AIM OF WORK

The aim of the work is to study the role of soluble HLA-G in acute myeloid leukemia patients.

SUBJECTS AND MATERIALS

MATERIALS

Patients were selected from Alexandria Armed Forces Hospital , Alexandria Main University Hospital and Maadi Armed Forces Medical Compound (Hematology department). The patients were selected during the period from May 2013- December 2013. This study included 45 subjects subdivided into the following groups:

1- 1st group consists of 15 healthy controls (group A).
2- 2nd group consists of 15 newly diagnosed acute myeloid leukemic patients (group B).
3- 3rd group consists of 15 relapsed and refractory acute myeloid leukemic patients (group C).

Exclusion criteria:

1- Age of patients less than16 years old and more than 70 years old.
2- Patients with hepatic or renal failure.
3- Concomitant chronic illness.
4- Pregnant females

All patients were subjected to:

A) History taking
B) Thorough clinical examination
C) Investigations:
    1- Complete blood count.
    2- Bone marrow examination.
    3- Immunophenotyping .
    4- Cytogenetics.
    5- SGOT, SGPT.
    6- Serum urea, serum creatinine.
    7- Detection of sHLA-G by ELISA.

Patients received conventional chemotherapy in form of 3+7 regimen (3 days doxorubicine 30 mg/m2 + 7 days cytarabine 100mg/m2) and first consolidation in form of cytarabine 1.5-3g/m^2 (HIDAC). Patients were reassessed via hematological, biochemical and bone marrow aspirates.

METHODS

Sample collection

Blood samples were obtained by venipuncture using sterile disposable plastic syringe. About 7 milliliters of venous blood were withdrawn aseptically after disinfection of the skin using 70% alcohol and betadine, and divided into the following:

-
- Three ml were delivered into a K_2 EDTA tube forTET-2 gene expression analysis using Real Time - PCR analysis.
- Five ml were delivered into a sodium heparin tube for cytogenetic analysis (karyotyping).

Other tests were done routinely on patients' admission into the ward during which study began and were all noted and included.

Informed written consent was obtained during the study as proposed by the medical ethics community in Alexandria Medical Faculty.

HLA –G by ELISA:

Sample collection

1. Serum – coagulation at room temperature 10-20 mins was done and then centrifugation 20-min at the speed of 2000-3000 r.m.p.
2. Plasma sue suited EDTA was used as an anticoagulant then mixed 10-20 mins, centrifuged 20 min at a speed of 2000-3000 r.m.p. The supernatant was removed.
3. Cell culture supernatant – detect secretory components was done and collected sue a sterile container, centrifugation 20- min at the speed of 2000-3000 r.m.p. The supernatant was removed and the composition of cells was detected where the cell suspension was then diluted with PBS (PH 7.2-7.4).When cell concentration reached 1 million/ ml, repeated freeze thaw cycles, cells were damaged and release of intracellular components which were centrifuged 20 min at the speed of 2000-3000 r.m.p. and the supernatant was removed.

Washing method

Manual washing method: the remaining liquid in the enzyme plates was shaken away and some bibulous papers were placed on the test – bed, and the plates are flapped upside down strongly. At least 0.35mlwas injected after dilution washing solution into the well, and marinated 1-2 minutes. This process was repeated.

Specimen requirements

1. Serum – coagulation at room temperature 10-20 mins was then centrifuged for 20 min at a speed of 2000-3000 r.p.m. and then removing the supernatant.
2. Plasma used suited EDTA as an anticoagulant was mixed for 10-20mins and then centrifuged for 20 min at the speed of 200-3000 r.p.m. and the supernatant as removed.

Assay procedure

1. Standard dilution

1200ng/l	Standard no.5	120u original standard + 120u standard diluents
600ng/l	Standard no.4	120u standard no. 5 + 120u standard diluents
300ng/l	Standard no.3	120u standard no. 4 + 120u standard diluents
150ng/l	Standard no.2	120u standard no. 3 + 120u standard diluents
75ng/l	Standard no.1	120u standard no. 2 + 120u standard diluents

2. The quantity of plates was duplicated for each standard and blank well depending of the quantities of the samples to be tested.
3. The samples were injected:
    - Blank wells no samples were added and HLA-G antibody labeled with biotin, streptavidin – HRP, only chromogen solution A and B as well as stop solution were allowed; other operations were the same.

- Standard wells: standard 50 ul were added as standard , streptavidin – HRP 50ul
- To be tested wells 40ul samples were added and the both HLA-G antibody 10ul and streptavidin –HRP 50ul were added. Then sealed the sealing memberance, and gently shaked and incubated 60 min at 37°C

4. Confection: 30 times diluted and 30 times washing concentration with distilled water as standby.
5. Washing: the memberance was removed carefully and drained the liquid and then shaked away the remaining water.
6. Chromogen solution A 50 ul was added then chromogen solution 50ul to each well. Gently mixed and incubated for 10 min at 37 °C away from light.
7. Stop: stop solution 50ul was added into each well to stop the reaction.
8. Final measurement: taken blank well as zero the optical density (OD) under 450nm wavelength was measured which was carried out within 10 min after adding the stop solution.
9. According to the standard's concentration and the corresponding OD values a standard curve linear regression equation was calculated and applied the OD values of the samples on the regression to calculate the sample's concentration.

Conventional cytogenetics (karyotyping):

Protocol:

Peripheral blood was collected under aseptic techniques and 5ml from each patient was collected into a sodium heparin tube and referred for cytogenetics.

