AIM OF THE WORK

The aim of the present work was to assess the immunogenicity of Hepatitis B vaccine in non immune children who have been treated with chemotherapy for acute lymphoblasic leukemia at Hematology Oncology Unit of Alexandria University Children's hospital after three doses of the vaccine.

SUBJECTS

This study was carried out on twenty five children with acute lymphoblastic leukemia (ALL) attending the hematology oncology clinic at Alexandria University Children's Hospital (AUCH), after completion of chemotherapy. These children were consecutively recruited between November 2011 and May 2012. Fifty healthy children were also included as controls. All children included in the study have been previously vaccinated with 3 doses of hepatitis B vaccine according to the schedule adopted by the Egyptian Ministry of Health (at 2, 4 and 6 months of age). Children with ALL who were found to be non immune to HB vaccine (assessed in a previous study) and were vaccinated with 3 doses of HB vaccine Engerix B (1/2 ml IM at 0, 1,2 months) and response to revaccination was assessed 1-6 months after the third dose.

Children were divided as follows:

1. Group I: 25 children with ALL who fulfilled inclusion and exclusion criteria of the study. There were 14 males and 11 females, their age ranged from 6.17 to 17.08 years with a mean of 12.99± 2.91 years. Group I was further subdivided according to immune status against HB vaccine into:
   - Group Ia: non-immune (anti-HBs titer <10mIU/mL).
   - Group Ib: immune (anti-HBs titer ≥10mIU/mL).
2. Group II: 50 normal children as control group. There were 22 males and 28 females, their age ranged from 7 to 17 years with a mean of 12±2.74 years. Group II was further subdivided according to immune status against HB vaccine into:
   - Group IIa: non-immune (anti-HBs titer <10mIU/mL).
   - Group IIb: immune (anti-HBs titer ≥10mIU/mL).

Informed consent was taken from parents of all children included in the study.

Inclusion Criteria

1. Age less than 18 years.
2. Documented complete active primary series of hepatitis B virus vaccination (revision of birth certificate or by parents' questionnaire), according to the recommended program applied by Ministry of Health (at 2, 4 and 6 months of age).
3. Ended chemotherapy.

Exclusion Criteria

1. Age less than one year at diagnosis of ALL.
2. Clinical or laboratory evidence of relapse.
3. Ongoing treatment with any immunosuppressive drugs such as interferon & corticosteroids or presence of any other condition affecting individual's immune response such as infections, autoimmune diseases, and chronic debilitating diseases.
4. History of HBV infection, symptoms or signs of liver disease by clinical examination.
5. Chronic liver or renal disease

METHODS

➢ Study design

The study was designed as a case control study.

➢ All included children were subjected to the following

A. History taking

Full history was taken through questioning children and their parents including:

1. Personal data: full name, sex, date of birth, and address.
2. Vaccination history: date of completion of HB vaccination and number of doses received.
3. History of increased risk of exposure to HBV infection as presence of chronically infected family member, surgical intervention, or number of blood products units (PRBCs, plasma and/or platelets) transfusion.
4. Data related to leukemia diagnosis: date of admission at hospital, age of the child at diagnosis of leukemia and at end of maintenance chemotherapy, type of leukemia, risk stratification, and protocol used for treatment. (from patients' record)
5. Data related to leukemia diagnosis: date of admission at hospital, age of the child at diagnosis of leukemia and at end of maintenance chemotherapy, type of leukemia, risk stratification, and protocol used for treatment. (from patients' record)
6. Revacination of all non immune leukemic cases with HB vaccine Energix B 1/2ml IM

B. Clinical examination

Systemic examination was done with special emphasis on signs of relapse (hepatosplenomegaly, lymphadenopathy, pallor, and/or purpura); and signs of liver disease (jaundice, dark urine and/or hepatomegaly).

C. Laboratory investigation

Done for leukemic children:

Anti-HBs Ab titer (by ELISA) 1-6 months after revaccination[150].

This laboratory investigation was done to the control group as well.

The individual leukemic patient results compared to their anti-HBs Ab titer before revaccination. The immune status of leukemic children was assessed in a previous study in our center conducted on 76 children who have completed chemotherapy for ALL and have been previously vaccinated with 3 doses of hepatitis B vaccine according to the schedule adopted by the Egyptian Ministry of Health (at 2, 4 and 6 months of age).

Thirty nine (51.3%) were found to be non-immune to HB vaccine (anti-HBs <10mIU/mL), and only 37 (48.7%) children had protective anti-HBs titer (anti-HBs ≥10mIU/mL). Their mean anti-HBs titer was 42.79 mIU/mL)[150].Anti-HBs titer was repeated to 25 children who were revaccinated with 3 doses of HB vaccine Energix B.

➢ Specimen collection and preparation

Aseptic venipuncture was done by our trained clinic's nurses. One sample of whole blood was withdrawn and collected in one tube. This specimen for assessment of antibodies to Surface Antigen of Hepatitis B Virus was centrifuged immediately after collection to obtain serum. The serum of each patient was put in an eppendorf tube, and kept frozen at -20 C. All samples were processed altogether after completion of collection.

➢ Laboratory assessment

I. **Quantitative anti-HBs titer** was processed manually then read by an ELISA reader. ELISA is a solid phase enzyme linked immunoabsorbant assay based on the principle of the double antigen sandwich technique for the detection of antibodies to HBsAg in human serum using commercial test kits supplied by (Dia.pro®, diagnistic bioprobes Srl.).

For quantitative anti-HBs test: During the assay, the test specimen and HRP-HBsAg conjugates are incubated simultaneously in the coated microwells forming sandwich complex conjugates. Unbounded conjugates composed are then removed by washing. The presence of the complex conjugates is shown by a blue color upon additional incubation with tetramethylbenzidine (TMB) substrate. The reaction is then stopped and absorbances are read using a spectrophotometer at 450 nm. The amount of color intensity can be measured and is proportional to the amount of antibody captured in the wells, and to the sample respectively.

Interpretation of the results: Samples with concentrations <10.0WHO mIU/ml are considered negative for anti-HBs. Quantitative determination of antibodies to anti-HBs titer is determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode. From the calibration curve the concentration of anti-HBs in each sample was expressed as mIU/mL.

➢ Statistical analysis

Data were fed to the computer using the *SPSS software package version 20.0 (*SPSS,Chicago, USA).

Qualitative data were described using number and percent. *Quantitative data* were described using median, minimum and maximum as well as mean and standard deviation.

Association between categorical variables was tested using *Chi-square test.* When more than 20% of the cells have expected count less than 5, correction for chi-square was conducted using Fisher's Exact test or Monte Carlo correction. Not normally distributed quantitative data were analyzed using Mann Whitney test for comparing two groups. Correlations between two quantitative variables were assessed using *Spearman's rho test.*

Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

RESULTS

A. Demographic data

Table (11): Comparison between the studied groups according to sex and age :

	Group I "Leukemic cases after revaccination" (n=25)		Group II "Healthy control" (n=50)		Test of Sig.	p
	No.	%	No.	%		
Sex						
Male	14	56.0	22	44.0	χ^2=0.962	0.327
Female	11	44.0	28	56.0		
Age(years)						
Range	6.17 – 17.08		7.0 – 17.0		t= 0.600	0.551
Mean ± SD.	12.99 ± 2.91		12.58 ± 2.74			
Median	13.92		12.50			

χ^2: Chi square test
*: Statistically significant at $p \leq 0.05$

In Group I (the 25 leukemic children), age ranged from 6.17 to 17.08 years with a mean of 12.99±2.91 years. There were 14 (56.0%) males and only 11 (44.0%) females in this group. In Group II (50 normal controls), age ranged from 7 to 17 years with a mean of 12.58±2.74 years. There were 22 (44%) males and 28 (56%) females. There were no statistical significant difference between both groups as regards sex and age (p=0.327,p=0.551) respectively. Table (11) fig (3 and 4).

