Aim of the Work

The aim of the work is to study the impact of haptoglobin gene polymorphism on phenotypic variability in patients with β thalassemia major in relation to iron overload and oxidative stress.

Subjects

This study was conducted among fifty patients with established β thalassemia, followed up in the Hematology Clinic of Student sporting Hospital, as well as the Hematology department , Medical Research institute from 1st March to 1st July 2013.

Twenty five normal apparently healthy individuals of matching age and sex with the previous group served as control. None of control subjects were thalassemia minor, and none had any history of previous blood transfusion, anemia, liver diseases, or active inflammatory conditions, and were not taking any medication.

Inclusion criteria

All patients fulfilled the following :-

- They were previously diagnosed as having βTM by clinical signs, hemoglobin electrophoresis and were followed up regularly in hematology clinic.
- On regular RBCs transfusion, all patients received approximately 300 to 350 ml of packed red blood cells at each transfusion (2 to 4-week intervals).
- All were adherent to iron chelation therapy using deferoxamine at a dose of 20 to 50 mg/kg/day.
- Blood samples were collected before blood transfusion, after an overnight fast.

Exclusion criteria

- Cases with apparent acute infection were excluded.
- The presence of an acute-phase reaction was excluded by measurement of serum C-Reactive Protein. Persons with C-reactive protein concentrations > 6 mg/L were excluded from the study.
- Patients on combined chelation therapy.

Consent

Informed consent was taken from patients or their parents for enrollment in the study. In accordance with the Declaration of Helsinki, the study was approved by the Medical Research Institute Ethics Committee.

Methods

All patients as well control subjects included in the study were subjected to the following:

1. Thorough history taking including age, sex, family history(as regards thalassemic family members),consanguinity with special emphasis on patient's first presentation, frequency of blood transfusion and chelation therapy and history of operative procedures(splenectomy).
2. Complete clinical examination with special emphasis on thalassemic manifestations, hepatomegaly, splenomegaly.
3. Laboratory investigations including:

Routine investigation including

- Complete Blood Count (CBC).Blood film and Reticulocytes count.[213]
- Hemoglobin electrophoresis.[214]
- Iron profile (serum iron,total iron binding capacity, transferrin saturation serum ferritin and) .[215-216]
- C- reactive protein.(CRP)[217]
- Coombs' test.(direct and indirect) [218]

Special laboratory tests**;**

- Serum haptoglobin (Hp).[219]
- Serum malondialdehyde (MDA).[220]
- Haptoglobin gene polymorphism by PCR.[221]

Sampling

10 mls of blood where obtained from each patient and control subject by sterile venipuncture and divided as follows:

1 ml blood in sterile EDTA vacutainer for DNA extraction for PCR.

3 mls blood in EDTA for CBC, Hb electrophoresis and direct Coombs' test.

6 mls of blood in plain tube were left to clot at room temperature and serum was separated for estimation of iron profile, serum ferritin, serum haptoglobin, ALT, CRP, indirect Coombs' test and serum MDA.

Complete Blood Count:

Using Mindray BC 8500 fully automated hematology analyzer measuring the following parameters: Hb, PCV, WBCs, count and differential, MCV, MCH, MCHC, and platelets count.

Hemoglobin Electrophoresis:

Was carried out on cellulose acetate membrane (Helena-laboratories, Beaumont,texas) in EDTA borate buffer at PH 8.4. It was done to confirm diagnosis of βTM

Serum ferritin, Serum iron, total iron binding capacity, alanine transaminase

They were determined by automated chemistry analyzer (Cobass 6000, Hitachi, Roche, Germany). While percent of transferrin saturation was calculated by dividing serum iron by TIBC.

C-reactive protein (CRP)

It was determined in serum samples by a slide agglutination method utilizing latex particles coated with goat IgG anti human CRP antibodies by VitroScient, Cairo, Egypt.

The test serum is mixed with vitro CRP latex reagent and allowed to react. If CRP concentration is greater than 6 mg/l a visible agglutination is observed, and if it less than 6 mg/l, then no agglutination is observed. Agglutination in the highest serum dilution corresponds to the amount of CRP in mg/l present in the specimen.

Concentration of CRP can be calculated as follows:

CRP (mg/l) =S×D

Where,

S= Sensitivity of the reagents.

D=Highest dilution of serum showing agglutination.

Coombs' test

Indirect and direct antiglobulin test were done using ID-Card "LISS/Coombs" Diamed Gmbh,Switzerland. With 6 micro tubes containing polyspecific AHG (rabbit anti-IgG and monoclonal anti-C3d ,cell line C 139-9)within the gel matrix.

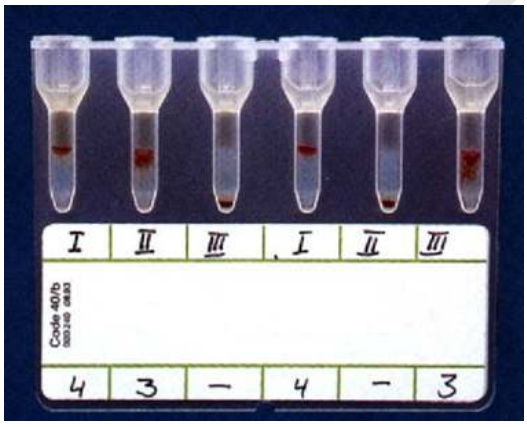

Figure 13. ID-Card "LISS/Coombs'.

Principle of Gel Technology

The gel system is based on the principle that the sephadex gel matrix acts as a sieve, through which agglutinated RBCs are too large to pass through and remain entrapped in the gel depending on their sizes. A negative reaction is seen as a clear pellet of cells settled at the bottom of microtube.

The gel card technique is sensitive and easier to perform and does not require technical skill, the interpretation of results is clear and simple and the difference between positive and negative results distinct, thereby overcoming the technical difficulties with conventional tube technique.

Grading of the reactions : can be done according to the distribution of RBCs agglutinates through the gel column.

4+
Agglutinated red blood cells form a line at the top of the gel microtube.

3+
Most agglutinated red blood cells remain in the upper half of the gel microtube.

2+
Agglutinated red blood cells are observed throughout the length of the microtube.
A small button of red blood cells may also be visible at the bottom of the gel microtube.

1+
Most agglutinated red blood cells remain in the lower half of the microtube.
A button of cells also be visible at the bottom of the gel microtube.

+/-
Most agglutinated red blood cells are in the lower third part of the gel microtube.

Negative (0)
All the red blood cells pass through and form a compact button at the bottom of the gel microtu

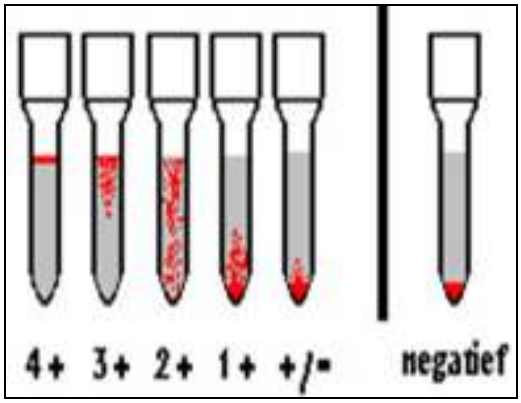

Figure 14. Grading of Coombs' reaction.