- Preparation of cell culture:

1. From each tube, 0.3-0.5 ml of blood was added to 5 ml of growth medium (RPMI) added 10% fetal calf serum and Phytohemagglutinin PHA.

-The cells were grown for 60-74 hours at 38° C with gentle inversion twice a day.

- Stopping the cell division at metaphase:

1. Arresting division was achieved by adding 0.05 mL (50 microliters of prewarmed 37°C Colcemid to the culture. It was mixed gently and put back into the incubator.

2. It was incubated for 30 to 60 minutes.

3. The blood and Colcemid solution were put into a conical centrifuge tube and centrifuge 500 - 900 rpm for 6 min.

4. The supernate was removed with a pasteur pipette.

- Hypotonic treatment of the red and white blood cells:

1. 1 mL of warmed 37°C hypotonic solution (KCl solution, 0.075M) was added to the tube, mixed by flicking the tube then, added another 9 mL of hypotonic solution .

2. The hypotonic solution was not being in contact with the cells for more than a total of 27 min.

3. The mixed solution was placed into the 37°C incubator for 17 min.

4. The fixative solution was prepared as follows: three parts of chilled absolute methanol was added to one part galacial acetic acid .

5. After 9 minutes, it was centrifuged for 6 minutes at 500 to 900 rpm.

6. The supernate was removed .

- Fixing the cells:

1. 5 mL of fixative solution was added to the centrifuge tube.

2. This solution of cells and fixative was placed into a refrigerator for 30 minutes. Then, the tube was centrifuged for 6 minutes at 500 to 900 rpm.

3. The supernate was removed and added another 6 mL of cold fixative and was mixed.

4. The tube was centrifuge for 6 minutes at 500 - 900 rpm.

5. The above two steps were repeat and then the supernate was removed.

- Making the chromosome slides:

1. The slide was cold.

2. Five or six slides were laid next to each other on paper toweling with no separation between them.

3. The entire contents of the centrifuge tube were withdrawn into a pasteur pipette.

4. From a height of about 18 inches, two or three drops of fluid onto each side were dropped.

5. The slides were allowed to dry thoroughly by placing them in the incubator (37°C) overnight.

6. The slide was stained by immersion in fresh Giemsa stain for 7 - 10 min.

7. The slides were removed from the stain and rinse in distilled water until all the excess stain was removed.[91]

Follow up and treatment outcome:

Response to induction therapy was assessed after 2 courses of chemotherapy. In accordance with standard criteria, complete remission (CR) was defined as less than 5% bone marrow blasts, an absolute neutrophil count of 1.0×10^9/L or more, a platelet count of 100×10^9/L or more, no blasts in the peripheral blood and no extramedullary leukemia. Therapeutic failures were classified as either refractory disease (RD) or early death, which was death before treatment. Relapse was defined as more than 5% BM blasts unrelated to recovery from the preceding course of chemotherapy or new extramedullary leukemia in patients with previously documented CR. [15]

STATISTICAL ANALYSIS

- IBM SPSS 20 was used to analyze data. Data were tested for normality using Kolmogorov- Smirnov test, Shapiro- Wilk test. Measurement data was presented by minimum, maximum, mean ± SD. T-test and F test in parametric data. Nonparametric data was presented by median value and range and nonparametric test (Mann-Whitney U and Kruskal Wallis) was used for comparing median value. Qualitative data was presented by percentages and tested by Pearson's Chi Square and Fisher Exact Test according to the categories and cells estimation %. Spearman bivariate correlation analysis was used for analyzing correlation. $P<0.05$ indicated statistical significance.

RESULTS

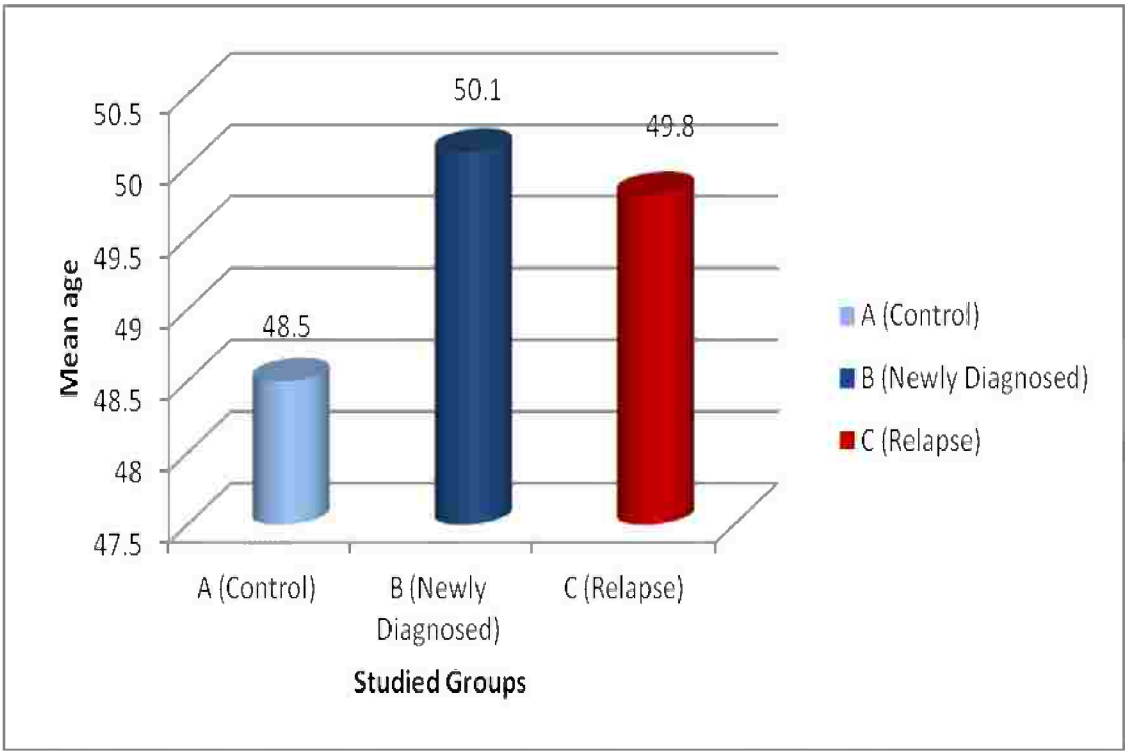
This study was carried out on thirty acute myeloid leukemia patients and fifteen healthy subjects as a control group. All patients and controls were matched in the demographic criteria in the form of age and sex. Controls enrolled had within normal range results for hematological and biochemical lab tests (CBC, liver and renal profiles).