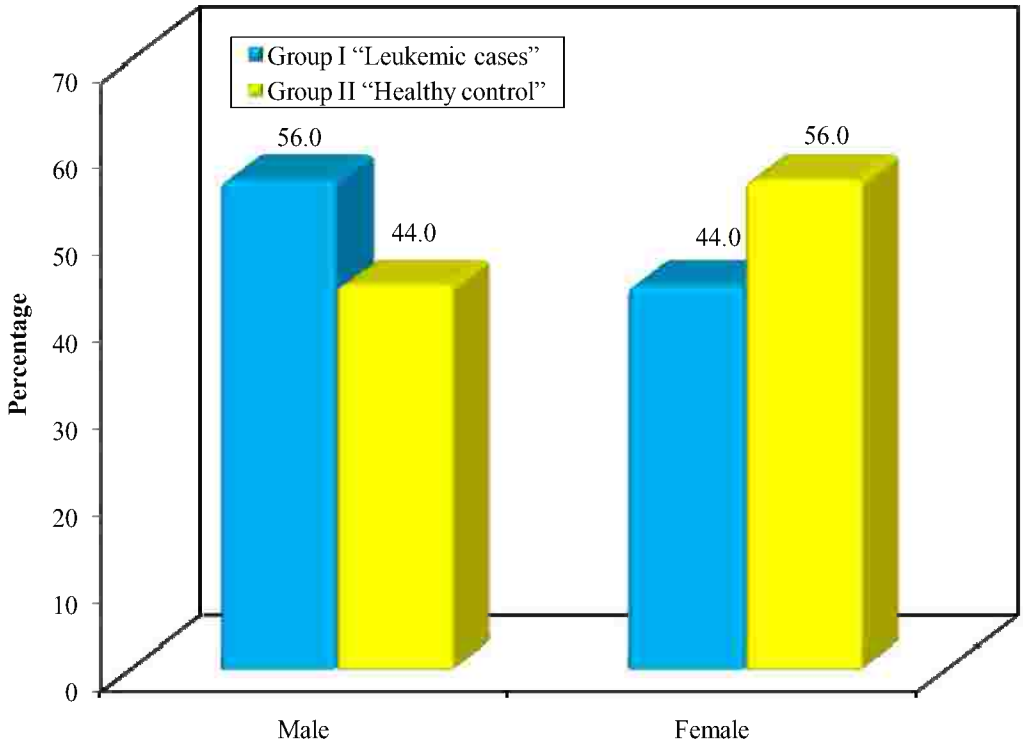

Figure (3): Comparison between the studied groups according to sex.

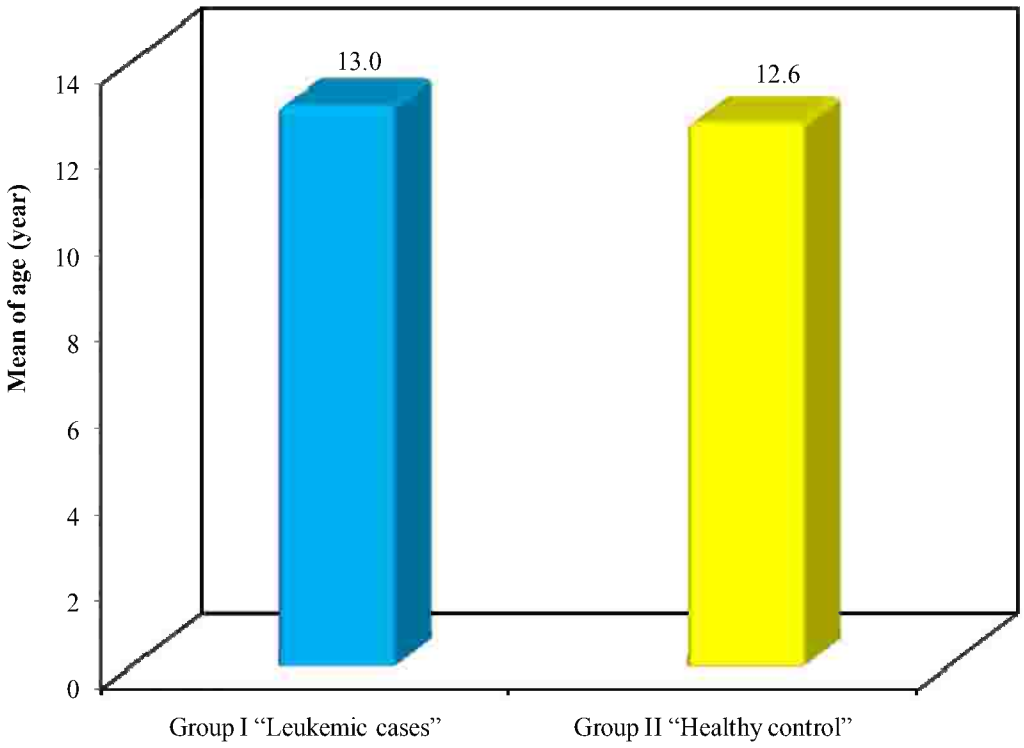

Figure (4): Comparison between the studied groups according to age.

Table (12): Comparison between group I and group II according to immune status to HB vaccine:

Immune status to HB vaccine	Group I "Leukemic cases after revaccination" (n=25)		Group II "Healthy control" (n=50)		χ^2	p
	No.	%	No.	%		
Non-immune <10 mIU/ml	4	16.0	13	26.0	0.951	0.330
Immune ≥10 mIU/ml	21	84.0	37	74.0		

χ^2: Chi square test
*: Statistically significant at $p \leq 0.05$

Among children of Group I(non immune children treated for leukemia after HB re-vaccination),4 (16%) patients were persistently non immune to HBV. The remaining 21 (84%) attained protective anti-HBs levels. In children of Group II (normal controls have been previously vaccinated with 3 doses of hepatitis B vaccine according to the schedule adopted by the Egyptian Ministry of Health at 2, 4 and 6 months of age) and did not receive any additional doses of HB vaccine, 37 (74%) children had anti-HBs titer ≥10mIU/mL and only 13 (26%) children were non-immune. The difference in the immune status between the two groups was not statistically significant, (p=0.330) as shown in table (12).Fig(5)

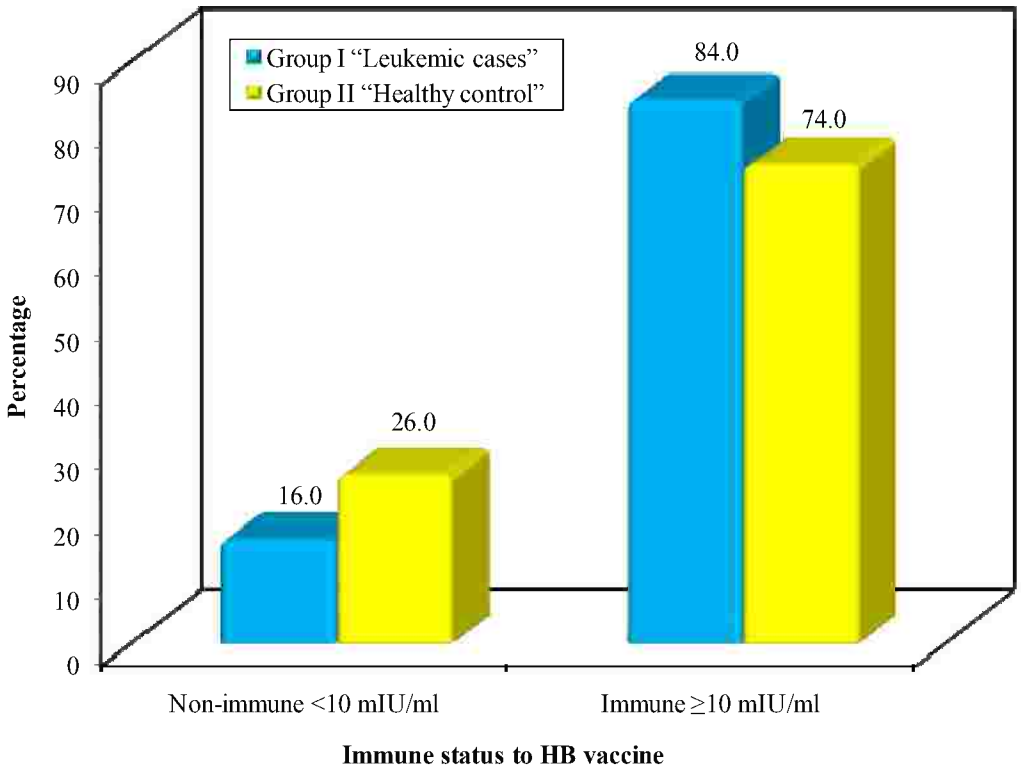

Figure (5): Comparison between group I and group II according to immune status to HB vaccine.

