AIM OF THE WORK

The aim of this study was to determine the prevalence of HLA alloimmunization in chronically transfused thalassemic children, compared to their splenectomised counterparts and its relation to febrile non hemolytic transfusion reactions. In addition, we compared the effect of leucoreduced packed RBCs by bed-side filtration and washed RBCs in the prevention of febrile non hemolytic transfusion reactions in all positive HLA antibodies patients and their impact on the rise of hemoglobin level after transfusion.

SUBJECTS

Sixty five thalassemic children were selected from the Hematology Clinic of Alexandria University Children's Hospital during the period from the 1st of January 2014 to March 2014 and twenty healthy volunteers, matched for age and sex, served as a control group.

Inclusion Criteria For Patients:

1. Patients diagnosed as β thalassemia major.
2. Age up to 14 years.
3. Patients on regular transfusion program.

Exclusion Criteria For Patients:

1. Patients maintained on blood transfusion for any other reason:
   - Aplastic anemia.
   - Leukemia.
   - Sickle cell anemia.
   - Hereditary spherocytosis.
   - G6PD
2. Patients maintained on washed or filtered RBCs from the start of transfusion.

All subjects were divided into three groups:

- **Group I:** Forty five cases of non splenectomised chronically transfused β thalassemia major children.

- **Group II:** Twenty cases of splenectomised chronically transfused β thalassemia major children.

- **Group III:** Twenty healthy children who were never received blood transfusion as a control group.

The parents of all subjects were asked to sign a written informed consent, after the nature and the aim of the study were explained to them. The study approval was obtained from the Ethics Committee of the Faculty of Medicine, Alexandria University.

METHODS

All patients (n=65) included in the present study were subjected to:

1. Detailed history of blood transfusions regarding the age of starting transfusion, frequency of transfusions and frequency of febrile reactions.
2. Qualitative estimation of anti- lymphocytotoxic antibodies by (ALA/LCA) ELISA kit.[73]
3. All thalassemic patients with positive HLA antibodies were subjected to two sessions of blood transfusion; once by bed-side leucofiltration and the other by washed RBCs, then observing the occurrence of FNHTRs and estimating the hemoglobin rise after transfusion by both methods.
4. Thirty LCA negative thalassemic patients suffering from FNHTRs were transfused only by washed RBCs observing its effect on elimination of these reactions.

The control group was subjected to:

1. Full history taking.
2. Qualitative estimation of ALA by (ALA/LCA) ELISA kit.

Specimen Collection and Storage:

2 milliliters of venous blood sample were aseptically withdrawn from every patient and emptied in a plain vaccutainer tube. Blood was allowed to clot for 30 minutes then centrifuged at 6000 rpm, at room temperature. The separated sera were frozen at -20˚C till the time of the assay.

Estimation of Anti-Lymphocytotoxic Antibodies

Method:

Anti lymphocytotoxic antibodies were determined by using an ELISA kit, Glory science Company lot no 10432, model no. EIA-1476, manufactured in USA.

Principle of the Test:

Human ALA/LCA kit uses purified human ALA/LCA antigen coating microtiter plate wells. Unknown sera are added to the wells and allowed to incubate followed by a washing step to remove unbound antibodies, and then secondary antibody labelled with peroxidase enzyme (HRP) is added followed by washing step. Tetramethylbenzidine (TMB) substrate solution is added. The enzyme-catalyzed reaction is terminated by the addition of sulphuric acid (H_2SO_4) solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The readings are compared with the cut off value to judge the presence of those antibodies.

Table (1): Composition of the kit

Reagent name	Quantity
Negative control	0.5ml
Positive control	0.5ml
HRP-Conjugate Reagent	6.0 ml
Sample diuent	6.0 ml
Chromogen Solution A	6.0 ml
Chromogen Solution B	6.0 ml
Stop Solution	6.0 ml
Wash Solution	20 ml
Microelisa Strip plate	
Closure plate membrane	

Preparation of Reagents:

1. **All Reagents** were brought to room temperature.
2. **Wash Buffer (30x):** The wash buffer was reconstituted by diluting 20 ml of the supplied wash buffer concentrate with 580 ml distilled water.
3. The rest of reagents were ready to use.

Test Protocol:

1. All the frozen samples were brought to room temperature.
2. All the samples were mixed by vortex.
3. Blank, positive control and negative control were run in duplicate.
4. 40 µl of sample diluent were added to all wells.
5. 10 µl of positive control, 10 µl of negative control and 10 µl of samples were added to the appropriate wells.
6. The plate was sealed with an adhesive film and incubated for 30 min at 37℃.
7. The plate was then washed 5 times.
8. 50 µl of HRP-Conjugate reagent were added to all wells except the blank wells.
9. The plate was sealed with an adhesive film, incubated for 30 min at 37℃ and washed 5 times.
10. 50 µl of Chromogen Solution A and 50 µl of chromogen solution B were added to every well.
11. The plate was kept in the dark for 15 min at 37˚C.
12. 50 µl of stop solution were added to each well, and finally the plate was read at 450 nm using ELISA plate reader, taking the blank well as zero.

