC). It was hypotfiesized that increased CD47 expression on human AML LSC contributes to pathogenesis by inhibiting them

"phagocytosis through the interaction of CD47 with an inhibitory receptor on phagocytes. It was found that CD47 was more highly expressed on AML LSC than their normal counterparts, and that increased CD47 expression predicted worse overall survival in AML patients"[16].

"By expressing CD47, AML cells trigger the "don't eat me" signal to macrophages via SIRPa engagement and can therefore escape the immune system by inhibiting phagocytosis"[16].

Also, Sallman [17] reported that "CD47 is a dominant macrophage checkpoint that is overexpressed on most cancer cell and increased CD47 expression has been associated with poorer prognosis".

"Several anti-human CD47 monoclonal antibodies have been generated including some capable of blocking the CD47-SIRPainteraction (B6H12.2 and BRIC126) and others unable to do so"[18]. "So, blocking monoclonal antibodies directed against CD47 enabled phagocytosis of AML LSC"[19].

Hu5F9-G4 (5F9), a first- in- class antibody targeting CD47, used as monotherapy or in combination with standard azacitidine was well tolerated and provided deep and durable response in patients with acute myeloid leukemia (AML) this is according to study presented at the 2019 European Hematology Association (EHA) Congress.

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## Conclusions:

Positive CD47 expression levels are associated with a poor outcome in AML patients and its expression can be easily determined in routine flow cytometric analysis. Therefore, it should be regularly investigated as a bad prognostic factor for assessment of AML patients.

**Ethical Approval and consent:**

The study was done after approval from the Ethical Committee Tanta University Hospitals.An informed written consent was obtained from the patients.

## References:

1. Ding L, Ley TJ, Larson DE, Miller CA, Koboldt DC, Welch JS, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. Nature. 2012;481:506-10.

2. Döhner H, Weisdorf DJ, Bloomfield CD. Acute Myeloid Leukemia. N Engl J Med. 2015;373:1136-52.

3. Oldenborg PA, Gresham HD, Lindberg FP. CD47-signal regulatory protein alpha (SIRPalpha) regulates Fcgamma and complement receptor-mediated phagocytosis. J Exp Med. 2001;193:855-62.

4. Roberts DD, Miller TW, Rogers NM, Yao M, Isenberg JS. The matricellular protein thrombospondin-1 globally regulates cardiovascular function and responses to stress via CD47. Matrix Biol. 2012;31:162-9.

5. Jaiswal S, Jamieson CH, Pang WW, Park CY, Chao MP, Majeti R, et al. CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. Cell. 2009;138:271-85.

6. Galli S, Zlobec I, Schürch C, Perren A, Ochsenbein AF, Banz Y. CD47 protein expression in acute myeloid leukemia: A tissue microarray-based analysis. Leuk Res. 2015;39:749-56.

7. Majeti R, Chao MP, Alizadeh AA, Pang WW, Jaiswal S, Gibbs KD, Jr., et al. CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. Cell. 2009;138:286-99.

8. Döhner H, Estey EH, Amadori S, Appelbaum FR, Büchner T, Burnett AK, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood. 2010;115:453-74.

9. Watson JV. The early fluidic and optical physics of cytometry. Cytometry. 1999;38:2-14.

10. Oldenborg PA, Zheleznyak A, Fang YF, Lagenaur CF, Gresham HD, Lindberg FP. Role of CD47 as a marker of self on red blood cells. Science. 2000;288:2051-4.

11. Deschler B, Lübbert M. Acute myeloid leukemia: epidemiology and etiology. Cancer. 2006;107:2099-107.

12. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin. 2009;59:225-49.

13. Liesveld JL, Lichtman MA. Acute Myelogenous Leukemia. In: Kaushansky K, Lichtman MA, Prchal JT, Levi MM, Press OW, Burns LJ, et al., editors. Williams Hematology, 9e. New York, NY: McGraw-Hill Education; 2006. p. 1183.

14. Mathur S. Clinical profile of acute leukemias: A study of 50 cases. Indian Pract. 1993;46:171-4.

15. Kassem H. CD47 Expression as a Possible Prognostic Tool in Egyptian AML Patients. Acta Sci Cancer Biol. 2017;1:25-30.

16. Kong F, Gao F, Li H, Liu H, Zhang Y, Zheng R, et al. CD47: a potential immunotherapy target for eliminating cancer cells. Clin Transl Oncol. 2016;18:1051-5.

17. Sallman DA, Donnellan WB, Asch AS, Lee DJ, Malki MA, Marcucci G, et al. The first-in-class anti-CD47 antibody Hu5F9-G4 is active and well tolerated alone or with azacitidine in AML and MDS patients: Initial phase 1b results. J Clin Oncol. 2019;37:7009-.
18. Subramanian S, Parthasarathy R, Sen S, Boder ET, Discher DE. Species- and cell type-specific interactions between CD47 and human SIRPalpha. Blood. 2006;107:2548-56.
19. Chao MP, Alizadeh AA, Tang C, Myklebust JH, Varghese B, Gill S, et al. Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma. Cell. 2010;142:699-713.