Table (13): Comparison between group I and group II according to mean anti-HBs titer:

	Group I "Leukemic cases after revaccination" (n=25)	Group II "Healthy control" (n=50)	Z	p
Anti HBs titer(mIU/ml)				
Range.	0.60 – 264.80	0.0 – 265.80		
Mean ± SD.	145.39 ± 102.15	85.74 ± 96.50	2.276*	0.023*
Median	166.10	42.85		

Z: Z for Mann Whitney test
*: Statistically significant at $p \leq 0.05$

In Group I, mean anti-HBs titer was significantly higher than mean anti-HBs in Group II (145.39 vs 85.74 mIU/mL), (*p*=.023). Table (13) fig(6).

So re-vaccination in non immune children treated for leukemia improve theire immune status to reach that of their normal counterparts even with statistically significant higher mean anti-HBs titer than normal controls who did not receive additional HB vaccine doses.

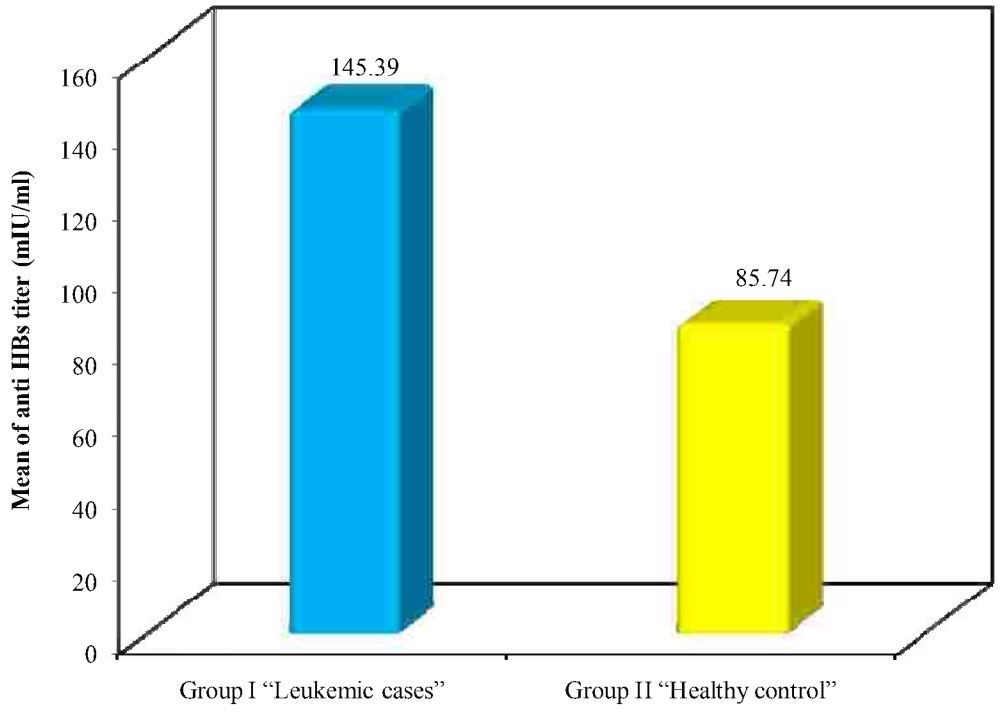

Figure (6): Comparison between group I and group II according to mean anti HBs titer.

B. Analysis of factors affecting the immune status of Group I subjects (leukemic children):

Group I was composed of 25 children who completed chemotherapy for ALL that were found to be non immune to HB vaccine (assessed in a previous study) and received 3 doses of HB vaccine Engerix B at 1/2 ml IM at 0, 1 ,2 months and response to re-vaccination was assessed 1-6 months after the third dose.

Group I was further subdivided into two subgroups according to immunity to HB vaccine. Non-immune children were 4 (16%) constituting Group Ia, and the 21 (84%) immune children constituted Group Ib. In Group Ia, mean age was 14.04±2.39 years, while it was 12.79±3.0 years in Group Ib. There was no significant difference between both groups regarding age or sex Table (14) fig (7and8)

Table (14): Comparison between Group Ia and Group Ib according to age and sex:

		Group Ia Non-immune n=4	**Group Ib** Immune n=21	Test of significance
Age (years)	Range	11.17-17.0	6.9-14.6	t=0.783 (p=0.442)
	Mean±SD	14.04±2.39	12.79±3.0	
	Median	14	13.92	
	Mean±SD	13.54±2.38	12.29±3.0	
	Median	13.5	13.42	
Sex	Males	3(75.0%)	11 (52.4%)	χ^2=0.698 (p=0.604)
	Females	1(25.0%)	10 (47.6%)	

χ^2: value for Chi square
t: Student t-test
*Significant $p \leq .05$

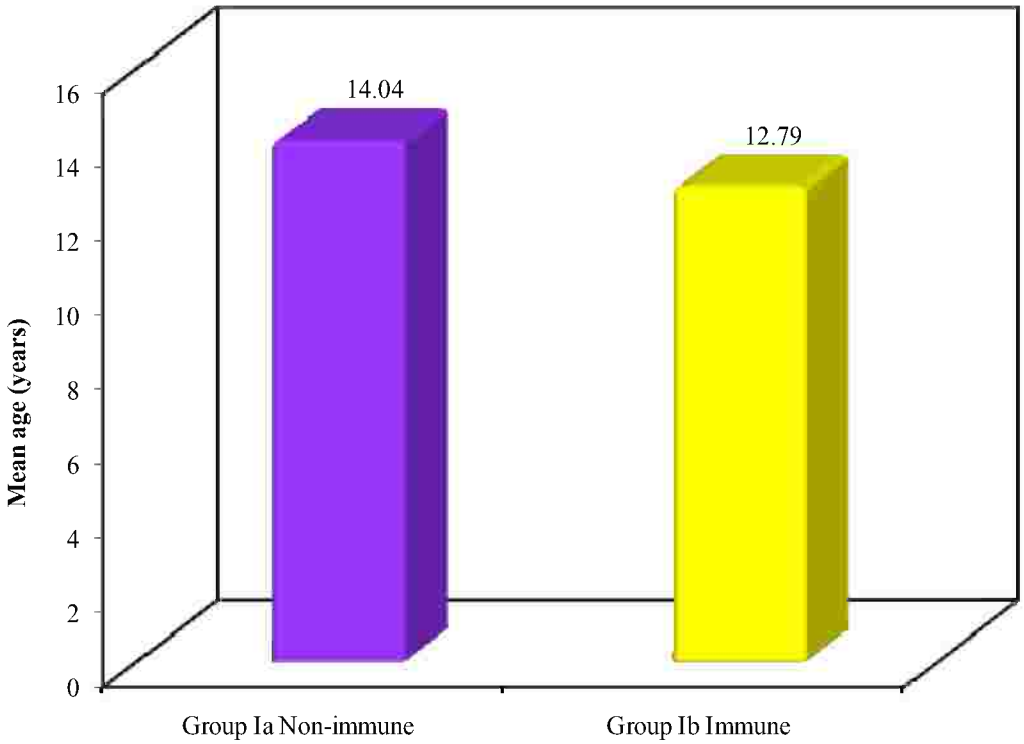

Figure (7): Comparison between Group Ia and Group Ib according to mean age.

