

## Original Research Article

### **Research of Natural Killer Cell (CD56+, CD16+, CD3-) and It's Activating Ligand (CD 112) in Acute Myeloid Leukemia**

#### **Abstract**

**Background:** Myeloid, erythroid, megakaryocytic, and monocytic cell lineage progenitors are all involved in the development of acute myeloid leukemia (AML), making AML a highly diverse collection of leukemias. This research was aimed to evaluate the impact of natural killer cell (CD56+, CD16+, CD3-) and its activating receptor ligand (CD 112) on AML and their clinicopathological significance.

**Methods:** This prospective randomized research was carried out on 40 newly diagnosed AML, and 20 apparently healthy subjects age and sex matched to cases group. (Determination of natural killer cell and activating receptor ligand).

**Results:** There was an evident decrease in percentage of NK cells in newly diagnosed AML cases. There was an evident increase in expression of CD112 in newly diagnosed AML cases. An evidently higher OS in cases with low NK cells expression with cutoff  $\leq 15.5$ .

**Conclusions:** There was an evident decrease in the level of NK cells and an evident increase in expression of CD112 in newly diagnosed AML cases. A lower percentage of NK cells and higher levels of expression of CD112 may predict better outcome of newly diagnosed AML cases.

**Keywords:** Natural Killer Cell, Activating Ligand (CD 112), Acute Myeloid Leukemia

## **Introduction:**

The malignant clonal hematopoietic disease known as acute myeloid leukemia (AML) is characterized by a halt in the differentiation of hematopoietic progenitor cells and an abnormality in the control of proliferation (1).

AML are malignant clones that originate in myeloid progenitor cells. Myeloblasts and other myeloid lineage embryonic cells proliferate uncontrollably as a consequence. It is possible for the malignant cells to gather in organs like the liver, spleen, and lymph glands after they have displaced the normal bone marrow (BM) cells and spread throughout the body. Upwards of 90% of adult leukemia cases are classified as AML (2).

The lymphoid cells that undertake innate immunity are known as natural killer (NK) cells, and they are a unique subgroup of lymphoid cells. NK cells are blood cells that originate in the bone marrow and become active when they meet target cells that produce ligands for NK cell receptors or when exposed to cytokines. (3)

Human NK cells account for 5% to 20% of all lymphocytes in the bloodstream. AML is just one of many blood tumors and is associated with NK cell dysfunction. Lack of human leukocyte antigen (HLA) expression on leukemia blasts which is the target for natural killer (NK) and natural killer-like T (NKT) cells. It is known that NK cells are ineffective against endogenous blast cells. (4)

Inhibitory receptors, including NKG2A and killer cell immunoglobulin like (KIR) family receptors, and activating receptors, like NK group 2D (NKG2D) and the natural cytotoxicity receptors (NCRs) family, regulate NK cell function. IN case of AML, cytotoxicity and cytokine release are diminished, activating receptors on NK cells are downregulated, and inhibitory receptors are upregulated. It has been hypothesized that cellular stress regulates the production of CD112 (nectin 2) and CD155 (poliovirus receptor, PVR), two receptors for DNAX accessory molecule 1 (DNAM-1). DNAM-1 ligands have been reported to bind

TACTILE CD96 and T cell immunoreceptor with Ig and ITIM domains (TIGIT), both of which are inhibiting receptors that control NK cell activity.

The loss of DNAM-1 on NK cells is correlated with CD112 expression on AML blasts, further supporting the idea that chronic ligand exposure can cause a decreased NK cell-activating receptor expression in AML cases. (5)

Because they produce ligands that engage with NK-cell-activating receptors, NK cells can easily kill AML cells. AML development cannot be successfully controlled by autologous NK cells, though. Downregulation of ligands for NK-cell activating receptors, release of soluble versions of these ligands, and upregulation of ligands for NK-cell inhibiting receptors are all proposed pathways for AML escape. Downregulation of NK cell stimulating receptors, including NKG2D and the natural cytotoxicity receptors (NCRs) NKp46 and NKp30 7, has also been related to reduced NK cell toxicity. (6)

The purpose of this research was to examine the clinical and pathological implications of natural killer cell (CD56+, CD16+, CD3-) and its activating receptor ligand (CD 112) expression in AML.