Serum Haptoglobin:

Principle of method

Hp was quantitatively determined by means of immune- nephelomerty automated chemistry analyzer (BN ProSpec system), Siemens. Using N anti sera (NAS HAPT), a liquid animal sera which produced by immunization of rabbits with highly purified human Hp.

Haptoglobin in serum form immune complexes in an immunochemical reaction with specific antibodies. These complexes scatter a beam of light passed through the sample; the intensity of the scattered light is proportional to the concentration of the haptoglobin in the serum. The result is evaluated by comparison with a standard of known concentration.

Sample

Human serum, stored no more than 7 days at 2 to 8 ^{o}C and up to 3 months if stored below – 20 ^{o}C within 24 hours after collection. Serum sample must be completely anti coagulated and after centrifuging must not contain any particles or trace of fibrin

Assay of sample

All the steps are performed automatically, as serum samples are diluted 1:20 with N diluent and measured. If the reading obtained is outside the measuring range, the assay can be repeated using higher or lower dilution of the sample.
The instrument automatically calculate and prints the concentration of serum haptoglobin.

Serum Malondialdehyde:

Determination of malondialdehyde (MDA) as thiobarbituric acid reactive substances (TBARS) according to the method of Draper and Hadley.MDA has been identified as the product of lipid peroxidation that reacts with TBA to give pink species absorbing at 532 nm.

Assay Principle

TBARS Assay is a tool for the direct quantitative measurement of MDA in biological samples. The unknown MDA containing samples are first reacted with TBA at 95°C and at low pH. After a brief incubation, the samples and standards can be read either spectrophotometrically or fluorometrically. The MDA content in unknown samples is determined by comparison with the predetermined MDA standard curve.

Reagents:

Sodium dodecylsulphate (SDS) 8.1 % in distilled water.
Acetic acid 20%. Its pH was adjusted to 3.5 with 1N sodium hydroxide.
- Thiobarbituric acid (TBA) 0.8 % in distilled water.
- n Butanol.
- Tetramethoxy propane (TMP) (Aldrich Chemical Co., Milwauke, Wisconsen, USA) as MDA standard (1,2,4,6,8,12 nmol/ml) prepared fresh in ethanol

Procedure:

An aliquot of 0.1ml of the sample was pipetted into a tube containing an equal volume of SDS solution. This was followed by the addition of 0.75ml acetic acid, 0.75ml, of TBA and 0.3ml of distilled water. The contents of the tubes were then mixed with a vortex. The tubes were incubated in a boiling water bath for 1 hour then cooled to room temperature. An aliquot of 0.5ml of distilled water was added to each tube followed by the addition of 2.5ml n butanol. The contents of the tubes were vigorously mixed with a vortex then rotated in a centrifuge at 2500xg for 10 minutes. Absorbance of the organic layer was read at 532nm in a spectronic 21 spectrophotometer against a blank prepared and treated exactly like the sample however, containing phosphate buffer solution instead of the sample.

The concentration (nmol/ml) of MDA in sample was obtained from a standard curve (Figure 15) made by preparing serial dilutions of tetramethoxypropane (TMP), 1,2,4,6,8,12 nmol/ml, in ethanol and treating them like the sample.

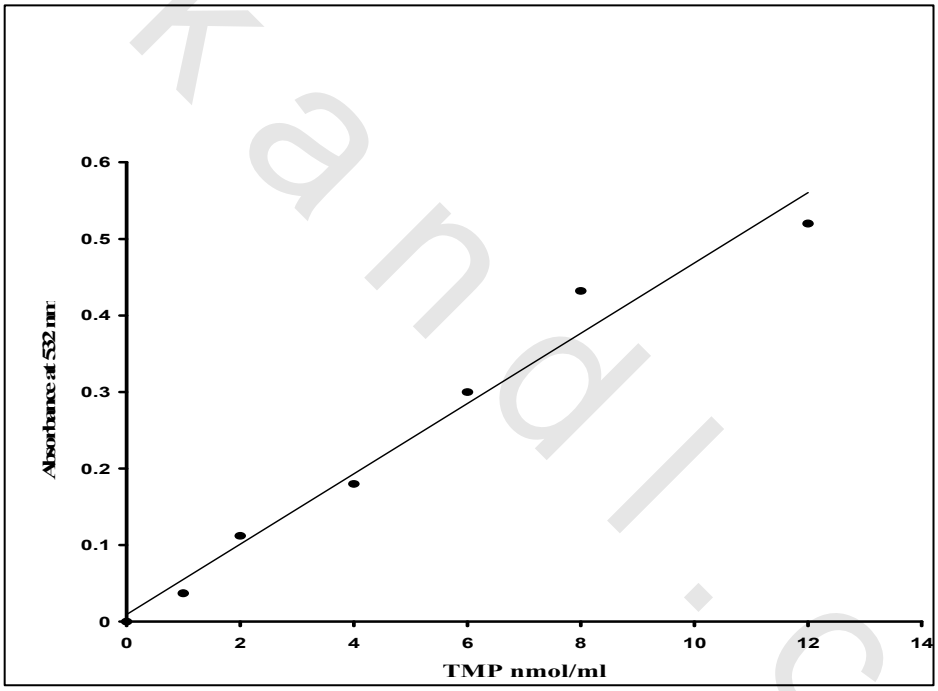

Figure (15): Stander curve of MDA

Genotyping of Hp Polymorphism by PCR

Venous EDTA blood samples are obtained from the study population and the nuclear DNA was isolated from blood using Fermentas whole blood gemonic DNA isolation kit (Fermentas, EU) according to the manufacturer instructions.

Genotyping of Haptoglobin was assayed by polymerase chain reaction (PCR) based DNA amplification of a 1757-bp Hp 1 allele-specific sequence and a 3481-bp Hp 2 allele-specific sequence using four primers set:

Primer A: 5-GAGGGGAGCTTGCCTTTCCATTG-3
Primer B: 5-GAGATTTTTGAGCCCTGGCTGGT-3
Primer C: 5-CCTGCCTCGTATTAACTGCACCAT-3
Primer D: 5-CCGAGTGCTCCACATAGCCATGT-3

Two sets of PCR reaction were performed

1- Using primer A and B for amplification of a 1757-bp Hp 1 allele-specific sequence and a 3481-bp Hp 2 allele-specific sequence (Annealing temp 63 C, 30 cycles).
2- Using primer C and D for amplification of 349-bp specific for Hp 2 allele (Annealing temp 61 C, 30 cycles) (Performed only for unconfirmed Hp 2-1 individuals).
3- After electrophoresis of the reaction products in 1 % agarose gel, Hp genotyping-specific banding patterns were obtained. With primers A and B, Hp 1-1 and Hp 2-2 genotypes were characterized by single bands representing the 1757 and 3481 bp products, respectively and Hp 2-1 genotype was characterized by the presence of both the 1757 and 3481 bp products. Primers C and D were used for detecting the 349 bp Hp-2 allele specific product.