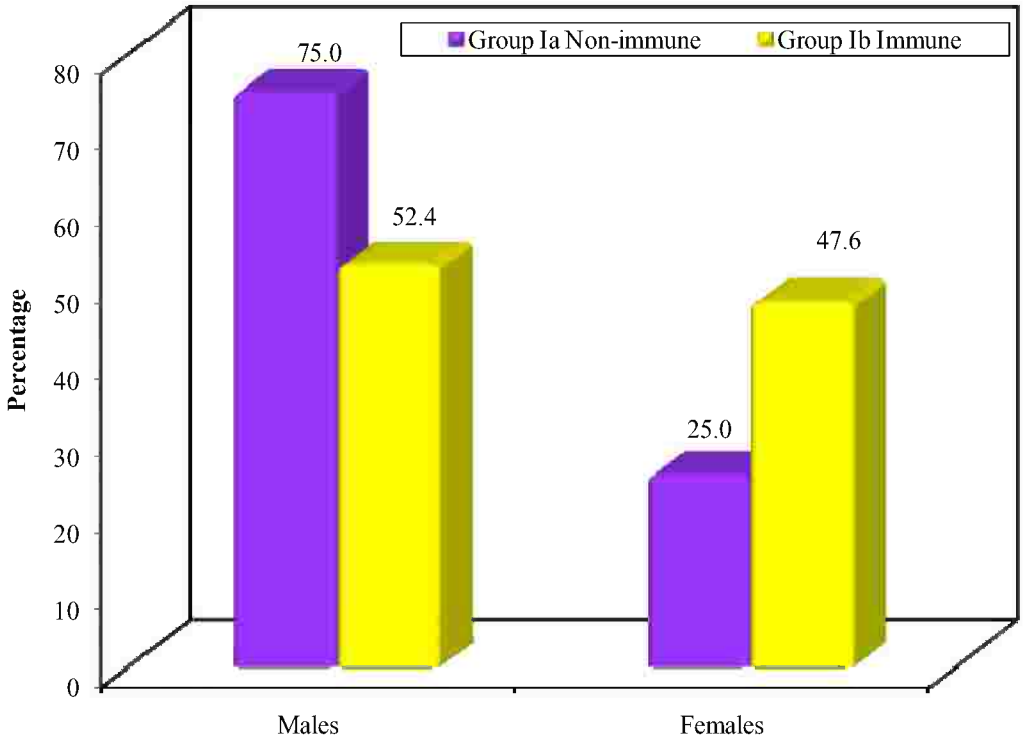

Figure (8): Comparison between Group Ia and Group Ib according to sex.

Table (15): Comparison between Group Ia and Group Ib according to age at diagnosis and post chemotherapy interval:

		Group Ia Non-immune n=4	**Group Ib** Immune n=21	
Age at diagnosis (years)	Range	2.75-6.08	1.0-12.75	Z=1.038 (p=0.299)
	Mean±SD	4.81±1.5	6.33±3.32	
	Median	5.6	4.1	
Post chemotherapy interval (years)	Range	3.17-4.68	1.33-6.0	t=0.855 (p=.0402)
	Mean±SD	3.82±0.75	3.12±1.58	
	Median	3.71	2.67	

Z: Z for Mann Whitney test
t: Student t-test
*: Statistically significant at $p \leq 0.05$

Age at diagnosis was slightly higher in GroupIb (p=0.299). In Group Ia, it ranged from 2.75 to 6.08 years with mean of 4.81±1.5 years, while in Group Ib, it ranged from 1.0 to 12.75 years with a mean of 6.33±3.32. Regarding post chemotherapy interval, it was almost the same in Group Ia and Group Ib, (p=0.402). Table (15) fig (9 and10).

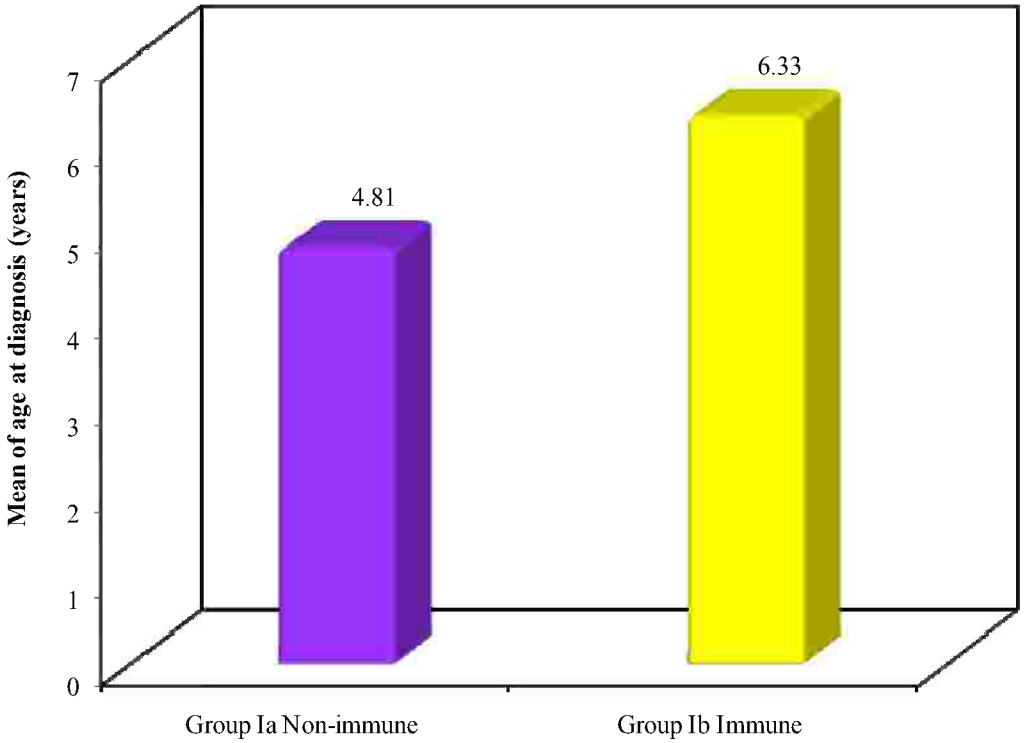

Figure (9): Comparison between Group Ia and Group Ib according to mean age at diagnosis.

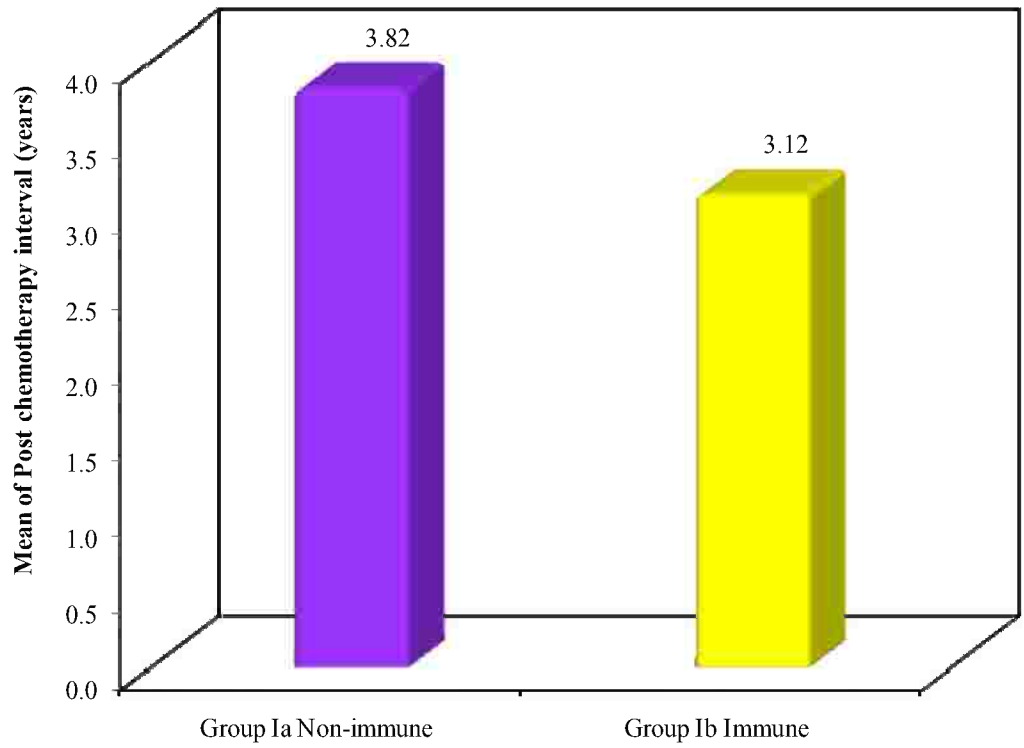

Figure (10): Comparison between Group Ia and Group Ib according to post chemotherapy interval

Moreover, when comparing mean anti-HBs titer of patients who have ended chemotherapy recently (≤2 year) to those who finished since a longer interval (>2 year), anti-HBs was higher in the former group (179.18 vs 129.49 mIU/mL), but the difference did not reach statistical significance, (p= 0.180) .Table (16) and fig (11).