Calculation of Results:

The test was considered valid if the absorbance reading average of the positive control wells is ≥ 1.00 and that of the negative control wells is ≤ 0.10.

The critical (cut off) value was calculated by the following equation:

Critical value = Absorbance reading average of negative control wells + 0.15.

- ALA negative samples were those samples with optical density (OD) less than the cut off value.
- ALA positive samples were those samples with OD more than the cut off value.

Steps of Washed RBCs Preparation [74]

Step 1: PRBCs units were centrifuged at 3000 rpm for 5 minutes.

Step 2: Plasma and buffy coat layer were removed.

Step 3: The red cells were resuspended in normal saline (0.9 % NaCl).

Step 4: This blood unit was centrifuged for 5 minutes at 2000 rpm and the supernatant was discarded.

Step 5: Steps 3 and 4 were repeated twice for a total of 3 washes or until the supernatant is clear.

Step 6: The supernatant were discarded.

Figure 12: Washed RBCs preparation

Leucocytes Filters [75]

Filters used in this study were Cellbarrier Plus (Figure 13), imported from B.Braun Avitum Company, lot number 13C18 and manufactured in Italy.

Criteria of these filters:

1- Disposable filters.
2- Made from unique microporous polyurethane filter material.
3- Pore sizes range from 11 to19 µm.
4- Filtrate 99.9% of the leucocytes from the PRBCs units.
5- Average residual leucocytic count is less than $1x10^6$ per unit.
6- After infiltration the product recovery is equal or more than 90%.
7- Transparent filter housing makes monitoring the filtration processes easy.
8- It costs 120 L.E.

Figure 13: Cellbarrier Plus leucocyte filter.[75]

Statistical Analysis of the Data: [76]

Data were fed to the computer and analyzed using IBM ***SPSS software package version 20.0.***[77]

Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Comparison between different groups regarding 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. The distributions of quantitative variables were tested for normality using ***Kolmogorov-Smirnov test, Shapiro-Wilk test and D'Agstino test, also Histogram and QQ plot were used for vision test.*** If it revealed normal data distribution, parametric tests were applied. If the data were abnormally distributed, non-parametric tests were used. For normally distributed data, comparison between different groups were analyzed using F-test (ANOVA) and Post Hoc test (Scheffe) for pair wise comparison, while for abnormally distributed data, comparison between two independent populations was done using Mann Whitney test. Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

RESULTS

This study was conducted on sixty five chronically transfused β thalassemia major children who were selected from the Hematology Clinic of Alexandria University Children's Hospital and twenty healthy volunteers served as a control group. Among the 65 children, twenty were splenectomised.

Comparison between the first two studied groups regarding age showed that median age was 10 years in group I ranging from 1.5 to 14 years and 11.5 years in group II ranging from 4 to14 years. There was no statistically significant difference between the two groups regarding age ($p = 0.091$). (Table 2)

Regarding gender, 28 were males (62.2%) in group I and 12 (60%) in group II while females were 17 (37.8%) in group I and 8 (40%) in group II. There was no statistically significant difference between the two studied groups regarding gender ($p = 0.865$). (Table 2)

Regarding age of starting transfusion, the median age was 9 months in group I ranging from 15 days to 96 months and 6 months in group II ranging from 10 days to 36 months. Therefore the age of starting transfusion was significantly higher in non splenectomised than splenectomised chronically transfused β thalassemia major children ($p = 0.031$). (Table 2)

Regarding frequency of requiring transfusions, the median was 1 month in group I ranging from 0.3 to 1 month and 1 month in group II ranging from 0.5 to 1 month. Therefore the frequency of requiring transfusions was significantly higher in non splenectomised than splenectomised children ($p = 0.036$). (Table 2)

Regarding frequency of FNHTRs, 2 patients (4.4%) in group I and 3 patients (15%) in group II didn't experience any, the occasional reactions were reported by 21 patients (46.7%) in group I and by 9 patients (45%) in group II, and the every time reactions were reported by 22 patients (48.9%) in group I and by 8 patients (40%) in group II. There was no statistically significant difference between the two studied groups regarding frequency of FNHTRs ($p = 0.463$). (Table 2)

Comparison between the three studied groups according to ALA results showed that percentages of patients with positive ALA results were significantly higher in group I (20%) as compared to group II (0%) and group III (0%) ($p = 0.048$) (Table 3, Figure 14).