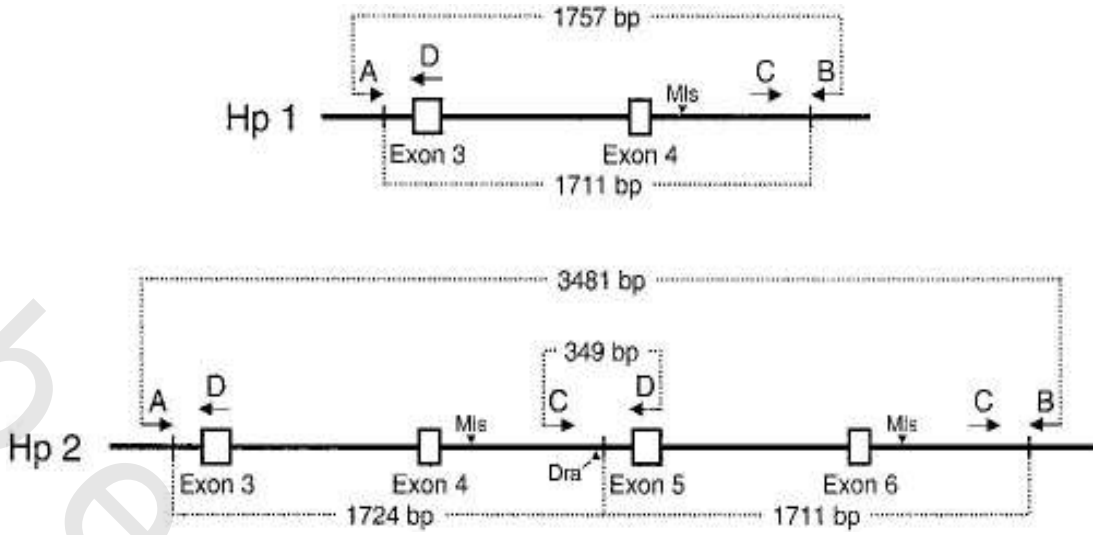

Figure 16. Partial structure of haptoglobin alleles Hp 1 and Hp 2.[221]

Hp 1 is represented by subtype Hp 1S, as indicated by the presence of the 1711-bp element. Hp 2 is represented by subtype Hp 2FS, as shown by the presence of the 1724-bp element followed by the 1711-bp element. The arrows, representing oligonucleotide primers A, B, C, and D, are located at positions next to the binding sites of the primers within the DNA sequence. The directions of the arrows indicate the 5→3orientation of the primers relative to the template DNA.

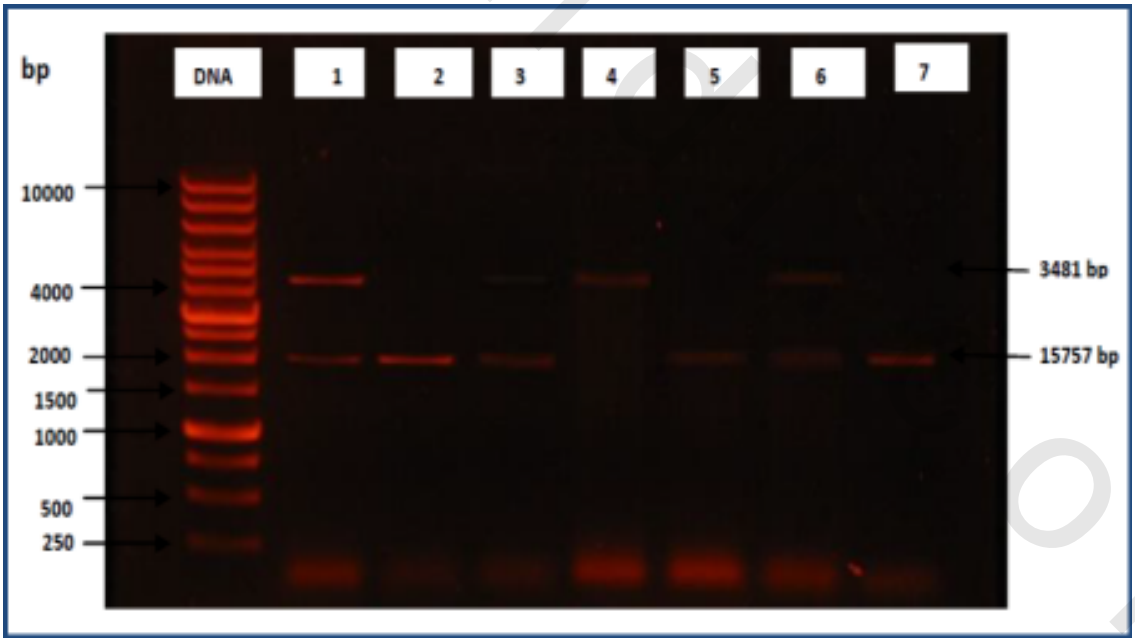

Figure17. Agarose gel electrophoresis of genotyping of Haptoglobin.

M lane is 1Kbp DNA ruler. Lane 2, 5 and 7 are for genotype Hp1-1, lane 4 is for genotype Hp2-2 and lane 1, 3 and 6 are for genotype Hp2-1.

Statistical analysis of the data (222-223)

Was done using IBM *SPSS software package version 20.0.* 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 reveals normal data distribution, parametric tests was applied. If the data were abnormally distributed, non-parametric tests were used. *For normally distributed data, comparison between two independent population were done using independent t-test. For abnormally distributed data, comparison between two independent population were 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

Demographic data: (Table 1)

This study included fifty βT patients. Their age ranged from 12.0-20.0 year with mean age of 16.38±2.65.The patients were 22 males (44%) and 28 females (56%). Twenty five healthy control group included, 10 males (40%) and 15 females(60%).their age ranging from 13 to 20 year with mean age of 16.40 ± 2.60. No significant differences related to age or gender was observed between patients and control groups.

A positive family history was found in 12 patients (24%) and positive consanguinity between parents was found in 16 patients (32%). As regards frequency of blood transfusion, 26 patients (52%) had regular blood transfusion every 2 weeks, 8 patients (16%) had transfusion every 3 weeks and 16 patients (32%) had less frequent blood transfusion every 4 weeks. The age at which first blood transfusion was given ranged from 6 to 36 months with a mean of 12.90±7.58 months.

All studied thalassemic patients in the present work were receiving DFO chelation therapy regularly, 16 of them (32%) were receiving it by subcutaneous infusion using electronic infusion pump over 8 to 12 hours ,3 to 5 days per week. 22 patients (44%) were receiving it by intramuscular route and 12 patients (24%) by intravenous route . Local reaction in the form of rash at site of injections was reported in 5 patients (10%), no anaphylaxis or visual disturbance were reported.