Table (16): Comparison of anti-HBs titer between children of ≤2 and >2 year post chemotherapy interval:

Anti-HBs titer (mIU/mL)	**Post chemotherapy interval ≤2year** n=8	**Post chemotherapy interval >2year** n=17	
Range	26.10-264.8	0.6-260.9	Z=1.340 (p=0.180)
Mean ± SD	179.18±95.83	129.49±103.89	

Z : Z for Mann Whitney test.
*: Statistically significant at p ≤ 0.05

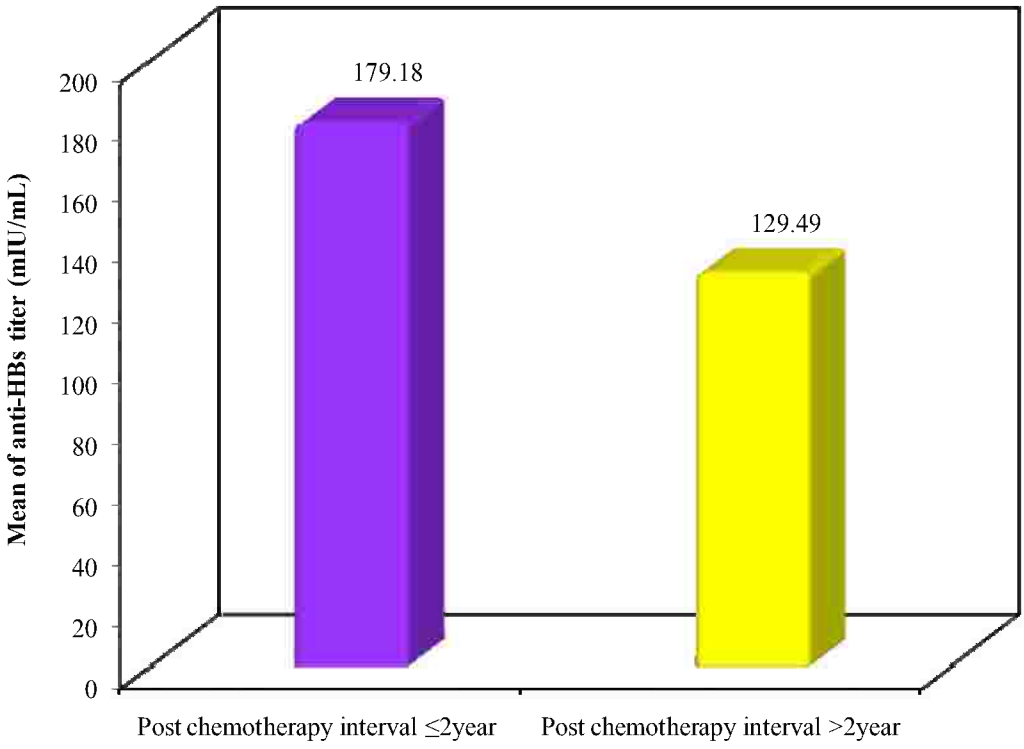

Figure (11): Comparison of anti-HBs titer between children of ≤2 and >2 year post chemotherapy interval.

Table (17): Comparison between Group Ia and Group Ib according to type of leukemia:

	Group Ia Non-immune n=4		Group Ib Immune n=21		Chi-square test (*p* value)
	No	%	No	%	
B-ALL	4	100	20	95.2	χ^2=0.198
T-ALL	0	0.0	1	4.8	^{FE}p =1.000

χ^2: Chi square test
FE: Fisher Exact test
*: Statistically significant at $p \leq 0.05$

In Group Ia 100% children's diagnosis was B-ALL, While in Group Ib 95.2% was B-ALL and only 4.8% was T-ALL.(*p* =1.000) Table(17) and fig(12).

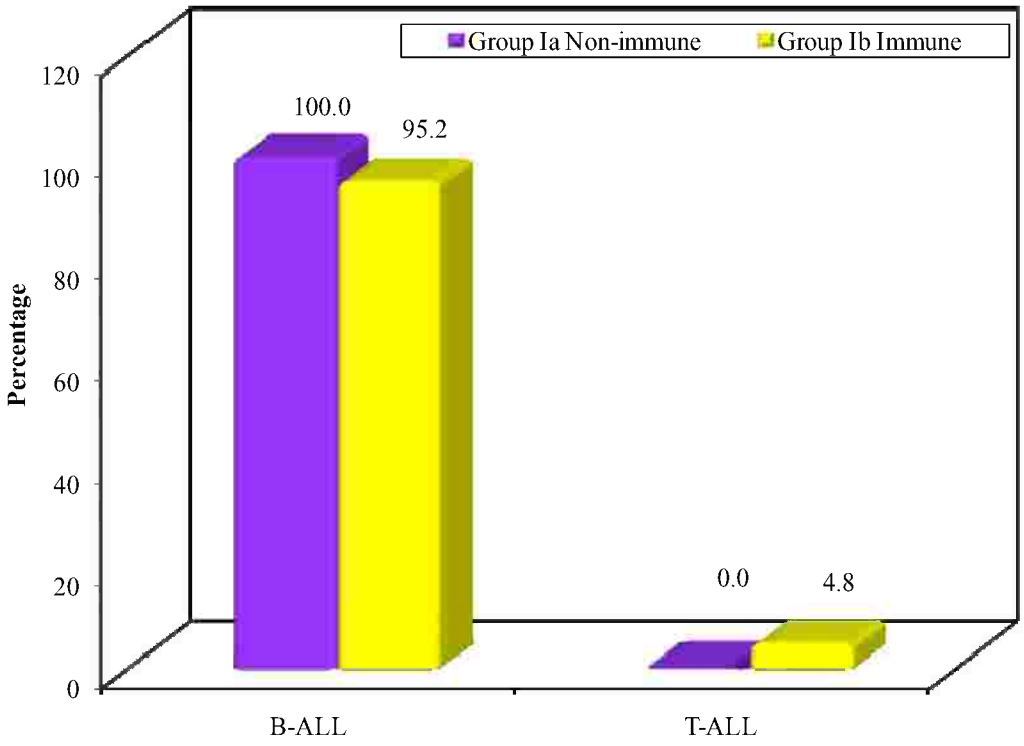

Figure (12): Comparison between Group Ia and Group Ib according to type of leukemia.

Table (18): Comparison between Group Ia and Group Ib according to the protocol used for treatment of leukemia:

	Group Ia Non-immune n=4		Group Ib Immune n=21		Chi-square test (*p* value)
	No	%	No	%	
Previously adopted protocol	3	75	9	42.9	χ^2=1.198
Modified CCG 1991 Standard risk	0	0	4	19.0	
Modified CCG 1961 High risk	1	25	8	38.1	^{MC}p =0.663

χ^2: Chi square test
MC: Monte Carlo test
*: Statistically significant at $p \leq 0.05$

Table (18) shows the immune status to HB vaccine in relation to protocol used for treatment of leukemia. In Group Ia 3 (75%)children were treated with previously adopted protocol, 1 (25%)with Modified CCG 1961 High risk protocol, and no one with Modified CCG 1991 Standard risk protocol.

In Group Ib the percentage of children treated with previously adopted protocol and with Modified CCG 1961 High risk was almost similar (42.9%) and (38.1) respectively. While only 4 (19%) were treated with Modified CCG 1991 Standard risk protocol. There was no statistical significant difference between immune status after re-vaccination and protocol used for treatment of leukemia. (p=0.663). Fig (13).