### **Patients and Methods:**

This prospective randomized research was carried out on 40 newly diagnosed AML, aged more than 18 years. In addition to 20 apparently healthy doner for stem cell transplantation subjects matched for age and sex with cases' group. This research was conducted at the clinical pathology department, Faculty of medicine, Tanta University Hospital during the period between 2019 and 2021.

Cases were followed up after remission induction for AML with the (7+3) protocol, which consists of 7 days of intravenous (IV) cytarabine and a single dosage of intravenous (IV) anthracycline in the first 3 days of treatment. Blood and bone marrow (BM) samples were

checked routinely for signs of disease after induction treatment and following BM recovery on day 28.

The research was conducted with permission from the medical institution's ethics board at Tanta University. All subjects voluntarily provided documented informed permission.

Exclusion criteria were any case with malignant diseases other than AML, less than 18 years, and treated AML cases.

All cases and controls were subjected to: Detailed history, clinical examination, and abdominal ultrasonography, routine laboratory investigations (CBC, Lactate dehydrogenase (LDH) and Erythrocyte sedimentation rate (ESR). Diagnosis of AML cases was based on morphological examination, cytochemical analysis, and immunophenotyping. Specific laboratory tests (Determination of natural killer cell and activating receptor ligand).

#### **Diagnosis of AML cases:**

Diagnosis of AML was based on morphological examination of bone marrow aspirate smears and the presence of blast cells  $\geq 20\%$  in BM film according to WHO proposal, together with the cytochemical stains included Myeloperoxidase (MPO, Nonspecific esterase ( $\alpha$ -naphthyl acetate esterase) and Sudan black and presence of immunopheno-typing results consistent with AML <sup>8</sup>.

Tests performed to diagnose AML: Giemsa stained BM aspirate smears for the existence of 20% of blasts BM. Analysis of stains taken from air-dried samples of BM for cytochemical markers. Myeloperoxidase (MPO), NSE, and Sudan black were the cytochemical stain. Becton Dickinson (BD) FACS Calibur immunophenotyping of blast cells in BM aspirate samples using the acute leukemia panel to validate diagnosis. Myeloid cell markers (CD 13, CD 33, CD 117, cyt Anti MPO), lymphoid cell markers (T-cell markers (CD 2, CD7, cyt CD3), and B-cell markers (HLA-DR, CD34) that are not lineage-specific. (CD 10, CD19, Anti TDT, cyt CD 79a). Identifying features of mononuclear cells (CD64, CD

14). Identification of erythrocytes (glycophorin A, CD 71). Antigen of megakaryocytes (CD61).

Sampling: Two ml of peripheral venous blood were collected into a labelled ethylenediaminetetra-acetic acid (EDTA) vacuum sealed tube for complete blood count and Giemsa-stained smears and were labelled. And one ml of peripheral blood was delivered into EDTA vacuum sealed tube and used for immunophenotypic determination. 2 ml of bone marrow aspiration sample was delivered into EDTA vacuum sealed tube and used for immunophenotypic determination. Two ml blood was collected into a plain tube and after centrifugation serum was separated for measurement of serum LDH, liver and renal tests.

Specific laboratory tests: By using Flow cytometry: Determination of natural killer cell (CD56+, CD 16+, CD3-) in peripheral blood samples-Determination of natural killer cell activating receptor ligand (CD 112) in bone marrow aspiration samples.

### **Flowcytometry**

Surface marker (CD112) procedure included labeling two tubes, one for a negative isotopic reference and one for the polyclonal antibodies, for each sample. The number of cells was concentrated to  $10^6$ .

The next steps were done to each sample: 100  $\mu$ l of BMA were put in the staining tube. 5  $\mu$ l of each antibody was added to the blood and mix well then incubated away from light at room temperature for 30 minutes. 2 ml of FACS lysing solution was added then incubated away from light at room temperature for 15 minutes. The samples were spined down at 1400 rpm for five minutes. The supernatant was discarded and saved the pellets. 2 ml of washing buffer (PBS) was added. The samples spined down at 1300 rpm for five minutes. The supernatant was discarded and saved the pellets. The pellets were resuspended in 500  $\mu$ l of washing buffer (PBS). The samples in the cytometer (Acquisition). Procedure for surface markers (Ckit of CD3/CD56+CD16). For each sample, two tubes were labelled, one for

negative isotopic control and the other for the monoclonal antibodies. The cell count was adjusted to  $10^6$ .