Table (1): Comparison between the two studied groups according to demographic data

Item	Cases (n = 50)		Control (n = 25)		Test of sig.	P
	No.	%	No.	%		
Sex						
Male	22	44.0	10	40.0	$\chi^2 = 0.109$	0.741
Female	28	56.0	15	60.0		
Age (years)						
Min. – Max	12.0-20.0		13.0 - 20.0			
Mean ± SD.	16.38±2.65		16.40 ± 2.60		Z = 0.051	0.959
Median	17.0		16.0			
Age of first blood transfusion (months)						
Min. – Max	6.0-36.0					
Mean ± SD.	12.90±7.58		-		-	-
Median	12.0					
Family history of thalassemia						
Negative	38	76.0	-	-	-	-
Positive	12	24.0	-	-		
Consanguinity						
Negative	34	68.0	-	-	-	-
Positive	16	32.0	-	-		
Frequency of blood transfusion (weeks)						
2weeks	26	52.0	-	-		
3weeks	8	16.0	-	-	-	-
4weeks	16	32.0	-	-		
Route of chelation therapy (DFO)						
Subcutaneous	16	32.0	-	-		
Intramuscular	22	44.0	-	-	-	-
Intravenous	12	24.0	-	-		

p: p value for comparing between the two studied groups
χ^2: Chi square test
Z: Z for Mann Whitney test

Anthropometric measurements: (Table 2),(figures 18-20)

The mean weight and BMI of thalassemic patients (Mean ± SD 45.06 ± 11.4 kg and 17.993± 3.07 kg/m² respectively) are significantly lower in patients than those in control group (Mean ± SD 55.52± 12.08 kg and 21.30 ± 2.49 kg/m² respectively) (P 0.001). Although mean height in thalassemic patients is lower than the control (Mean ± SD.156.98±8.88 and 160.12±10.70) it doesn't reach the significant level (P 0.182).

Table (2): Comparison between the two studied groups according to anthropometrics.

Item	Cases (n = 50)	Control (n = 25)	T	P
Weight (kg)				
Min. – Max	28.0-68.0	35.0-80.0		
Mean ± SD.	45.06±11.40	55.52±12.08	3.673*	<0.001*
Median	45.50	54.0		
Height (cm)				
Min. – Max	137.0-173.0	140.0-180.0		
Mean ± SD.	156.98±8.88	160.12±10.70	1.347	0.182
Median	159.50	162.0		
BMI (kg/m^2)				
Min. – Max	13.10-24.80	16.0-26.0		
Mean ± SD.	17.99±3.07	21.30±2.49	4.682*	<0.001*
Median	17.65	21.50		

t: Student t-test

*: Statistically significant at $p \leq 0.05$

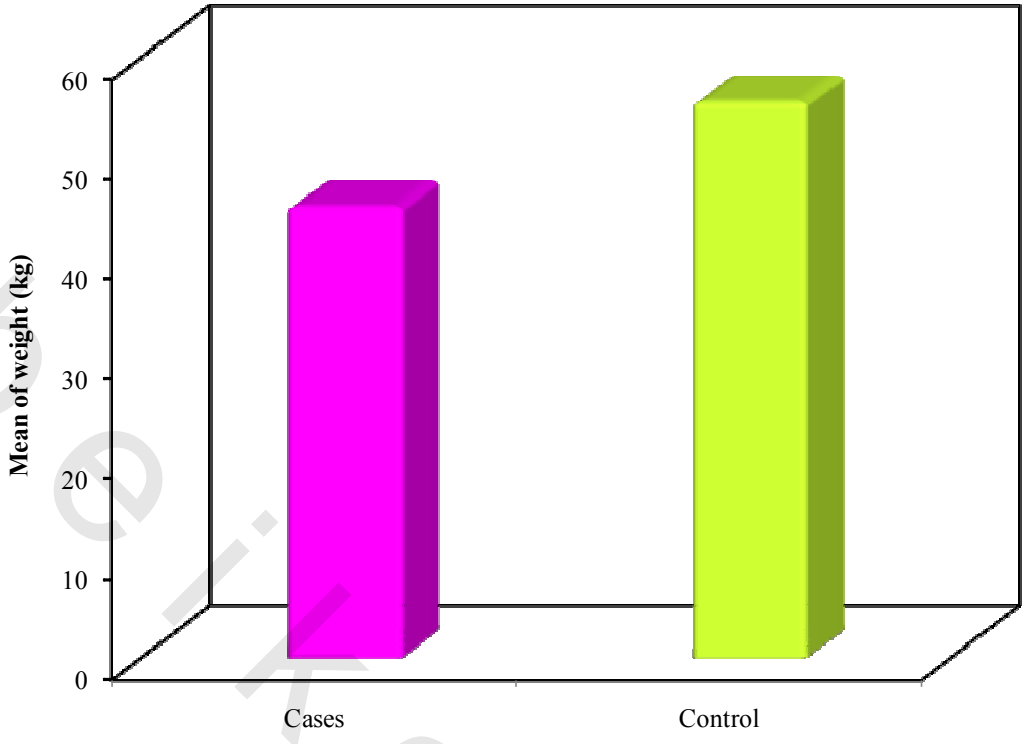

Figure (18): Comparison between the two studied groups according to weight (kg).

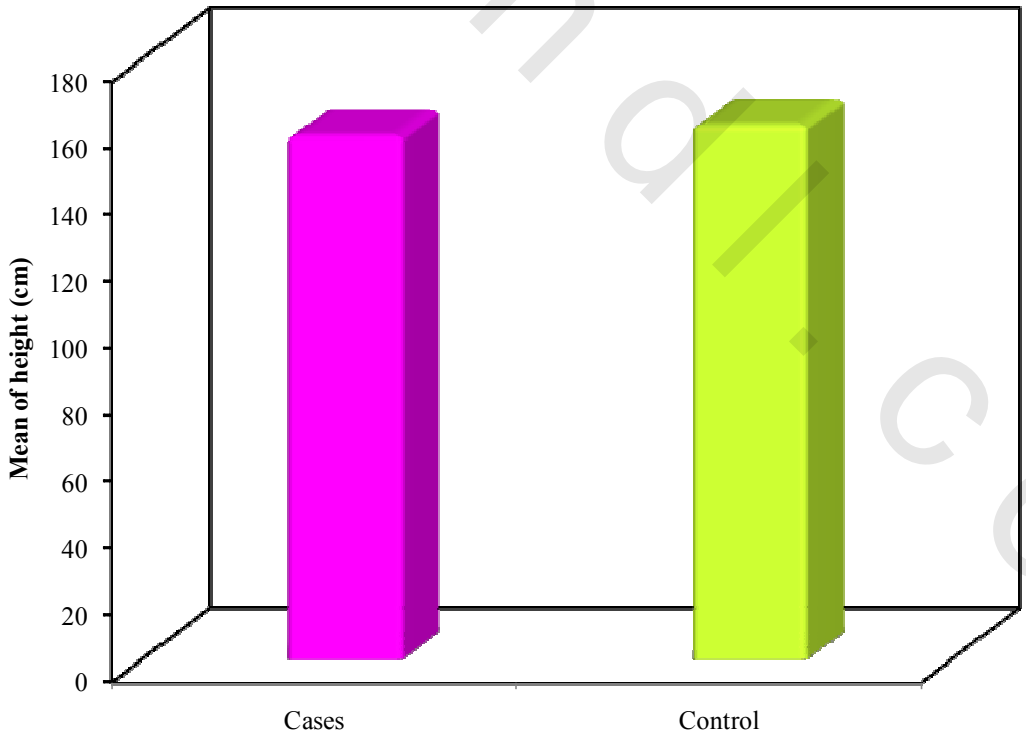

Figure (19): Comparison between the two studied groups according to height (cm)