Patients and controls were matched regarding age and gender therefore no statistically significant difference was found between the two groups ($p = 0.203$, $p = 0.545$ respectively). (Table 3)

Table (2): Characteristics of the studied cases

		Group I (n=45)	Group II (n=20)	Test statistic	*p*
Age (years)	***Median (Min- Max)***	10 (1.5- 14)	11.5 (4-14)	***Z**** = 568.5	0.091
Gender	**Male *(%)***	28 (62.2)	12 (60.0)	**χ2†**= 0.029	0.865
	Female *(%)*	17 (37.8)	8 (40)		
Age of starting transfusion (Months)	***Median (Min- Max)***	9 (0.5-96.0)	6 (0.3-36.0)	***Z*** = 4.650	0.031*
Frequency of transfusions (Per Months)	***Median (Min- Max)***	1 (0.3.-1)	1 (0.5-1)	***Z*** = 4.419	0.036*
Frequency of FNHTRs	**None *(%)***	2 (4.4)	3 (15)	***MCp*$^{\$}$**= 0.463	
	Occasional *(%)*	21 (46.7)	9 (45)		
	Every time *(%)*	22 (48.9)	8 (40)		

*Mann-Whitney test (*Z*)
†Chisquare test (χ2)
$ Monte Carlo test

Table (3): Comparison between the studied groups according to age, gender and ALA results

	Patients				Controls		Test of sig.	p
	Group I		Group II		Group III			
	n.	%	n.	%	n.	%		
Age (years)								
Median	10.0		11.5		9.0		$^{KW}\chi^2$=3.193	0.203
(Min. – Max.)	1.50 – 14.0		4.0 – 14.0		3.0 – 13.0			
Gender								
Male	28	62.2	12	60	15	75.0	χ^2=1.215	0.545
Female	17	37.8	8	40	5	25.0		
ALA results								
Positive ALA	9	20.0	0	0.0	0	0.0	χ^2=7.966*	^{MC}p= 0.014*
Negative ALA	36	80.0	20	100	20	100		
$^{FE}p_1$			0.048*		0.048*			

$^{KW}\chi^2$: Chi square for Kruskal Wallis test
χ^2: value for Chi square test
MC: p value for Monte Carlo test for comparing between 3 groups
FE: Fisher Exact test
p_1 : p value for comparing between group I and each other group
*: Statistically significant at $p \leq 0.05$

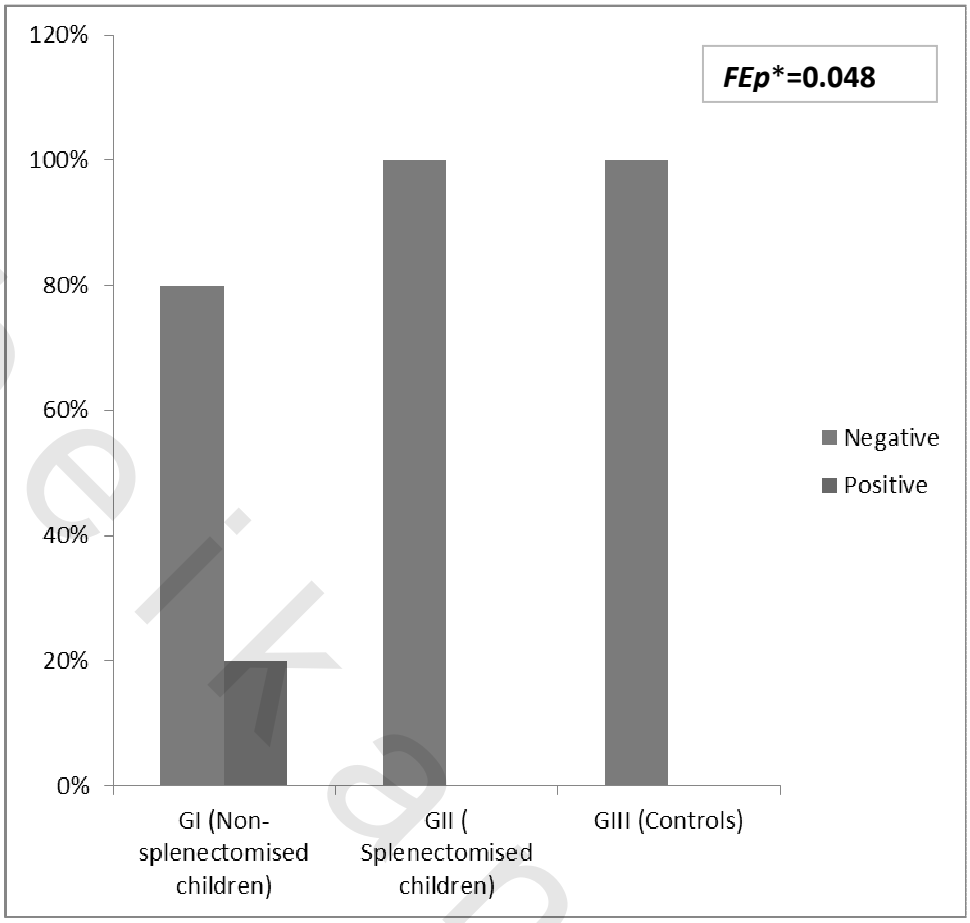

Figure 14: Bar chart showing presence of ALA among group I, II and III.