Age and sex

Table (8) and figure (16) show comparison between the three studied groups regarding age and sex. Age in group A ranged from 19 to 65 years with a mean value of 48.5 ± 16.98 years, in group B, it ranged from 20 to 66 years with mean value of 50.1 ± 17.5 years and in group C it ranged from 19 to 67 years with mean value of 49.8 ± 19.5 years. Males in group A were 7(46.67%), in group B 6 (40%) and in group C 8(53.33%). Females in group A were 8(53.33%), in group B 9(60%) and in group C 7(46.67%). There was no statistical significant difference between the three studied groups regarding age (p=0.968) and sex (p=0.765).

Table 8: Comparison between the three studied groups regarding age and sex

	Group A Controls		**Group B New diagnosed AML**		**Group C Relapsed AML**		**Test**
Age (years)							
Range	19-65		20-66		19-67		F=0.03
Mean	48.5		50.1		49.8		
S.D.	16.98		17.5		19.5		P=0.968
Sex							
Male	7	46.67	6	40.00	8	53.33	X^2 =0.53
Female	8	53.33	9	60.00	7	46.67	P=0.765



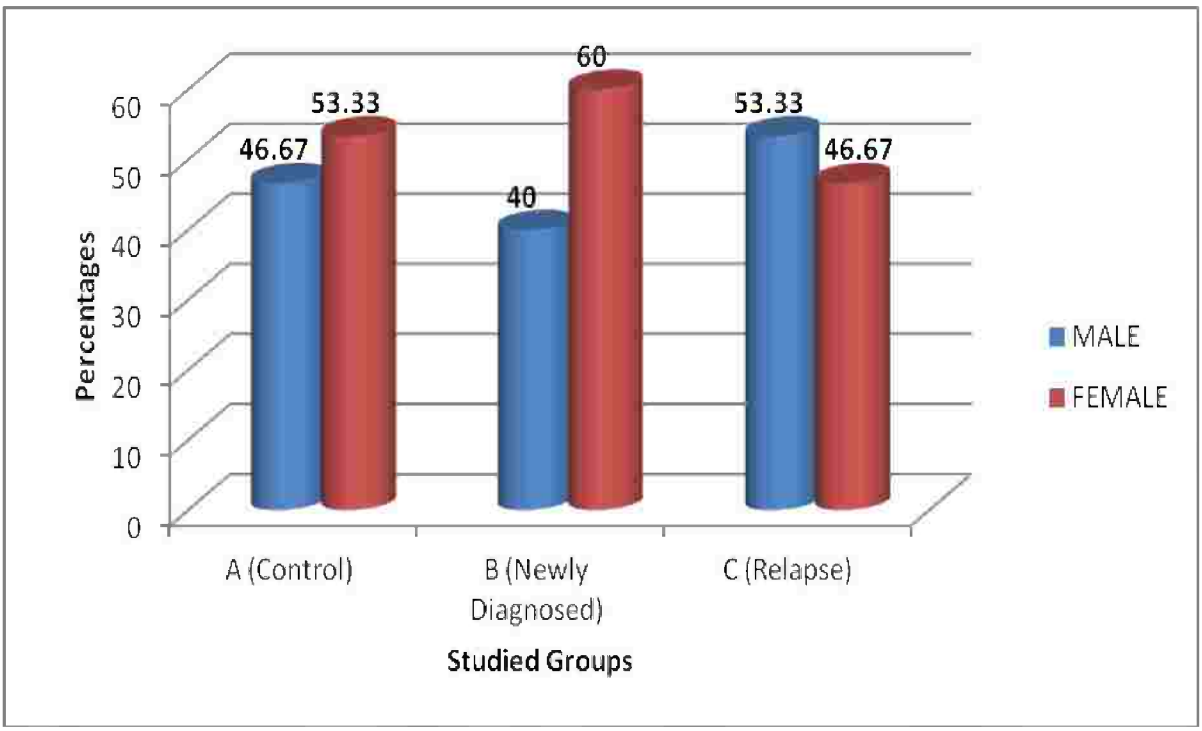


Figure 16: Comparison between the three studied groups regarding age and gender

Table (9) and figure (17) show Comparison between the two studied AML patients groups regarding FAB classification. M4 was the commonest subtype in both groups (6 cases, 40%). There was no statistical significant difference between two studied patients groups regarding FAB subtypes (p =0.365).