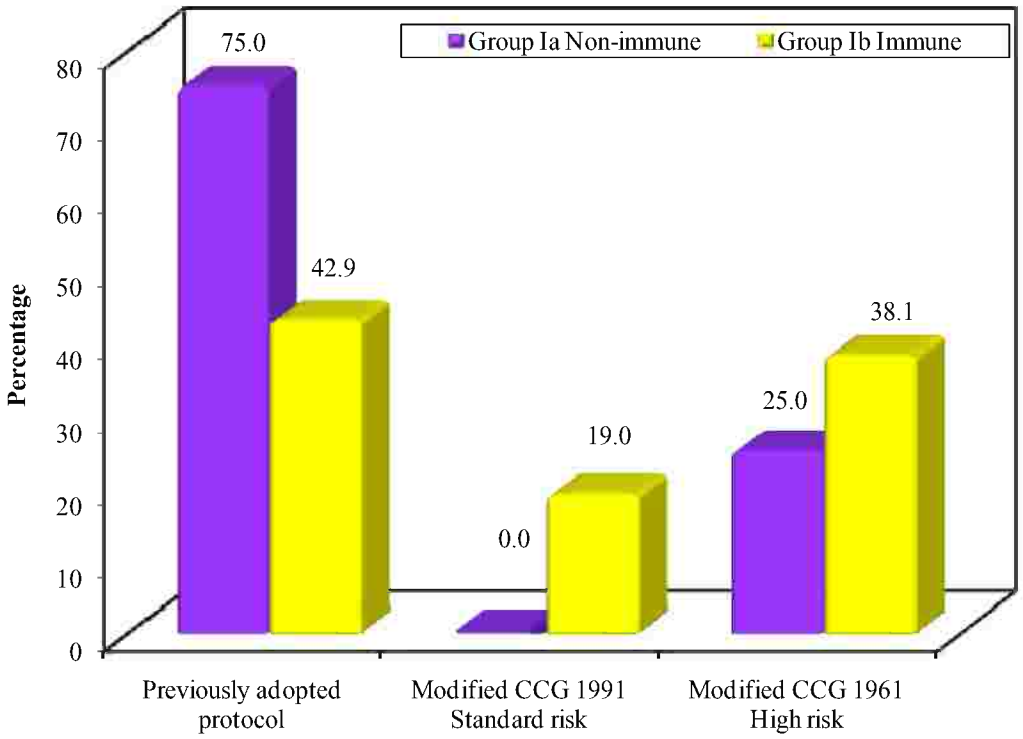

Figure (13): Comparison between Group Ia and Group Ib according to the protocol used for treatment of leukemia.

Table (19): Comparison between Group Ia and Group Ib according to the number of blood units transfused since diagnosis of leukemia:

		Group Ia Non-immune n=4		Group Ib Immune n=21		Chi-square test (*p* value)
		No	%	No	%	
Number of units of blood products transfused	<5	1	25	7	33.3	χ^2=1.328 MC p =.0.642
	5- <10	3	75	9	42.9	
	≥10	0	0	5	23.8	

χ^2: Chi square test
MC: Monte Carlo test
*: Statistically significant at p ≤ 0.05

Table (19) shows comparison between both groups according to the number of blood units transfused (including PRBCs, plasma, and platelets units) since diagnosis of leukemia. Nearly one third of the patients in both groups have received less than 5 units of blood products. Up to 23.8% of Group Ib children have received ≥10 blood units during treatment. There was no significant difference between both groups regarding the number of blood units transfused (*p*=0.642).Fig (14).

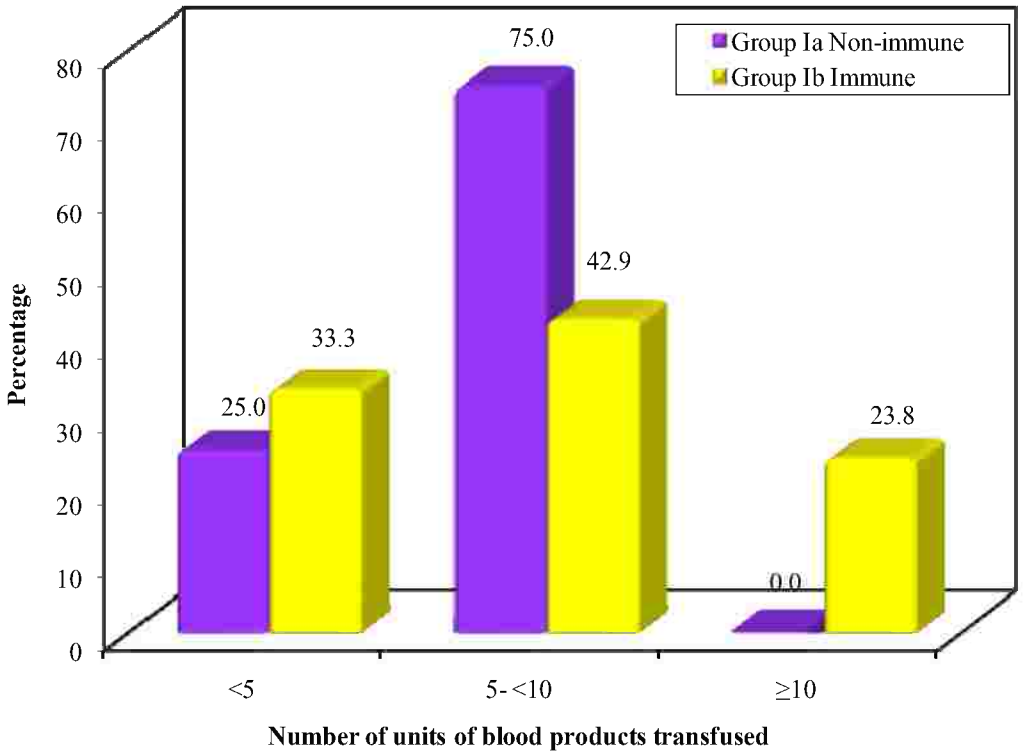

Figure (14): Comparison between Group Ia and Group Ib according to the number of blood units transfused since diagnosis of leukemia.

Table (20): Comparison between anti HBs titers before and after re-vaccination in group I:

	Before re-vaccination	After re-vaccination	Z	p
Anti HBs titer (mIu/ml)				
Range	0.0 – 9.30	0.60 – 264.80		
Mean ± SD.	2.38 ± 2.90	145.39 ± 102.15	4.346*	<0.001*
Median	0.80	166.10		
% of seroconverison	86%			

Z: Z for Wilcoxon signed ranks test
*: Statistically significant at $p \leq 0.05$

Anti-HBs levels were significantly increased after 3 doses of HB vaccine and 86% of previously non immune children treated for leukemia turned immune. ($p<0.001$).Table (20) fig (15).

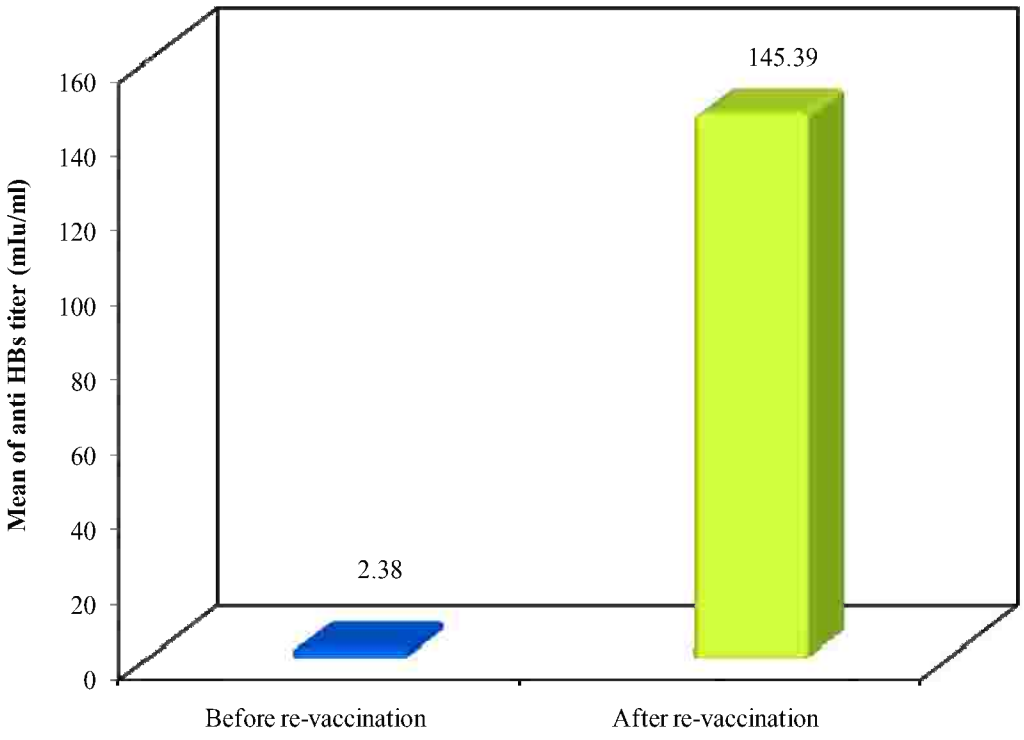

Figure (15): Comparison between anti HBs titers before and after re-vaccination in group I.