Statistical analysis obtained for ALA results shows that the sixty five thalassemic patients were divided into 9 positive ALA cases (13%) and 56 negative ALA cases (87%). (Table 4, Figure 15)

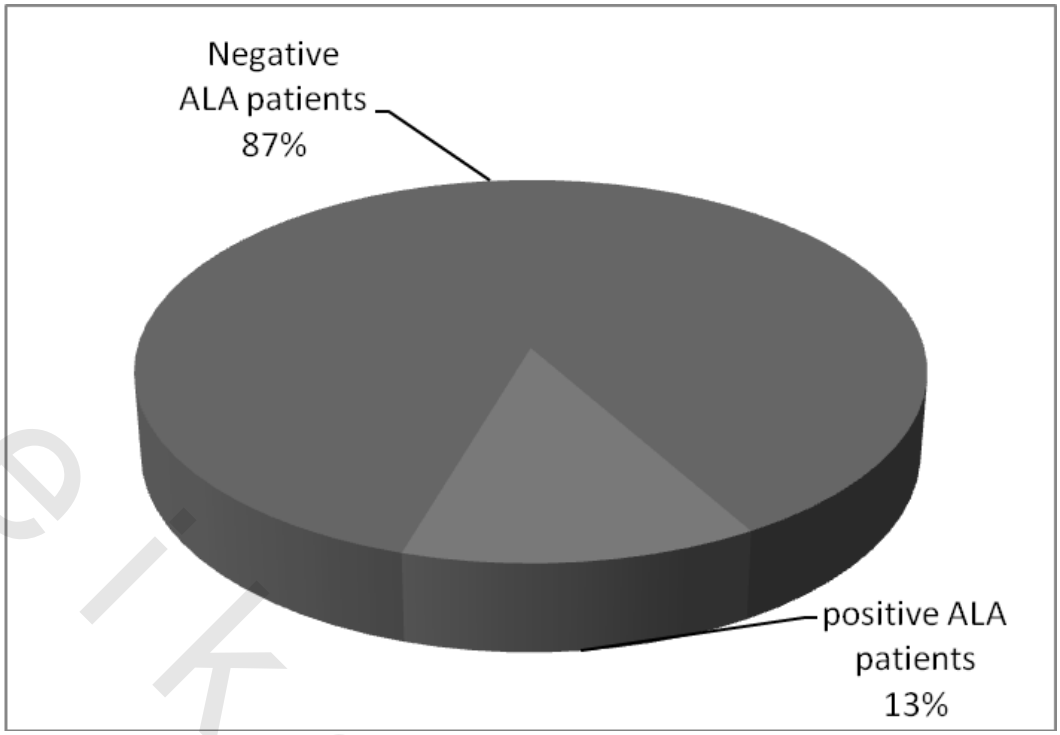

Figure (15): Distribution of studied cases according to ALA result

Comparison between ALA positive and negative groups showed that males were 8 (88.9%) in ALA positive group and 32 (57.1%) in ALA negative group while females were 1 (11.1%) in ALA positive group and 24 (42.9%) in ALA negative group. There was no statistically significant difference between the two studied groups regarding gender (p = 0.137).(Table 4)

Regarding age, the median was 11 years in the ALA positive group ranging from 1.5 years to 14 years and 10 years in the ALA negative group ranging from 2 years to 14 year. There was no statistically significant difference between the two groups regarding age (p = 0.746).(Table 4)

Table (4): Comparison between ALA positive and negative cases regarding gender and age

	Patients				Test of sig.	p
	ALA Positive (n=9) (13%)		ALA Negative (n=56) (87%)			
	No.	%	No	%		
Gender						
Male	8	88.9	32	57.1	$\chi^2 = 3.302$	$^{FE}p = 0.137$
Female	1	11.1	24	42.9		
Age (years)						
Median	11.0		10.0		t= 0.326	0.746
Min. – Max	1.50 – 14.0		2.0 – 14.0			

p: p value for comparing between the two studied groups
χ^2: vale for Chi square
FE: Fisher Exact test
t: Student t-test

Comparison between ALA positive and negative groups showed that the median age of starting transfusion was 10 months in the ALA positive group ranging from 4 months to 30 months and 6 months in the ALA negative group ranging from 0.3 month to 96 months. There was no statistically significant difference between the two groups regarding age of starting transfusion (p = 0.338). (Table 5, Figure 16)

As for the frequency of transfusions, the median was 1 month in the ALA positive group ranging from 0.3 to 1 month and 1 month in the ALA negative group ranging from 0.3 to 1 month. There was no statistically significant difference between the two groups regarding frequency of transfusions (p = 0.362). (Table 5, Figure 17)

Regarding the frequency of FNHTRs, reactions were absent in 1 patient (11.1%) of the ALA positive group and in 4 patients (7.2 %) of the ALA negative group, the occasional reactions were reported by 5 patients (55.6%) of the ALA positive group and by 25 patients (44.6%) of the ALA negative group, and the every time reactions were reported by 3 patients (33.3%) of the ALA positive group and by 27 patients (48.2%) of the ALA negative group. There was no statistically significant difference between the studied groups regarding frequency of adverse reactions (p =0.887). (Table 5, Figure 18)

Table (5): Comparison between ALA positive and negative cases regarding age of starting transfusion, frequency of transfusions and frequency of adverse reactions.