Table 9: Comparison between different studied patients groups regarding FAB classification

Parameter	Group B Newly diagnosed AML		Group C Relapsed AML	
	No.	%	No.	%
M2	2	13.3	2	13.3
M3	3	20.0	2	13.3
M4	6	40.0	6	40.0
M5	2	13.3	2	13.3
M6	2	13.3	3	20.0
Test of significance	X^2= 0.98 P=0.365			

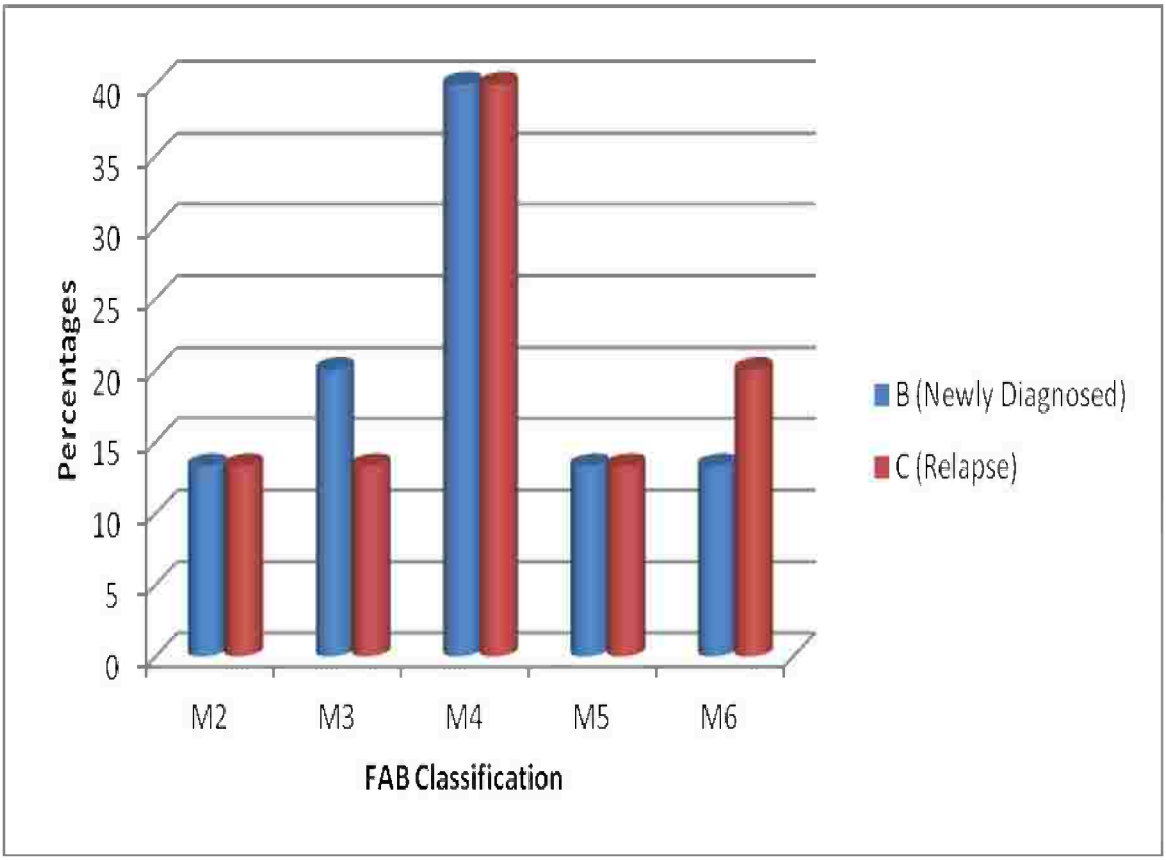


Figure 17: Comparison between different studied patients groups regarding FAB classification

Complete blood count

Table (10) shows comparison between the three studied groups regarding blood picture. WBCs in group A ranged from 4 to 11 $x10^9$/l with a mean value of 9.8 ± 2.58 $x10^9$/l, in group B it ranged from 2.3 to 130 $x10^9$/l with a mean value of 41.77 ± 43.30 $x10^9$/l and in group C it ranged from 1.2 to 185 $x10^9$/l with a mean value of 34.12 ± 57.51 $x10^9$/l. Hb in group A ranged 12-15 g/dl with a mean value of 12.3±1.57, in group B, it ranged from 4.0 to 12.6 g/dl with a mean value of 6.97 ± 2.06 g/dl and in group C it ranged from 9.2 to 11.3 g/dl with a mean value of 10.07 ± 2.10 g/dl. Platelets in group A ranged from 150-342 $x10^9$/l with a mean value of 184 ± 11.5 $x10^9$/l, in group B it ranged from 20 to 55 $x10^9$/l with a mean value of 30.8 ± 61.69 $x10^9$/l and in group C it ranged from 12 - 249 $x10^9$/l with a mean value of 67.53

± 61.38x10^9/l. There was statistically significant difference between the three studied groups regarding hemoglobin (p<0.001) and platelets (p= 0.001).