Table (21): Distribution of group I as regards response to HB re-vaccination:

	No.	%
Status		
Non responder "Anti-HBs <10 mIU/ml"	4	16.0
Hypo responder "Anti-HBs 10- 100 mIU/ml"	6	24.0
Good responder "Anti-HBs ≥100 mIU/ml"	15	60.0

Table (21) show distribution of Group I as regards the response to HB re-vaccination 4 (16%) showed anti-HBs titer <10 mIU/ml (non responders), 6 (24%) showed titer10-100 mIU/ml(hypo responders), and 15 (60%) showed titer≥100mIU/ml (good reponders). Fig(16).

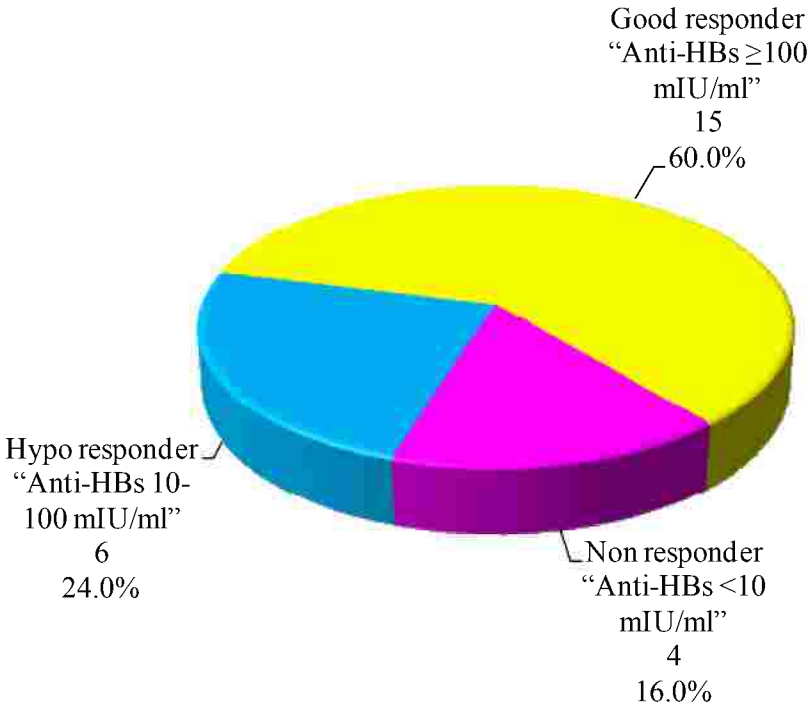

Figure (16): Distribution of group I as regards response to HB re-vaccination.

C. Analysis of factors affecting the immune status of Group II subjects (normal controls):

Group II was composed of 50 normal children vaccinated against HBV as infants and did not receive additional HB vaccine doses. Group II children were subdivided into two subgroups according to immunity to HB vaccine.

Non-immune children were 13 (26%) constituting Group IIa, and the 37 (74%) immune children constituted Group IIb as shown in table (22) . In Group IIa, age was ranging from 10 to 17 years with a mean of 14.54years, while in Group IIb the range was from 7 to 17 years with mean age of 11.89 years. Age and post vaccination interval were significantly higher in Group IIa, (p=0.002 for both). There was no significant difference between both groups regarding the sex (p=0.856). Table (22) fig (17and18).

Table (22): Comparison between non-immune and immune subjects of Group II according to sex, age, and post vaccination interval:

	Group IIa "Non-immune anti HBs <10 mIU/ml" (n=13)		**Group IIb "Immune anti HBs ≥10 mIU/ml" (n=37)**		**Test of sig.**	**p**
	No.	**%**	**No.**	**%**		
Sex						
Male	6	46.2	16	43.2	χ^2=0.033	0.856
Female	7	53.8	21	56.8		
Age(years)						
Min. – Max.	10.0 – 17.0		7.0 – 17.0			
Mean ± SD.	14.54 ± 2.47		11.89 ± 2.51		t=3.279*	0.002*
Median	15.0		12.0			
Post vaccination interval(years)						
Min. – Max.	9.50 – 16.50		6.50 – 16.50			
Mean ± SD.	14.04 ± 2.47		11.39 ± 2.51		t=3.279*	0.002*
Median	14.50		11.50			

χ^2: Chi square test
t: Student t-test
*: Statistically significant at $p \leq 0.05$

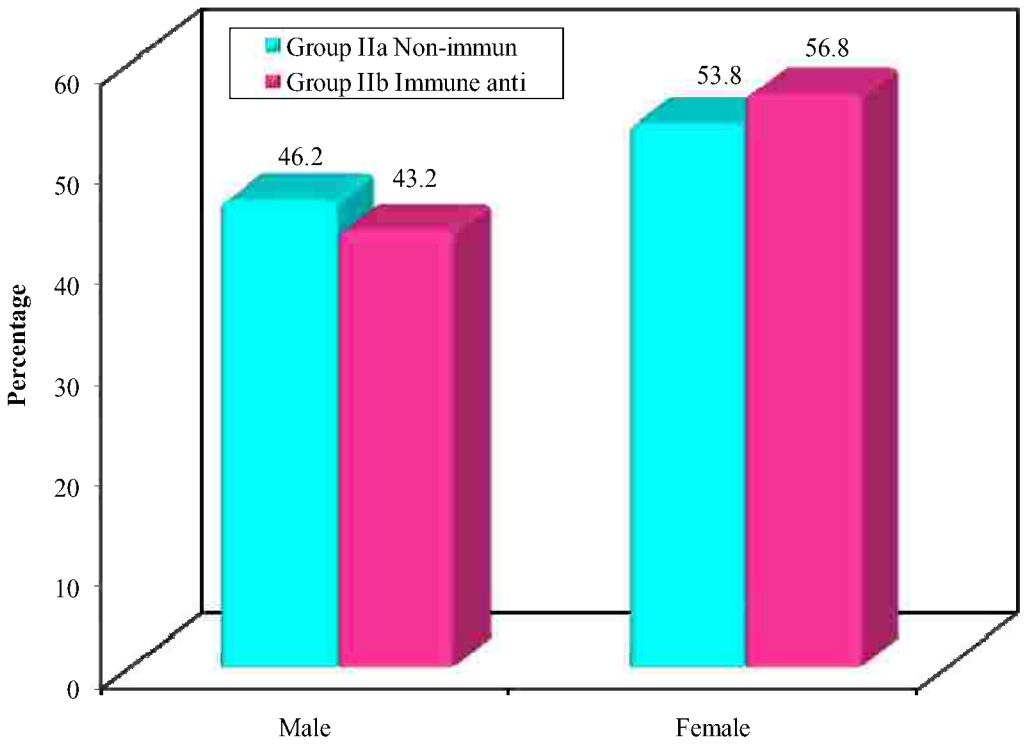

Figure (17): Comparison between non-immune and immune subjects of Group II according to sex.