		ALA Positive (n=9)	ALA Negative (n=56)	Statistical Test	*p*
Age of starting transfusion (Months)	***Median (Min- Max)***	10 (4-30)	6 (0.3-96)	***Z*** = 302	0.338
Frequency of transfusions (Per Months)	***Median (Min- Max)***	1 (0.3-1)	1 (0.3-1)	***Z*** = 210	0.362
Frequency of FNHTRs	**None *(%)***	1 (11.1)	4 (7.2)	***MCp*= 0.887**	
	Occasional *(%)*	5 (55.6)	25 (44.6)		
	Every time *(%)*	3 (33.3)	27 (48.2)		

(*Z*): Mann-Whitney test
MC: p value for Monte Carlo test

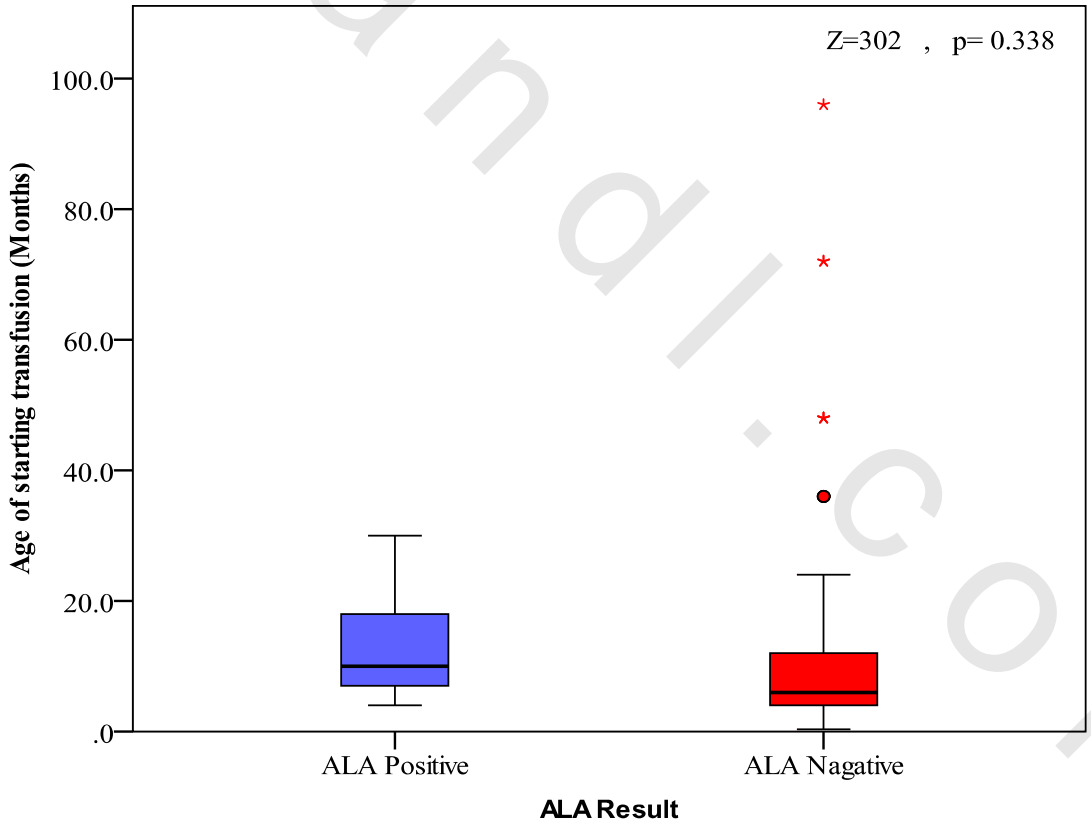

Figure 16: Box plot for the median age of starting transfusion and its range in ALA positive and negative thalassemia major children