Table 10: Comparison between the three studied groups regarding blood picture

Parameter	Group A Controls	Group B Newly diagnosed AML	Group C Relapsed AML
WBCs (x10^9/l)			
Range	4-11	1.2 – 130	1.2-185
Mean	9.8	41.77	34.12
S.D.	2.58	43.30	57.51
Median	11.0	5.7	8.0
H=2.9; P=0.233			
Hb (g/dl)			
Range	12-15	4.0 - 12.6	9.2–11.3
Mean	12.3^{bc}	6.91^{ac}	10.07^{ab}
S.D.	1.57	2.06	2.10
F=32.066, P=0.000*			
Platelets (x10^9/l)			
Range	150 – 342	20 – 55	12 – 249
Mean	184	39.8	67.53
S.D.	76.87	11.02	61.38
Median	148.0^{bc}	44.0	48.0
H=25.6; P=0.001*			

H: Kruskal-Wallis test

* P < 0.05 (significant)

Liver function tests

Table (11) shows comparison between the three studied groups regarding liver function. AST in group A ranged from 15 to 38 R & F unit with a mean value of 28 ± 6.46, in group B it ranged from 29 to 40 R & F unit with a mean value of 35.20 ± 4.02 and in group C it ranged from 25 to 46 with a mean value of 33.70 ± 7.35. ALT in group A ranged from 11 to -32 R & F unit with a mean value of 20 ± 7.42, in group B it ranged from 13 to 40 R & F unit with a mean value of 21.60 ± 7.52 and in group C it ranged from 19 to 39 R & F unit with a mean value of 28.70 ± 7.35. There was statistically significant difference between the three studied groups regarding liver function tests (p = 0.008 and 0.012 for AST and ALT respectively).

Table 11: Comparison between the three studied groups regarding liver function

Parameter	Group A Controls	Group B New diagnosed AML	Group C Relapsed AML	Test of significance
AST (R & F)				
Range	15-38	29-40	25-46	F=5.5
Mean	28.00 [bc]	35.20	33.70	P=0.008*
S.D.	6.46	4.02	7.35	
ALT (R & F)				
Range	11-32	13-40	19-39	F=4.9
Mean	20.00	21.60	28.70 [ab]	P=0.012*
S.D.	7.42	7.52	7.35	

F: One Way ANOVA * P < 0.05 (significant)

Renal function tests

Table (12) shows comparison between the three studied groups regarding kidney function tests. Urea in group A ranged from 3 to 11 with a mean value of 7.34 ± 2.38 mg/dl, in group B it ranged from 5.6 to 13 mg/dl with a mean value of 9.92 ± 2.03 and in group C it ranged from 12.1 to 18.1 mg/dl with a mean value of 15.52 ± 1.67. Creatinine in group A ranged from 0.4 to 0.8 mg/dl with a mean value of 0.61 ± 0.14, in group B it ranged from 0.3 to 1.2 mg/dl with a mean value of 0.54 ± 0.27 and in group C it ranged from 0.35-0.9 mg/dl with a mean value of 0.62 ± 0.17. There was statistically significant difference between the three studied groups regarding blood urea only (p =0.001 and 0.639 for blood urea and serum creatinine respectively).

Table 12: Comparison between the three studied groups regarding kidney function tests

Parameter	Group A Controls	Group B Newly diagnosed AML	Group C Relapsed AML	p
Blood urea (mg/dl)				
Range	3-11	5.6-13	12.1-18.1	F=49.1 P=0.001*
Mean	7.34[bc]	9.92 [ac]	15.52 [ab]	
S.D.	2.38	2.03	1.67	
Serum creatinine (mg/dl)				
Range	0.4-0.8	0.3-1.2	0.35-0.9	F= 0.45 P=0.639
Mean	0.61	0.54	0.62	
S.D.	0.14	0.27	0.17	

F: One Way ANOVA * P < 0.05 (significant)

Bone marrow blasts percent

Table (13) show comparison between different studied groups regarding bone marrow (BM) blasts. BM blasts in group B ranged from 22 to 90% with a mean value of 51.40±22 and in group C it ranged from 10 to 82 % with a mean value of 30.06±24.69. There was no statistically significant difference between the two AML studied groups regarding BM blasts (p =0.571).

Table 13: Comparison between AML studied groups regarding bone marrow blasts

Parameter	Group B Newly diagnosed AML	Group C Relapsed AML
BM blasts (%)		
Range	22 - 90	10 - 82
Mean	51.40	30.06
S.D.	22.00	24.69
Median	34.0	36.0
Test of significance	Z=1.54 ; P=0.571	

Z: Mann-Whitney test

sHLA-G

Table (14) and figure 18 show comparison between the three studied groups regarding sHLA-G. sHLA-G in group A ranged from 224.3 to 413.6 ng/L with a mean value of 329.8 ± 57.54 ng/L and in group B, it ranged from 275.9 to 873.7 ng/L with a mean value of 451.15 ± 163.99ng/L and in group C, it ranged from 372.29 to 783.05 ng/L with a mean value of 551.63 ± 109.08 ng/L. There was statistically significant difference between new and relapsed AML cases when compared with

controls (p=0.001). There was statistically significant increase in sHLA-G in new cases compared to relapsed AML cases.

Table 14: Comparison between the three studied groups regarding sHLA-G

Parameter	Group A Controls	Group B Newly diagnosed AML	Group C Relapsed AML
sHLA-G (ng/L)			
Range	224.3 - 413.6	275.9 - 873.7	372.29 - 783.05
Mean	329.8[bc]	451.15 [ac]	551.63 [ab]
S.D.	57.54	163.99	109.18
F= 12.21 P=0.001*			

F: One Way ANOVA * P < 0.05 (significant)