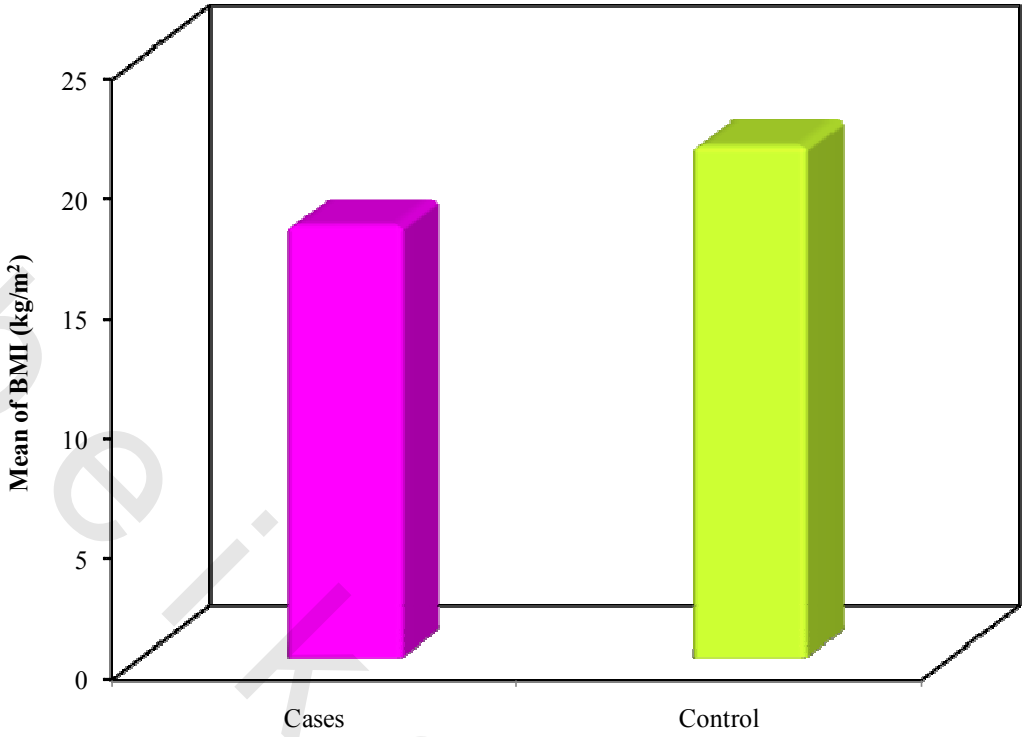

Figure (20): Comparison between the two studied groups according to BMI (kg/m2).

Clinical examination: (Table 3),(Figure 21)

On clinical examination, forty two (84%) patients had hepatomegaly, twenty two (44%) patients had undergone splenectomy, while 18 patients (36%) had splenomegaly. As regards hepatitis, sixteen patients (32%) patients had hepatitis C infection.

Table (3): Distribution of studied cases according to clinical findings.

Item	Negative		Positive	
	No.	%	No.	%
Hepatomegaly	8	16.0	42	84.0
Splenomegaly	10	20.0	18	36.0
Splenectomy	28	56.0	22	44.0
Hepatitis C	34	68.0	16	32.0

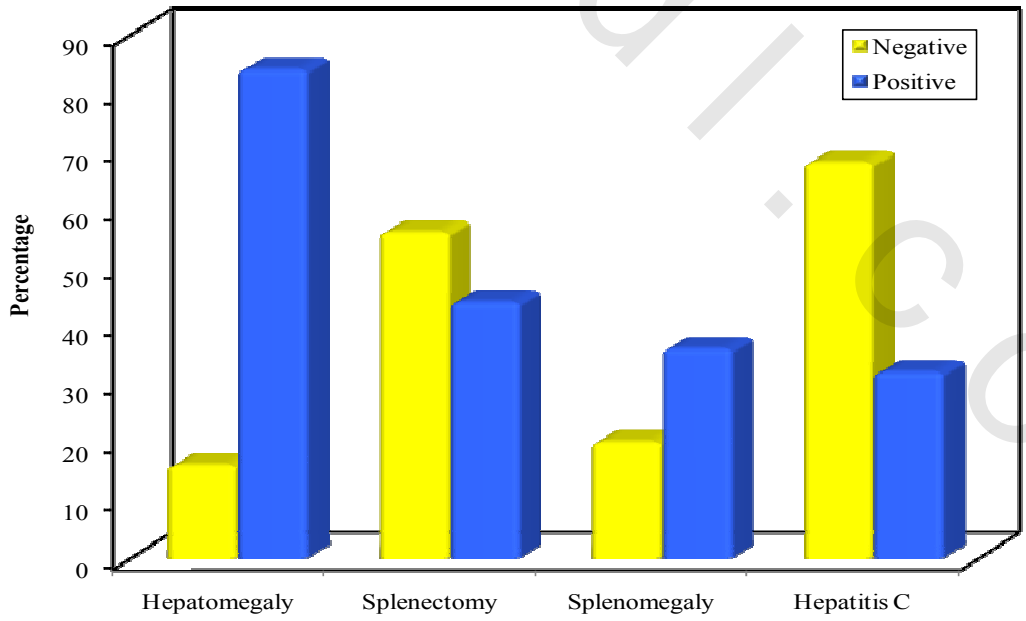

Figure (21): Distribution of studied cases according to clinical findings.

Hematological parameters : (Table 4),(Figures 22-28)

Table 4 showed the hematological data of patients compared to the controls.As regards CBC results, the mean Hb, PCV, MCV,MCH and RBCs (Mean ± SD 6.69±1.22, 21.19±3.94, 72.73±8.67, 23.12±3.18 and 2.94±0.71 respectively) are significantly lower in thalassemic group than in control group (Mean ± SD 13.18±0.89, 40.97±3.02, 82.28±4.98, 28.65±2.63and 5.03±0.38 respectively), ($P < 0.001$) .

In addition, the mean platelets and reticulocytes counts (Mean ± SD 457.28±263.80, 124.66 ± 69.43) are significantly higher in the thalassemic group than the control group (Mean ± SD 243.80±89.33, 39.44 ± 15.15), (P 0.001). While the mean MCHC and N/LR were not significantly differ between thalassemic and control groups (P 0.406 and 0.835 respectively).

Table (4): Comparison between the two studied groups according to CBC.