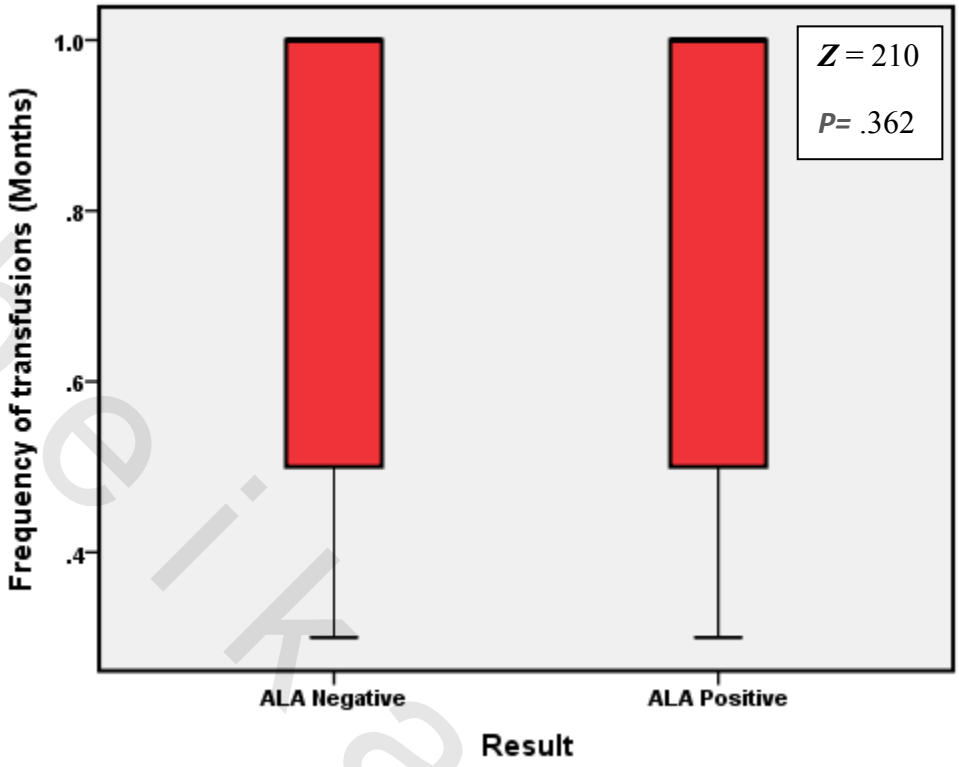

Figure 17: Box plot for the frequency of transfusions in ALA positive and negative thalassemia major children

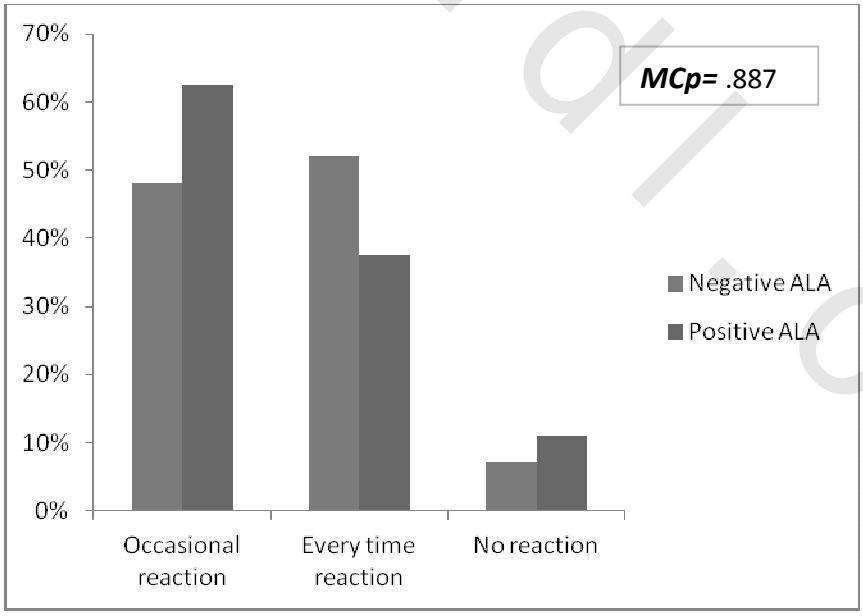

Figure 18: Bar chart showing the frequency of FNHTRs among ALA positive and negative thalassemia major children

One of our ALA positive patients died during our study and so the comparison between transfusing washed and filtered RBCs was performed on 8 positive cases, our results showed that after using washed RBCs 7 out of 8 (87.5%) ALA positive cases did not experience any FNHTRs, however 1 out of 8 (12.5%) ALA positive cases still suffered from FNHTRs. Using filtered RBCs 8 (100%) ALA positive cases did not experience any reactions. Therefore, there was no statistically significant difference between the two blood products regarding their ability to eliminate FNHTRs (p =1.000). (Table 6, Figure 19)

Table (6): Comparison between washed and filtered RBCs regarding their ability to eliminate the FNHTRs (n = 8)

	Washed RBCS		Filtered RBCs		χ^2	^{FE}p
	n	%	n	%		
FNHTRs:Absent	7	87.5	8	100.0	1.067	1.000
FNHTRs:Present	1	12.5	0	0.0		