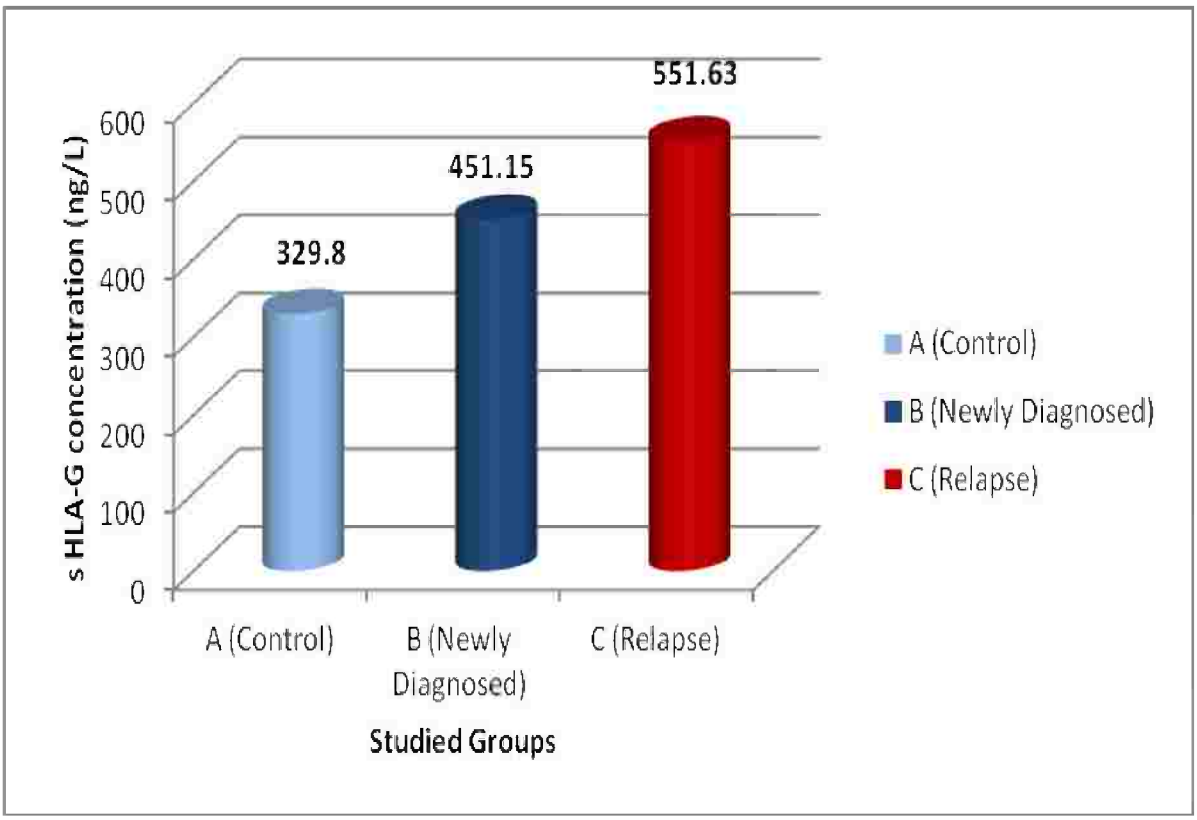


Figure 18:Comparison between the three studied groups regarding sHLA-G

Cytogenetic study

Table (15) and figure 19 shows comparison between the two studied patients groups regarding cytogenetic findings.

Table 15: Comparison between the two studied patients groups regarding cytogenetic study

Karyotyping *	Group B Newly diagnosed AML		Group C Relapsed AML	
	No.	%	No.	%
Normal	6	60	4	40
Deletion 20q	1	50	1	50
Trisomy 8	1	50	1	50
MCP	0.854			
* 16 patients' samples failed during culture				

MCP: P value based on Mont Carlo exact probability

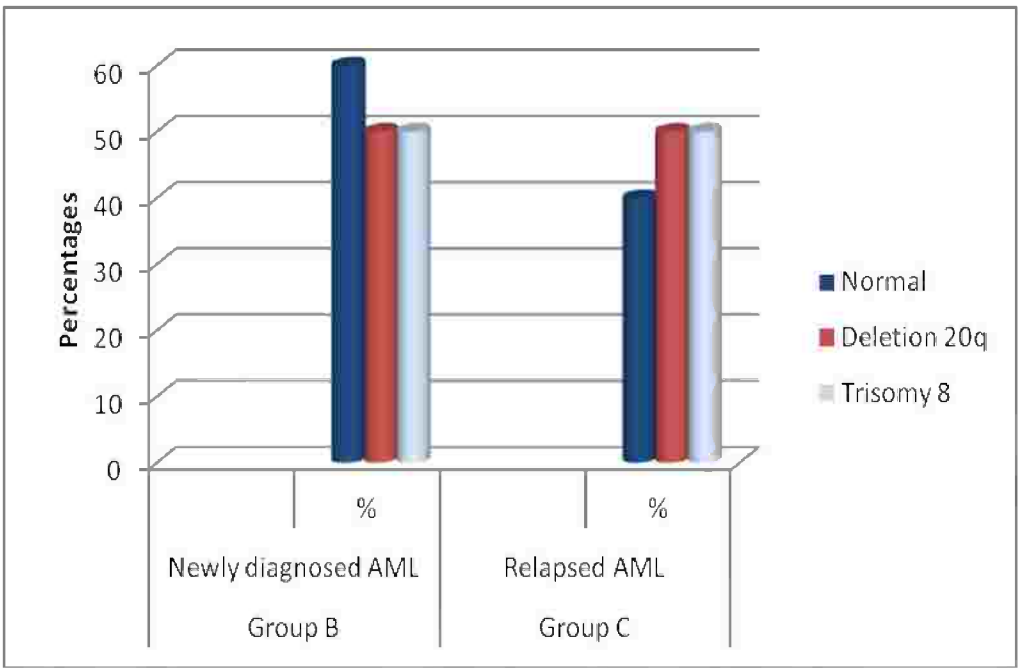


Figure 19: Comparison between the two studied patients groups regarding cytogenetic findings

As regarding molecular findings, 2 patients had FLT3-ITD positive; one was a newly diagnosed AML (female, 49 years old HLA-G 335.57) and the other relapsed case (male, 55 years old, HLA-G 637.34). 2 patients had P53; one patient was newly diagnosed (female, 50 years of age HLA-G 458.34) and the other relapsed (female, 66 years, HLA-G 528.33)..