	Cases (n=50)	Control (n=25)	Test of sig.	P
Hb (gm/dl)				
Min. – Max	3.50-10.0	11.70-14.70		
Mean ± SD.	6.69±1.22	13.18±0.89	t = 23.615*	<0.001*
Median	6.65	13.0		
PCV (%)				
Min. – Max	9.90-30.50	35.0-45.60		
Mean ± SD.	21.19±3.94	40.97±3.02	t = 22.031*	<0.001*
Median	21.15	41.50		
MCV (fl)				
Min. – Max	53.40-88.20	75.0-100.0		
Mean ± SD.	72.73±8.67	82.28±4.98	t = 6.044*	<0.001*
Median	74.25	81.0		
MCH (pg)				
Min. – Max	16.30-27.70	20.40-32.30		
Mean ± SD.	23.12±3.18	28.65±2.63	t = 7.505*	<0.001*
Median	23.95	28.60		
MCHC (gm/dl)				
Min. – Max	23.70-38.10	30.10 – 36.0		
Mean ± SD.	31.93±2.75	32.34 ± 1.57	t = 0.835	0.406
Median	32.70	32.0		
RBC_S ($x10^{12}$/l)				
Min. – Max	1.35-4.50	4.28-5.70		
Mean ± SD.	2.94±0.71	5.03±0.38	t = 16.650*	<0.001*
Median	2.86	5.10		
Retic count($x10^9$/l)				
Min. – Max.	54.0 – 364.0	22.0 – 77.0		
Mean ± SD.	124.66 ± 69.43	39.44 ± 15.15	Z=6.812*	<0.001*
Median	98.0	43.0		
WBCs ($x10^9$/l)				
Min. – Max	3.84 – 9.73	4.0-9.80		
Mean ± SD.	6.64 ± 1.65	6.83±1.64	Z = 0.326	0.744
Median	6.55	7.10		
NLR				
Min. – Max.	0.48 – 3.20	0.70 – 2.10		
Mean ± SD.	1.52 ± 0.66	1.49 ± 0.39	t=0.210	0.835
Median	1.42	1.60		
Plts ($x10^9$/l)				
Min. – Max	133.0-1056.0	131.0-455.0		
Mean ± SD.	457.28±263.80	243.80±89.33	Z = 3.962*	<0.001*
Median	354.50	225.0		

p: p value for comparing between the two studied groups
t: Student t-test Z: Z for Mann Whitney test
*: Statistically significant at $p \leq 0.0$

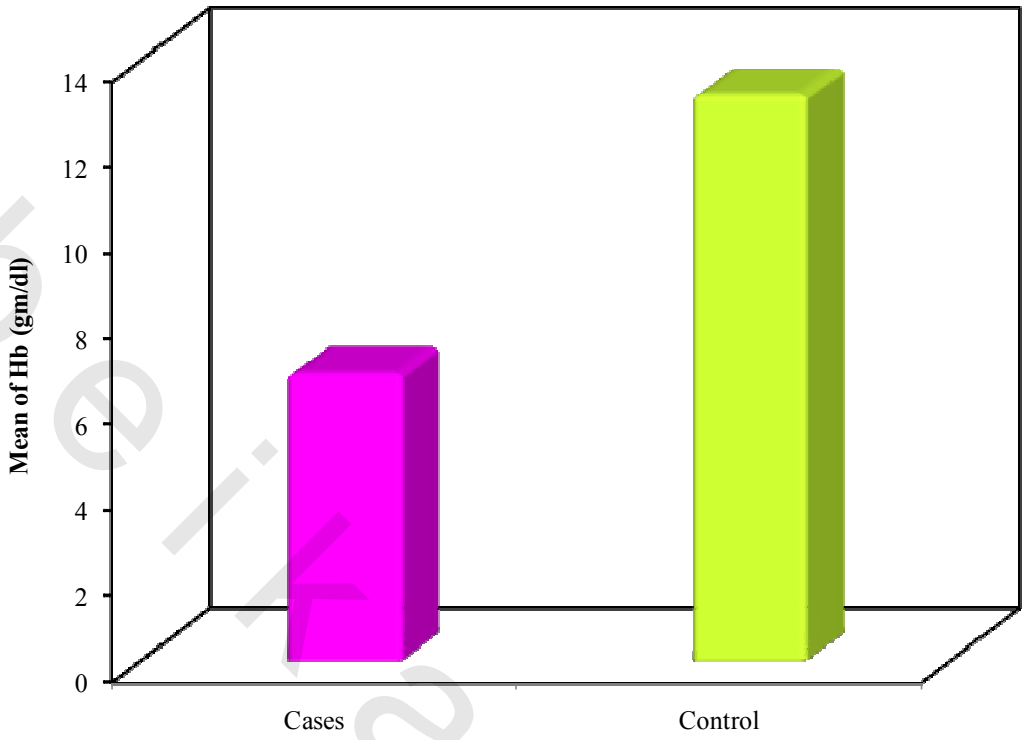

Figure (22):Comparison between the two studied groups according to Hb(gm/dl).

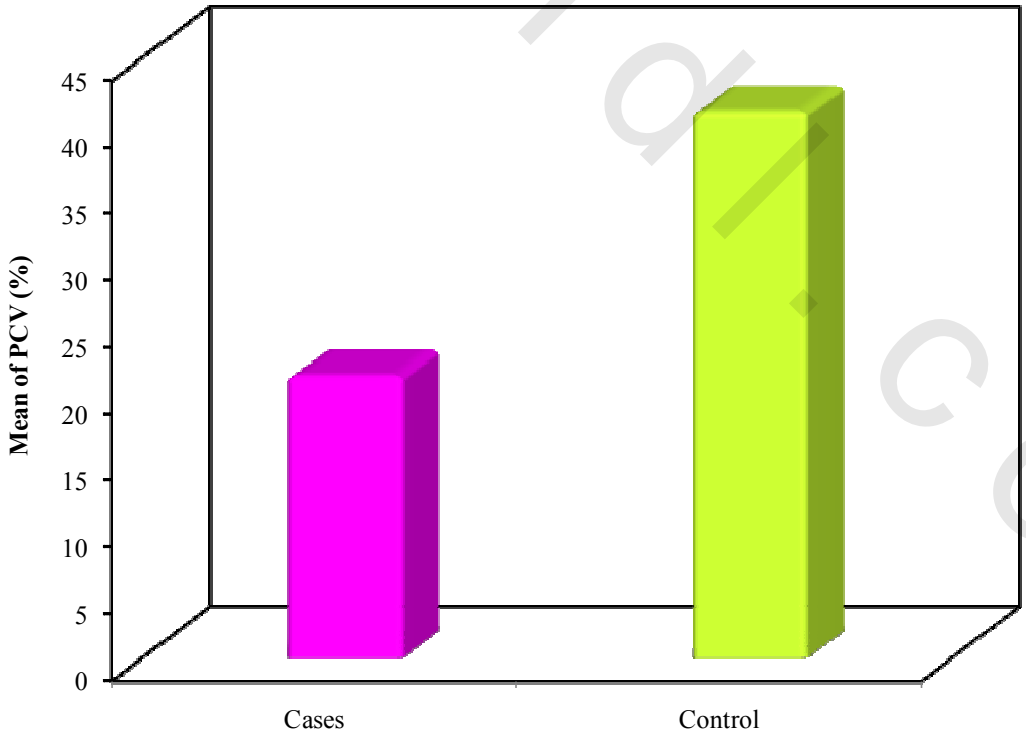

Figure (23):Comparison between the two studied groups according to PCV (%).