χ^2: value for Chi square
FE: Fisher Exact test

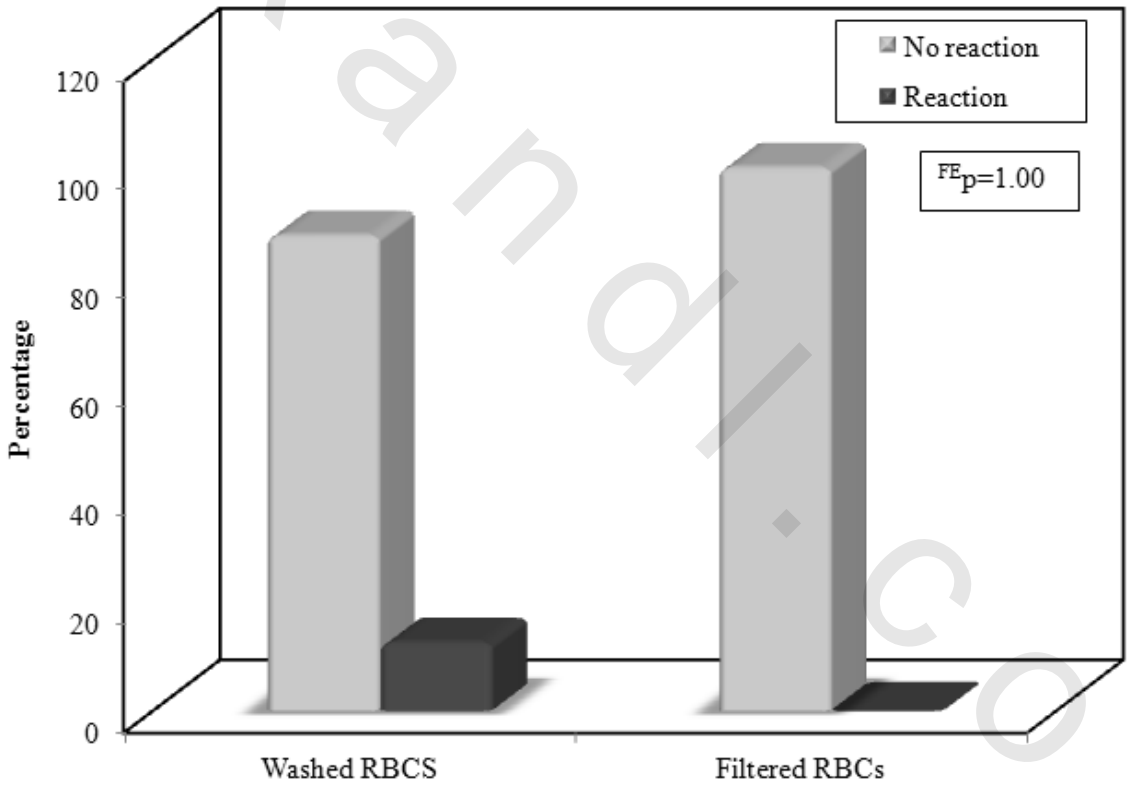

Figure (19): Comparison between washed and filtered RBCs regarding the decrease in FNHTRs.

Regarding the hemoglobin rise after transfusion, it showed that the median was 1.65 g/dl ranging from 0.60 to 2.30 g/dl after transfusing the washed RBCs and 1.65 g/dl ranging from 1.40 to 2.0 g/dl after transfusing the filtered RBCs. There was no statistically significant difference between the two blood products regarding the hemoglobin rise (p =0. 409). (Table 7, Figure 20)

Table (7): Comparison between washed and filtered RBCs transfusions regarding hemoglobin rise (n = 8)

Hb rise (g/dl)	Washed RBCs	Filtered RBCs	t	p
Median. Min. – Max	1.65 0.60 – 2.30	1.65 1.40 – 2.0	0.878	0.409