The next steps were done to each sample: 100  $\mu$ l of PB were put in the staining tube. Adding the aliquots of the antibodies (10  $\mu$ l of each antibody) to the blood and mix well. Incubation in the dark at room temperature for 30 minutes. 2 ml of FACS lysing solution was added then incubated away from light at room temperature for 1 five minutes. The samples were spined down at 1400 rpm for five minutes. The supernatant was discarded and saved the pellets. 2 ml of washing buffer (PBS) was added. The samples were spined down at 1300 rpm for five minutes. The supernatant was discarded and saved the pellets. The pellets were resuspended in 500  $\mu$ l of washing buffer (PBS). The samples were run in the cytometer (Acquisition).

**Flowcytometry technique outlines:** FACS caliber flowcytometry from Becton Dickinson was used for analysis. Automated software was used for data retrieval and analysis. The instrument setting was set following instructions by the manufacturer. Quality control was followed to exclude nonspecific binding and autofluorescence. 10.000 events (cells) at least were passed through the laser for each case and then the blast cells were gated (enclosed by a line to isolate them from the rest of the cells in the basic histogram) for immunophenotyping. Light scatter histogram, forward light scatter versus side scatter, was used to delineate cell populations of interest (Lymphocytes) and or (Myeloblasts) by bitmap drawing (gating). With the help of the cursor location from the isotopic controls in the gated fluorescence dot plot, we could identify 98% of positives.

#### **Sample Size Calculation:**

Forty cases are needed to have a confidence level of 95%, that is the real value within  $\pm 5\%$  of the measured value.

#### **Statistical analysis**

IBM's statistical analysis program, SPSS 20.0, was used to process the data that was inputted into the computer. (Armonk, NY: IBM Corp). Quantitative and qualitative information was

represented using numbers and percentages, and contrasted using the Chi-squared test. To make sure everything was distributed normally, we ran the Shapiro-Wilk test. Mean and standard deviation were used to characterize the quantitative data, with comparisons made using the Student t-test. The receiver operating characteristic curve (ROC) curve allows also a comparison of performance between two tests. Linear correlation coefficient was used for detection of correlation between two qualitative variables in one group. Kaplan-Meier, Cox regression were used to determine the evident association between Disease free survival (DFS) and Overall survival (OS). A 5% threshold of significance was applied to the results.

### Results:

Results obtained from the present research revealed that was no detectable evident difference between cases and controls in respect to age and gender ( $P>0.05$ ). There was an evident decrease in hemoglobin level and platelet count in AML cases compared to the controls. There was an evident increase in TLC, Blast % in BM, LDH and ESR 1st hour ( $P<0.001$ ) in AML cases compared to controls, there was an evident decrease as regard the expression of NK cells in AML cases as compared to controls ( $P=0.018$ ). There was an evident increase in CD112 in AML cases as compared to controls with P value ( $<0.001$ ). Table 1

**Table 1: Comparison between the two studied groups according to demographic data, laboratory parameters, NK cells and CD112**

		Cases (n = 40)		Control (n = 20)		P
		No.	%	No.	%	
Sex	Male	24	60.0	14	70.0	0.449
	Female	16	40.0	6	30.0	
Age (years)		46.50 ± 13.90		39.90 ± 13.20		0.083
<b>Laboratory parameters</b>						
Hb ( gm/dl)		8.39 ± 1.35		13.22 ± 1.35		<0.001*
PLT ( ×10 <sup>3</sup> /cmm)		76.3 ± 22.8		279.7 ± 73.36		<0.001*
WBC(×10 <sup>3</sup> /cmm)		39.0(18.0 – 77.50)		7.30(5.55 – 9.45)		<0.001*
Blast%(BM)		53.50(41.5 – 70.50)		1.75(0.75 – 2.50)		<0.001*
LDH (IU/L)		712.5(519.0 – 910.5)		177.5(149.0 – 200.0)		<0.001*
ESR 1 <sup>st</sup> h (mm /h)		95.0(85.0 – 112.5)		15.0(10.0 – 22.5)		<0.001*
NK cells (%)		12.65 ± 7.73		15.67 ± 5.22		0.018*
CD112 (%)		51.88 ± 26.10		13.09 ± 7.50		<0.001*