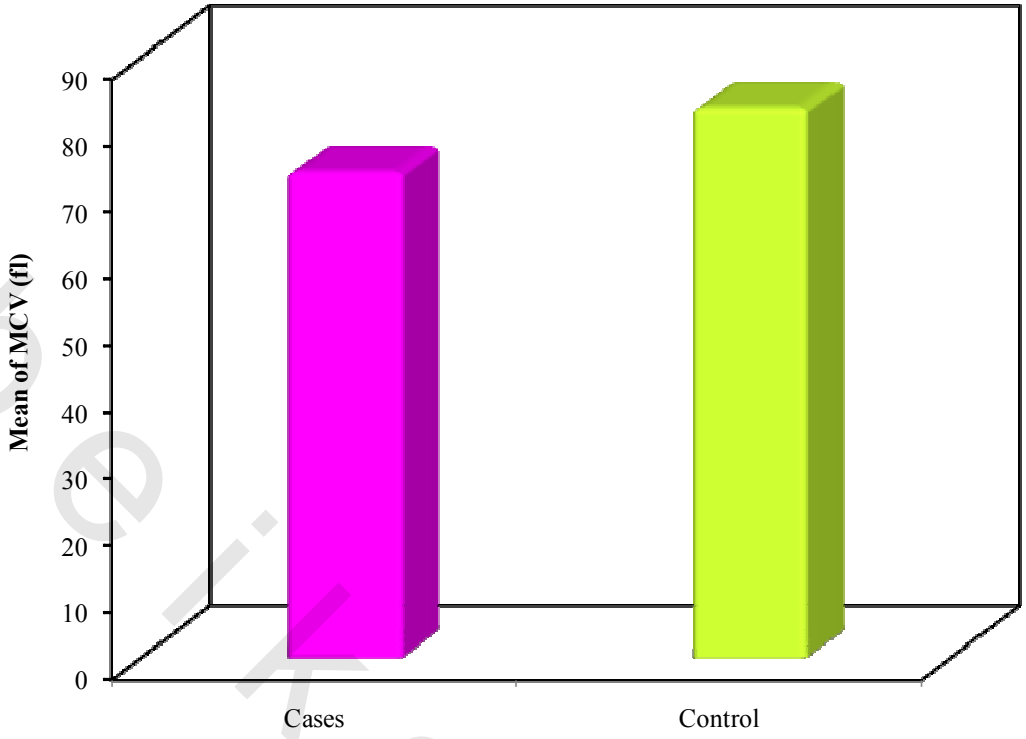

Figure (24):Comparison between the two studied groups according to MCV (fl).

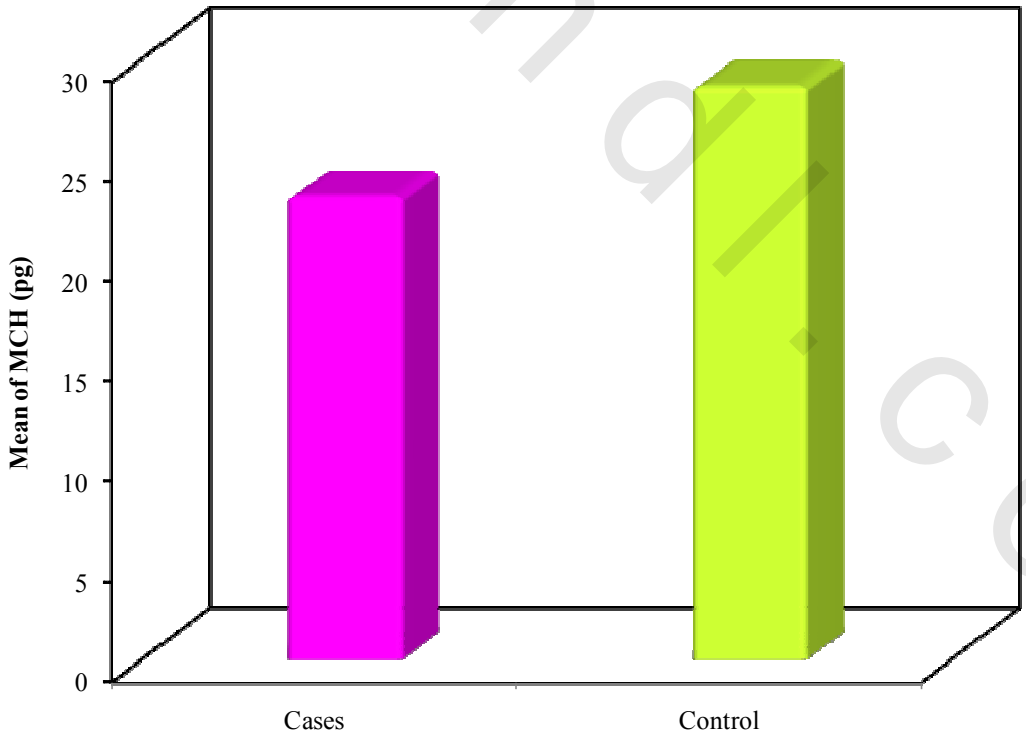

Figure (25):Comparison between the two studied groups according to MCH (pg).

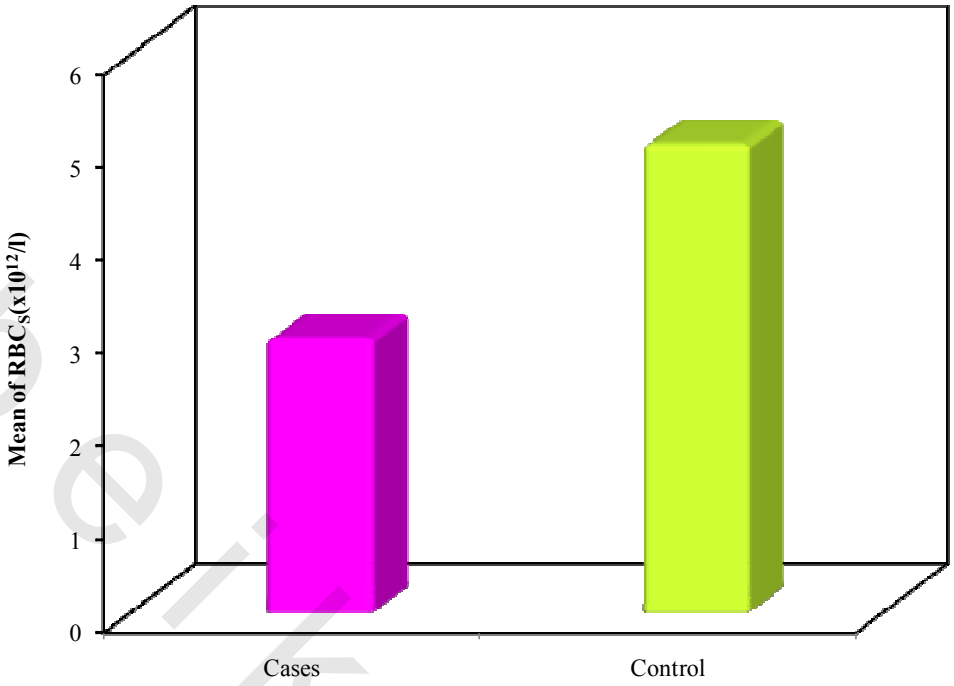

Figure (26): Comparison between the two studied groups according to RBCS ($x10^{12}$/l).