ROC curve to determine the sensitivity and specificity of sHLA-G in detection of the disease.

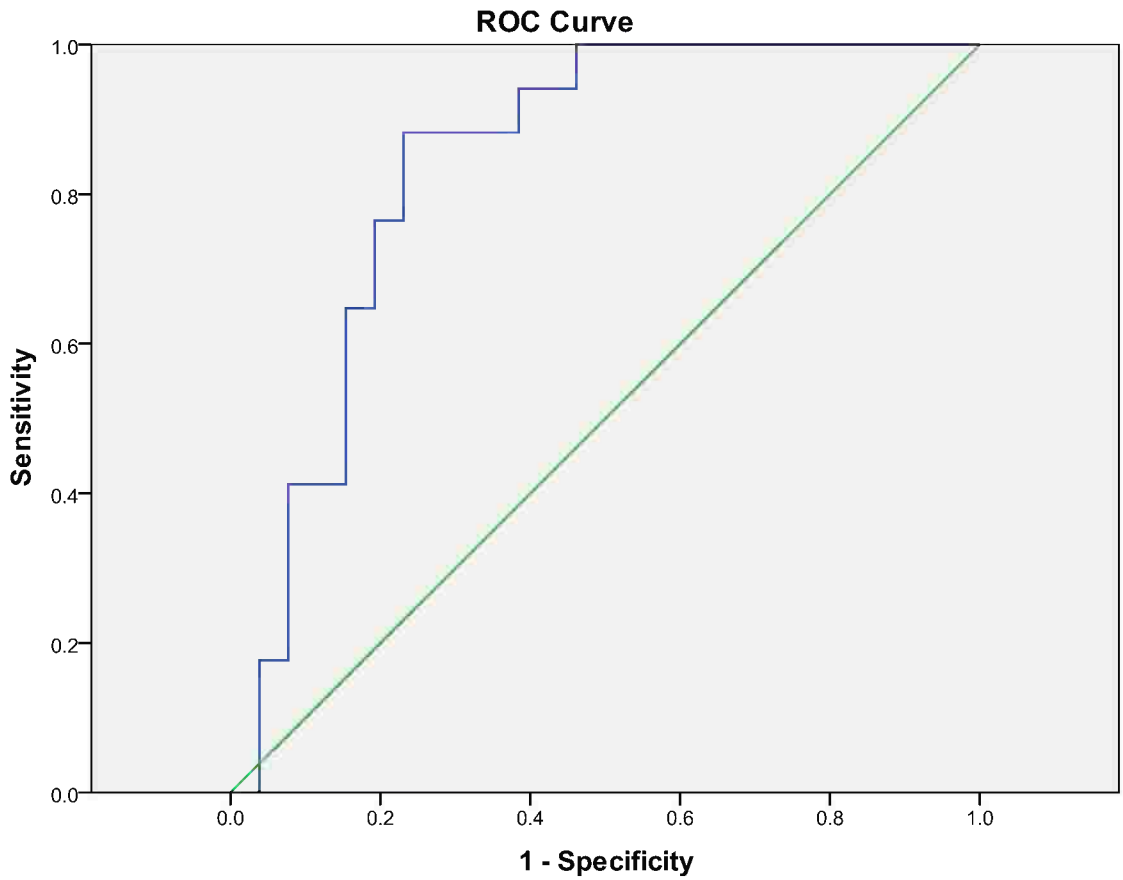


Figure 20: ROC curve to determine the sensitivity and specificity of sHLA-G in detection the disease

Table 16: Area Under the Curve

Test Result Variable(s):sHLA-G

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.839	.061	.000	.719	.960

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Table 17: Coordinates of the Curve

Test Result Variable(s):sHLA-G

Positive if Greater Than or Equal To[a]	Sensitivity	Specificity
368.845	1.000	.62

a. The smallest cutoff value is the minimum observed test value minus 1, and the largest cutoff value is the maximum observed test value plus 1. All the other cutoff values are the averages of two consecutive ordered observed test values.

The ROC curve show the sensitivity and specificity of sHLA-G in detection the incidence of AML cases. The area under the curve if wide shows that there is a significant sensitivity for the HLA-G to detect AML cases. Thus, the area under the curve was 0.839, if this area was more than 0.50; the HLA-G was significantly to detect AML. The cutoff value of sHLA-G was 368.84, this value show sensitivity 100.0% and specificity 62.0%. In this study this cutoff value was considered the level at which HLA-G was considered within normal. Using this cutoff point it was noted

that 24/30 (80%) of patients had levels higher than the cutoff point among all AML patients. The newly diagnosed AML were 10/24 whereas the relapsed AML were 14/24.

sHLA-G and gender:

Table (18) shows relation between sHLA-G and gender. There was no statistical significant difference between sHLA-G and gender in the same group ($P > 0.05$). Comparing males and females in the three groups together revealed statistically significant increase in sHLA-G in both males and females of newly diagnosed and relapsed group when compared to controls.