Data are presented as mean ± SD or frequency (%), \*: Statistically evident at  $p \leq 0.05$

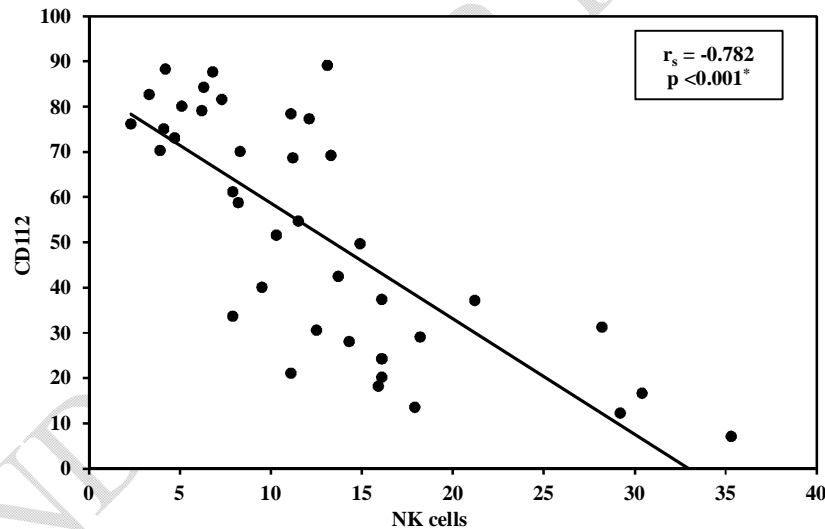
There was no difference in the percentage of NK cells and CD112 in favourable (M2-M4) and unfavourable (M0-M5-M6) ( $P=0.341$  and  $0.122$  respectively). Table 2

**Table 2: Comparison between Favourable & Unfavourable according to NK cells and CD112 in AML patients**

	Favorable (M2 – M4) (n = 22)	Unfavorable (M0 – M5 – M6) (n = 16)	P. value
<b>NK cells</b>	11.27 ± 6.30	14.93 ± 9.33	0.341
<b>CD 112</b>	57.88 ± 27.68	43.79 ± 22.55	0.122

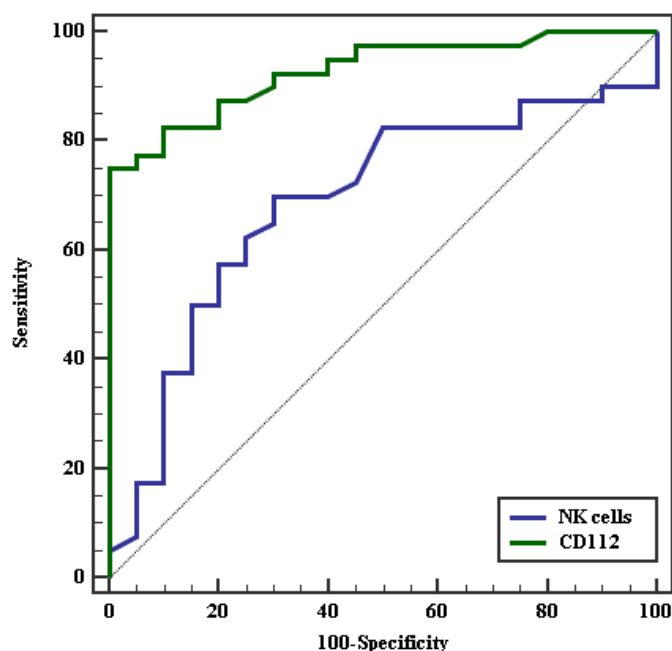
Data are presented as mean ± SD or frequency (%), \*: Statistically evident at  $p \leq 0.05$

There was non-evident correlation between Hb, Plt, WBC, LDH, Blast (BM) and NK cell expression and between ESR and NK cell expression ( $P>0.05$ ). There was non-evident correlation between Hb, Plt, WBC, LDH, Blast (BM) and CD112 expression between ESR and CD112 expression ( $P>0.05$ ). An evident inverse correlation between NK cells expression and CD112 expression with P value ( $<0.001$ ). Figure 1