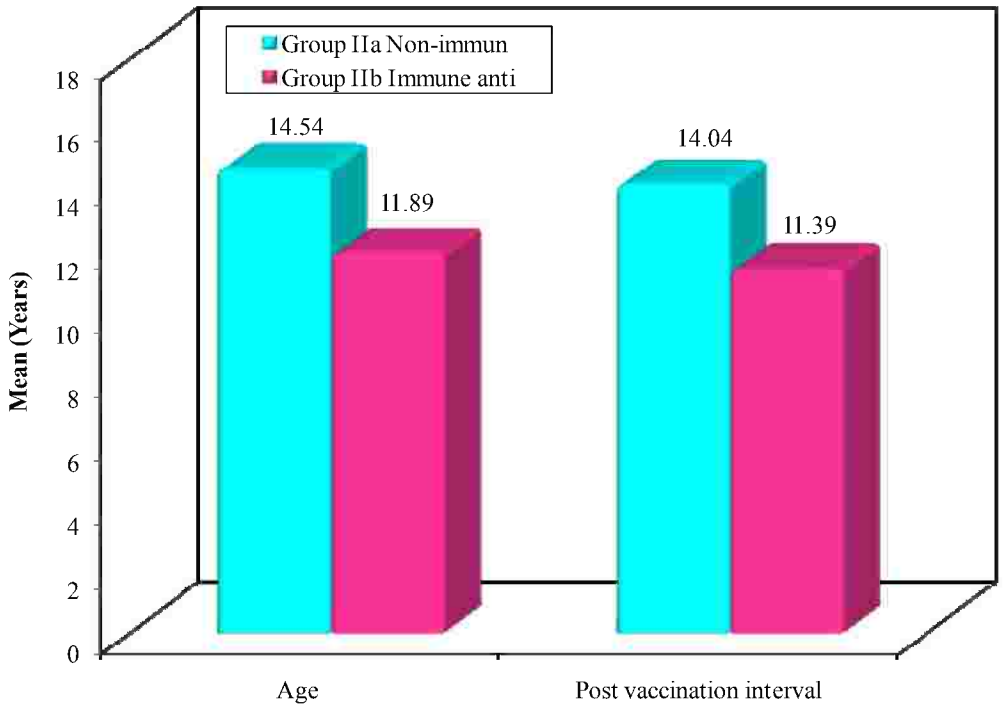

Figure (18): Comparison between non-immune and immune subjects of Group II according to age and post vaccination interval.

Using Spearman's Rho correlation study, the following correlations were found among Group I subjects:

- Negative correlation between age at the time of the study and anti-HBs titer in **group II** (r_s= -0.326*, ***p*=.021***), table (26) fig (23).
- Positive correlation between **age at diagnosis** in **group I** and **age at the time of the study** (r_s= 0.710*, ***p*<.0001***), table (26) fig (24).
- Non significant negative correlation between post chemotherapy interval in **group I** and anti-HBs titer (r_s= -.336, p=.100).
- Non significant negative correlation between number of blood unit transfusions in **group I** and anti-HBs titer (r_s=-.026, p=.901).
- Non significant negative correlation between age at diagnosis in **group I** and anti-HBs titer (r_s=-.025, p=.904), .

Table (23): Correlation between age at time of the study with Anti-HBs titer in GroupII and age at diagnosis in GroupI:

	Age at time of the study	
	r	**p**
Anti-HBs titer in Group II "Healthy control" (n = 50)	-0.326*	0.021*
Age at diagnosis in Group I "Leukemic cases" (n = 25)	0.710*	<0.001*

r: Pearson coefficient
*: Statistically significant at $p \leq 0.05$

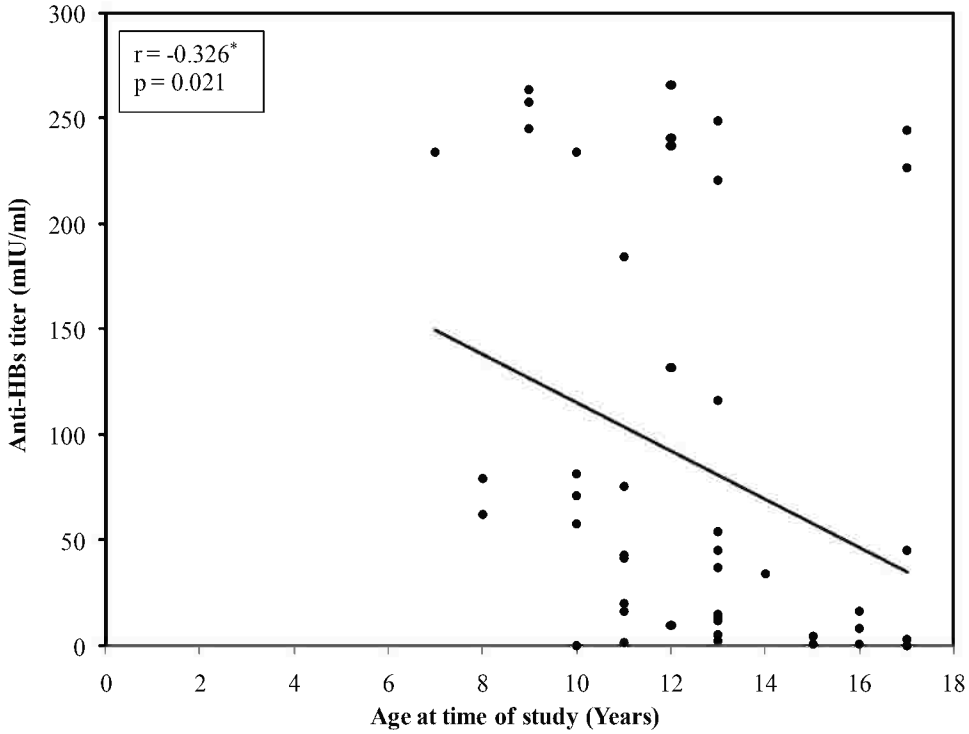

Figure (19): Correlation between age at time of study with Anti-HBs titer (mIU/ml) in Group II

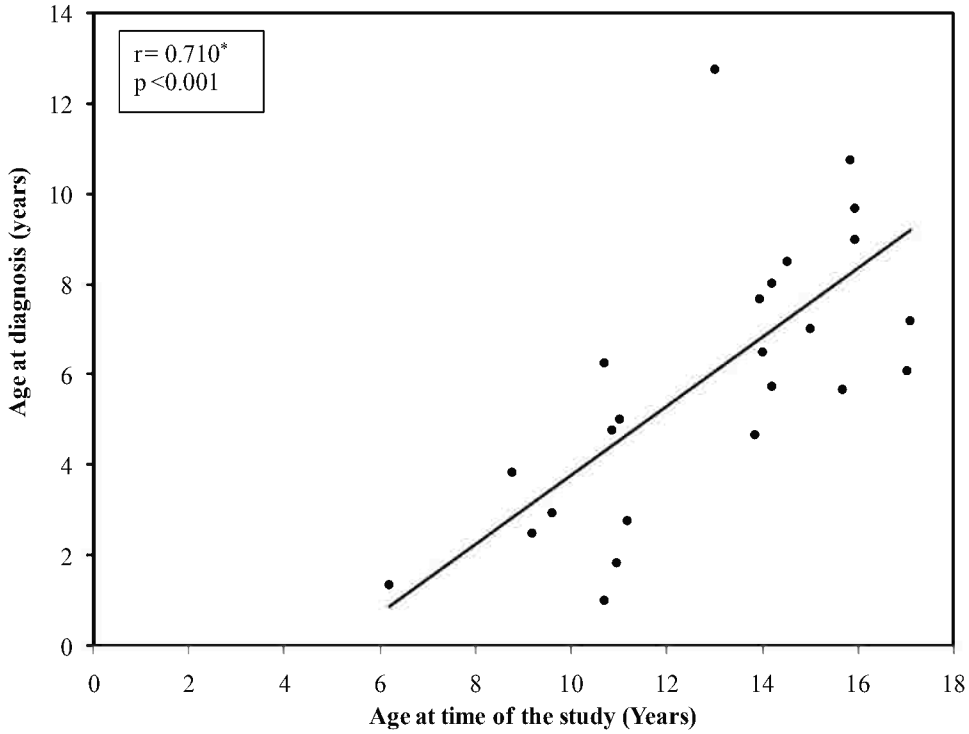

Figure (20): Correlation between age at time of the study with and age at diagnosis in Group I