Table 18: Relation between sHLA-G and gender

s-HLA-G	Controls		Newly diagnosed AML		Relapsed AML	
	Male **n=7**	Female **n=8**	Male **n=6**	Female **n=9**	Male **n=8**	Female **n=7**
Range	258.7-413.6	224.3-374.6	275.91-639.0	275.91-873.7	387.21-783.05	372.29-675.2
Mean	330.962 [bc]	328.428 [bc]	445.633	455.906	568.650	540.386
S.D.	48.258	61.332	140.919	204.586	135.372	105.278
t (P)	0.09 (0.930)		0.11 (0.913)		0.46 (0.655)	
F (P); males	7.7 (0.004)*					
F (P);females	4.6 (0.022)*					

t: independent samples t-test

F: One Way ANOVA * $P < 0.05$ (significant)

Correlation between sHLA-G, age, WBCs and bone marrow blast percentage:

Table (20) shows correlation between sHLA-G and age, WBCs and bone marrow blast percentage. There was no statistically significant difference between sHLA-G with neither age nor WBCs (P = 0.365 and 0.126 respectively) while there was statistically significant difference between sHLA-G and bone marrow blasts percentage (P= 0.021).

Table 19: Correlation between sHLA-G and age and blast percentage.

sHLA-G	r	P
Age	0.125	0.365
WBCs	0.232	0.126
Bone marrow blasts percentage	0.521	0.021*

P is considered significant if <0.05

sHLA-G and cytogenetics

Table (19) and figure 21 show comparison between normal and abnormal cytogenetics as regards level of sHLA-G. Two patients had trisomy 8 (1 newly diagnosed and 1 relapsed) and 2 had deletion 20q (1 newly diagnosed and 1 relapsed).

Table 20: Comparison between sHLA-G as regards cytogenetics

sHLA-G	Normal N=10	20q del N=2	Trisomy 8 N=2
Range	224.3-458.34	275.91-277	356-789.5
Mean	365.5	276.456	561.6
S.D.	160.3	0.769	115.6

HLA-G and FAB classification

Table (21) and figure 22 show comparison between sHLA-G as regards FAB classification. There was no statistically significant difference between sHLA-G and different FAB subtypes (P =0.685) with the highest mean values was observed in M6 (605.3±116.5) while the lowest mean value was observed in M2 (365.2±98.9).

Table 21: Comparison between sHLA-G and FAB subtypes

sHLA-G (ng/l)	**Range**	**Mean ±S.D.**
M2 (n=4)	224.3-685.0	365.2±98.9
M3 (n=5)	281.3-564.6	402.6±125.0
M4 (n=12)	352.6-780.0	526.1±105.1
M5 (n=4)	412-873.7	553.6±124.3
M6 (n=5)	522-870.0	605.3±116.5
F=2.96 P=0.685		

P is considered significant if <0.05

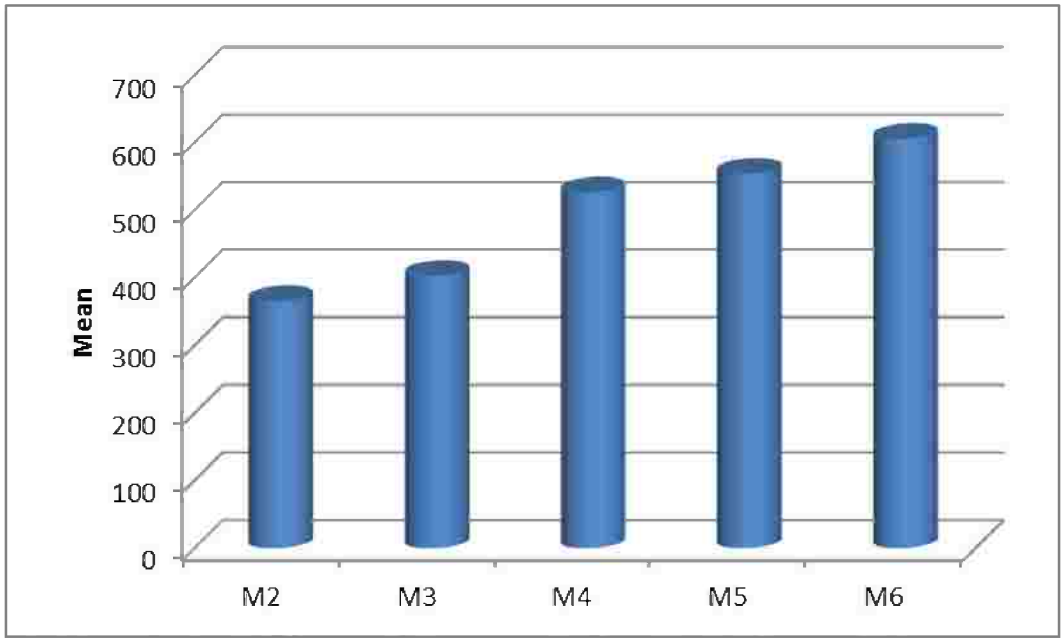


Figure 21: Comparison between sHLA-G and FAB subtypes

Table (22) shows comparison between sHLA-G and treatment response. There was no statistically significant difference between sHLA-G and response to therapy (P =0.158).The lowest mean value of sHLA-G was observed in patients in complete remission (359.67±58.29) when compared with relapsed patients (404.565±160.48) or those refractory to chemotherapy (302.8195±61.862).

Table 22: Comparison between sHLA-G and response to therapy

	sHLA-G				
	No	Mean	SD	Range	Median
Refractory	5	402.3	95.6	258-639.63	389.2
Complete remission	7	359.67	58.29	258.7-450.31	351.650
Relapse	18	404.565	160.48	224.3-873.7	391.796.5
H=14.1;P=0.158					

P value significant if <0.05