**Figure 1: Correlation between NK cells and CD112 and in patients group (n= 40)**

NK cell could evidently discriminate AML cases with area with area under curve (AUC) of 0.688, at cut-off  $\leq 15.5$ , with sensitivity of 70.0%, specificity of 65.0%, positive predictive value (PPV) of 80% and negative predictive value (NPV) of 52%. CD112 could evidently discriminate AML cases with area with AUC of 0.929, at cut-off  $>18.2$ , with sensitivity of 87.5%, specificity of 75.0%, PPV of 87.5% and NPV of 75%. Figure 2



**Figure 2: ROC curve for NK cells and CD112 discriminate AML patients**

Data from this research showed that cases with complete remission were associated with evidently lower levels of NK cells expression ( $\leq 15.5$ ). Moreover, relapsed and dead cases had evidently higher mean levels of NK cells expression ( $>15.5$ ). Cases with complete remission were associated with evidently higher levels of CD112 expression ( $>18.2$ ). Moreover, all cases with evidently lower mean levels of CD112 expression ( $\leq 18.2$ ) were dead. Table 3

**Table 3: Relation between NK cells and outcome in patients group (n = 40)**

Outcome	NK cells				Mean $\pm$ SD.	p
	$\leq 15.5$ (n = 28)		$>15.5$ (n = 12)			
	No.	%	No.	%		
Complete remission	15	53.6	0	0.0	7.65 $\pm$ 3.68	0.001*
Relapse	9	32.1	4	33.3	12.76 $\pm$ 6.29	
Death	4	14.3	8	66.7	18.77 $\pm$ 8.75	
Outcome	CD112				Mean $\pm$ SD.	p
	$\leq 18.2$ (n = 5)		$>18.2$ (n = 35)			
	No.	%	No.	%		
Complete remission	0	0.0	15	42.9	79.19 $\pm$ 6.67	$<0.001^*$
Relapse	0	0.0	13	37.1	46.40 $\pm$ 17.31	

<b>Death</b>	5	100.0	7	20.0	23.67±10.54	
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\*: Statistically evident at  $p \leq 0.05$

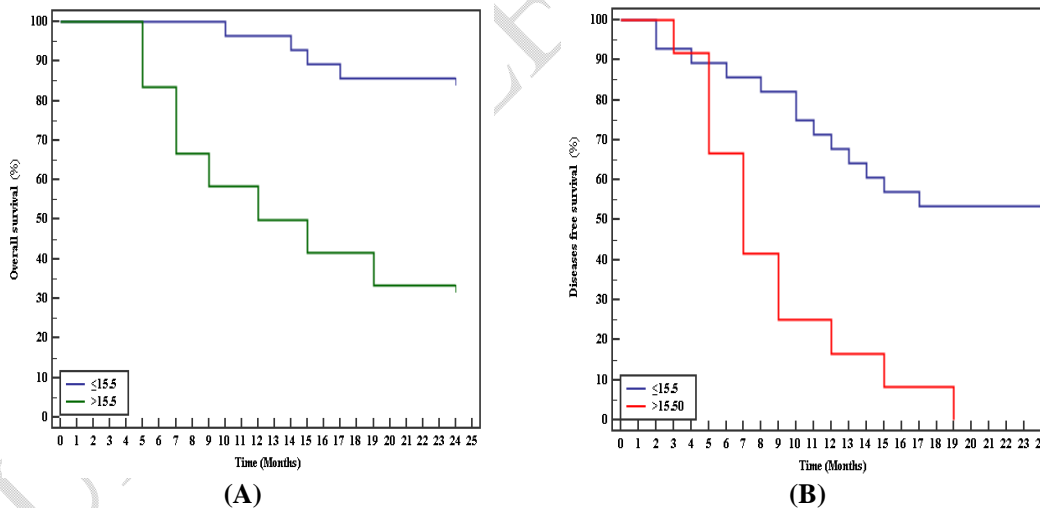
There was an evident difference between low levels and high levels of expression of NK cells with P value ( $<0.001$ ) for both (OS, DFS). Kaplan-Meier survival curve shows an evident higher OS in cases with low NK cells expression with cutoff  $\leq 15.5$  (p value  $<0.001$ ). Table 4