t: Paired t-test

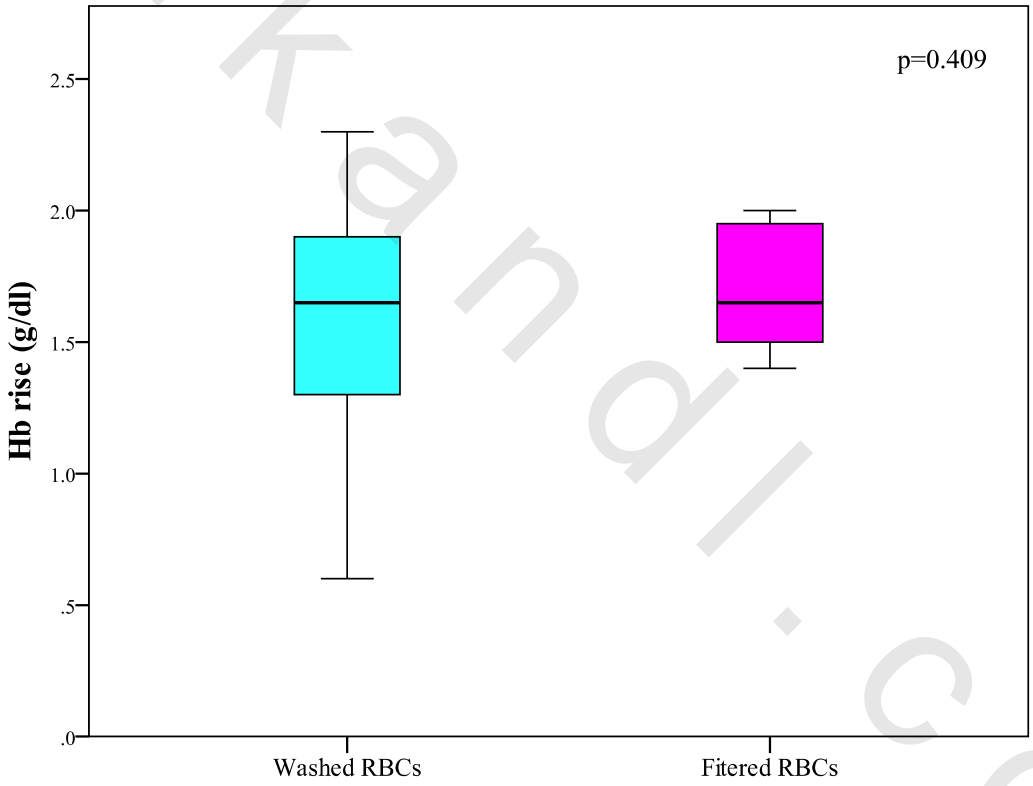

Figure (20): Comparison between washed and filtered RBCs regarding Hb rise.

Thirty HLA negative thalassemic patients suffering from FNHTRs were transfused by washed RBCs observing its effect on elimination of these reaction, we noticed that all the patients did not experience any FNHTRs after transfusions. (Table 8, Figure 21)

Table (8): Distribution of 30 HLA negative thalassemic patients according to occurrence of FNHTRs after transfusions by washed RBCs.

	n	%
Washed RBCs		
FNHTRs: Present	0	0.0
FNHTRs: Absent	30	100.0

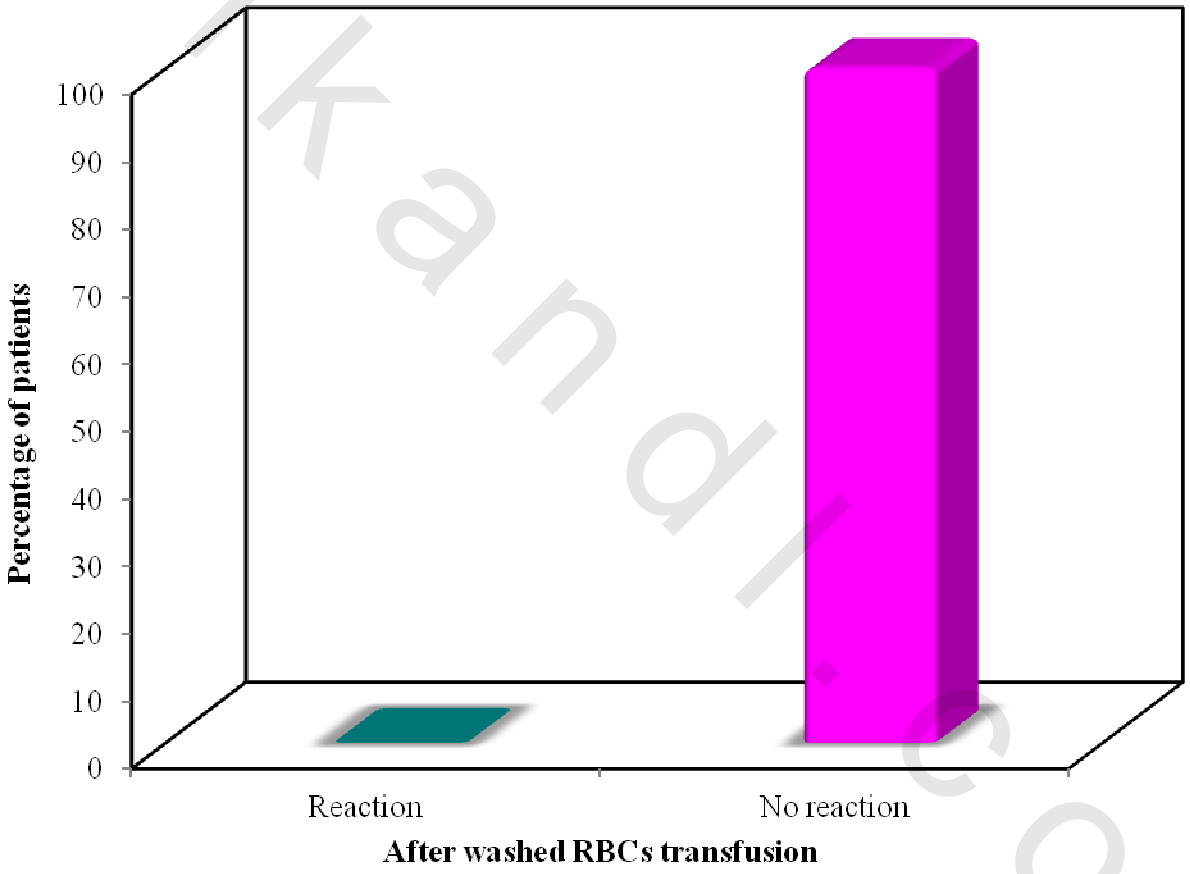

Figure (21): Distribution of the cases according to occurrence of FNHTRs after washed RBCs transfusions.