**Table 4: Kaplan-Meier curve for OS with NK cells and DFS with NK cells**

NK cells	Mean	%	p
$\leq 15.5$	22.571	85.7	$<0.001^*$
$>15.5$	14.583	33.33	
Diseases free survival			
$\leq 15.5$	17.286	53.6	$<0.001^*$
$>15.5$	8.583	0.0	

\*: Statistically evident at  $p \leq 0.05$

OS and diseases free survival was evidently higher with NK cells expression  $\leq 15.5$  (p value  $<0.001$ ). Figure 3



**Figure 3 : Kaplan-Meier curve for (A) OS with NK cells and (B) DFS with NK cells**

The cases with high NK cells expression had a higher relative risk of mortality when compared to those with low relative expression (hazard ratio (HR) = 1.142; 95% confidence interval (CI) = (1.066 – 1.222),  $p < 0.001$ ). Cases with elevated WBC count had a higher relative risk of mortality (hazard ratio (HR) = 1.018; 95% confidence interval (CI) = (1.001 –

1.035), p=0.032). The cases with low CD112 expression had an higher relative risk of mortality when compared to those with high relative expression (hazard ratio (HR) = 0.880; 95% confidence interval (CI) = (0.817 – 0.947), P= 0.001). Table 5

**Table 5: Univariate and multivariate COX regression analysis for the parameters affecting OS**

	Univariate		Multivariate	
	P	HR (LL – UL 95% C.I)	P	HR (LL – UL 95% C.I)
<b>Sex</b>	0.093	2.679(0.848 – 8.465)		
<b>Age (years)</b>	0.199	1.031(0.984 – 1.080)		
<b>Fever</b>	0.310	2.889(0.373 – 22.392)		
<b>Bleeding</b>	0.202	3.794(0.489 – 29.414)		
<b>HSM</b>	0.943	1.043(0.331 – 3.287)		
<b>LN</b>	0.713	0.798(0.240 – 2.652)		
<b>FAB</b>	0.310	2.889(0.373 – 22.392)		
<b>Blast</b>	0.123	1.025(0.993 – 1.059)		
<b>Hb</b>	0.069	1.524(0.968 – 2.399)		
<b>PLT</b>	0.107	1.003(0.999 – 1.006)		
<b>WBC (×103)</b>	0.032*	1.018(1.001 – 1.035)	0.183	1.019(0.991 – 1.049)
<b>LDH</b>	0.130	1.001(1.0 – 1.002)		
<b>ESR</b>	0.107	0.972(0.940 – 1.006)		
<b>NK cells</b>	<0.001*	1.142(1.066 – 1.222)	0.684	1.024(0.913 – 1.148)
<b>CD112</b>	0.001*	0.880(0.817 – 0.947)	0.010*	0.895(0.823 – 0.973)

HR: Hazard ratio, C.I: Confidence interval, LL: Lower limit , UL: Upper Limit, \*: Statistically evident at p ≤ 0.05

## Discussion

Since both the innate and acquired immune systems can recognize acute myeloid leukemia (AML) cells and eliminate them or maintain a balance by suppressing tumor proliferation and metastasis, the clinical application of natural killer (NK) and natural killer-like T (NKT) cells in leukemia treatment is a topic of interest and is currently subject of intensive research.<sup>9</sup>

CD112 is one of 6 activating NK receptor ligands (NKRLs) (MICA, MICAB, CD155, CD112, ULBP1, and ULBP2/5/6). AML blasts displayed heterogeneous expression of NKRLs., CD112 was most frequently expressed<sup>10</sup>.

In this work, there was an evident decrease as regard the expression of NK cells in AML cases as compared to controls (P=0.018). In agreement with our research, When comparing AML and myelodysplastic syndrome (MDS) cases, Aggarwal et al. 11 found that the proportion of NKT cells was lower in AML cases.

Results presented in this research revealed that there was an evident increase in CD112 in AML cases as compared to controls with P value (<0.001). Mastaglio et al.,<sup>12</sup> was in agreement with this research who described a heterogeneous expression of both activating and inhibitory NKRLs on AML blasts at diagnosis. ULBP1, MICA, and CD112 were the most frequently expressed with high percentage expression of CD112 (80.3%) on AML blasts.

This work showed the comparison between NK cells and CD112 according to (Favorable FAB) & (Unfavorable FAB) AML cases and showed that there was a decrease in NK cells percentage in favorable than unfavorable. However, this decrease was not statistically evident. Also, the results showed that there was increase in CD112 percentage in favorable than unfavourable. However, this increase was not statistically evident. This may be due to low numbers of cases and other contributory factors.

In the present work, there was non-evident correlation between Hb, Plt , WBC ,LDH ,Blast (BM) and NK cell expression . On the other hand, there was non-evident correlation between ESR and NK cell expression.

Non-evident correlation was observed between Hb, Plt , WBC ,LDH ,Blast (BM) and CD112 expression . On the other hand, CD112 expression showed non-evident correlation with ESR.

Regarding correlation between the markers (NK cells and CD112) an evident inverse correlation between NK cells expression and CD112 expression with P value ( $<0.001$ ). These results agreed with Khaznadar et al.,<sup>13</sup> who reported that NK cells from AML cases had a decreased expression that inversely correlated with CD 112 expression on autologous AML blasts.

In the current research, using the ROC curve for NK cell  $\leq 15.5$  was set as cut off and AUC: 0.688 with sensitivity and specificity 70.0% and 65.0% respectively. Also using the ROC curve for CD112  $>18.2$  was set as cut off and AUC: 0.929 with sensitivity and specificity 87.5% and 75.0% respectively.

In their research, Ferrera and colleagues investigated Remission induction for AML using the (7+3) protocol, which consists of 7 days of IV AraC and a single dosage of Adriamycin on days 1, 3, and 14. BM samples were checked routinely for signs of disease after induction treatment. Day 28 was used as the primary endpoint for follow-up.

Our research showed that cases with complete remission were associated with evidently lower levels of NK cells expression. Moreover, relapsed and dead cases had evidently higher mean levels of NK cells expression.

Data from this research showed that cases with complete remission were associated with evidently higher levels of CD112 expression. Moreover, relapsed and dead cases had evidently lower mean levels of CD112 expression.

OS and DFS were calculated by tracking cases for a full two years after initiation of treatment and documenting when they went into remission, relapsed, died, or were last seen alive.

By analysis of survival and using Kaplan-meier curve, as regard NK cell it has been found that there was an evident correlation between low levels and high levels of expression of NK cells with P value ( $<0.001$ ) for both (OS , DFS). Kaplan-Meier survival curve shows an evident higher OS in cases with low NK cells expression with cutoff  $\leq 15.5$ . Aggarwal et al.,

<sup>11</sup> was in agreement with these findings who reported that NK cell number is evidently correlated with survival and prognosis of studied AML cases as we concluded that a lower percentage of NK (CD65dim) cells at time of diagnosis came with better survival.

Park et al., (2018) concluded that cases with AML who had a low NK cell proportion (9.4%) were more likely to experience adverse genetic abnormalities (P=0.0244) and relapse (P=0.0567), while those with a high NK cell proportion (>16.6%) had a higher risk of dying before remission (P=0.0438) and this was in line with our findings

By analysis of survival and using Kaplan- meier curve, as regard CD112 it has been found that there was an evident correlation between high levels and low levels of expression of CD112 with P value ( <0.001\*) for both (OS , DFS). Kaplan-Meier survival curve shows an evident higher OS in cases with high CD112 expression with cutoff >18.2. Mastaglio et al., <sup>12</sup> was in agreement with our research who reported that Expression of CD112 was substantially linked to better 2-year OS (51.4% vs 11.4%), DFS (42.5% vs 10.0%), and decreased relapse (44.1% vs 78.6%).

Consistent with the findings of this research, Boeck et al. (15) postulated that a more expression of pattern of NKRL expression (CD112) was linked to better OS and could serve as a novel prognostic tool for cases with recently identified AML.

### **Conclusions:**

There was an evident decrease in the level of NK cells and an evident increase in expression of CD112 in newly diagnosed AML cases. Also, there was an evident inverse correlation between NK cells expression and CD112 expression. Lower percentage of NK cells and higher levels of expression of CD112 may predict better outcome of newly diagnosed AML cases

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