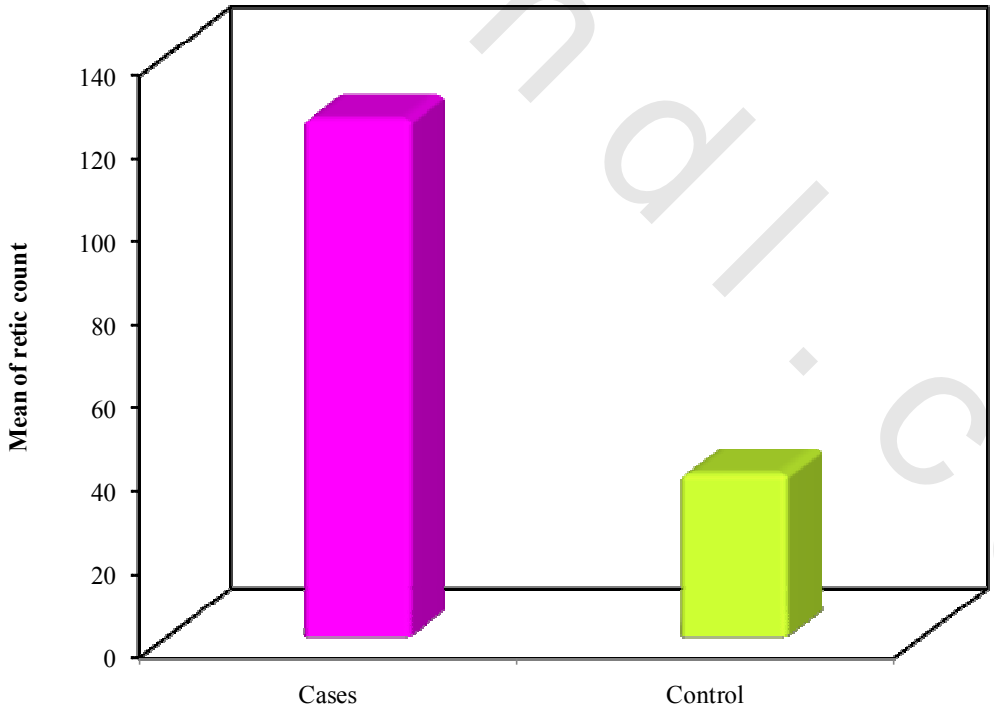

Figure (27): Comparison between the two studied groups according to retic count ($x10^{9}$/l) .

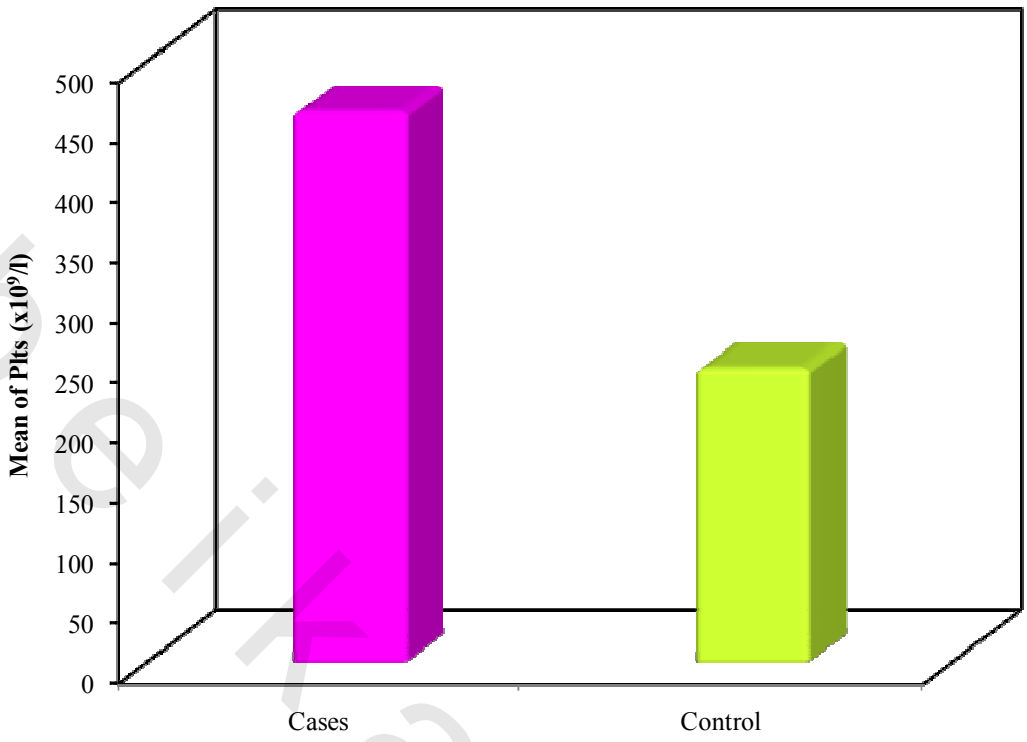

Figure (28): Comparison between the two studied groups according to Platelets ($x10^9$/l).

Red Blood Cells (RBCs) morphology in stained film: (Figure 29)

The RBCs showed hypochromia, microcytosis, anisocytosis, poikylocytosis, basophilic stippling, pappenheimer bodies, target cells and circulating erythroblasts were found in some cases.

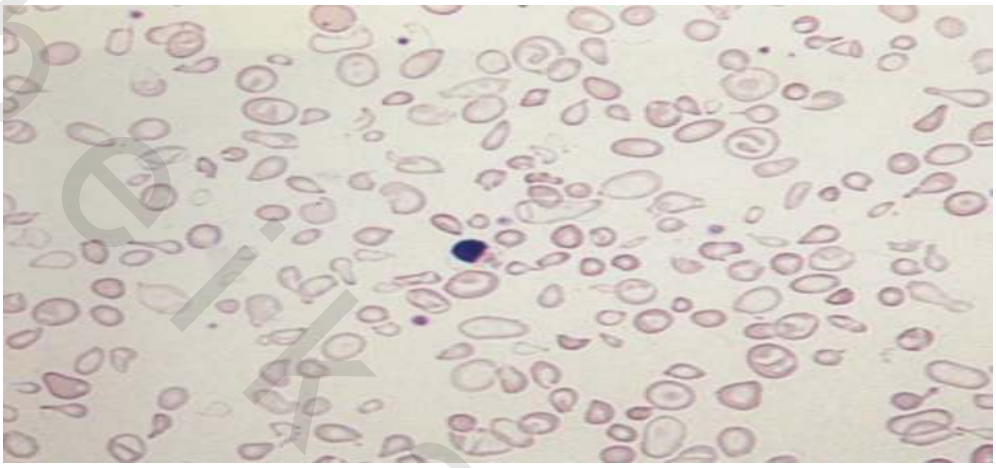

Figure 29: Blood film showing red blood cells morphology in thalassemia.

ABO blood group and Coombs' : (Table 5)

As regards blood group, there was no significance difference between thalassemic patients and control group. Among the thalassemic group eight patients (16%) were Coombs' positive, seven of them were DAT positive and one indirect.

Table (5): Comparison between the two studied groups according to ABO blood group and Coombs' test.

Item	Cases (n = 50)		Control (n = 25)		χ^2	P
	No.	%	No.	%		
ABO Blood groups						
A	18	36.0	8	32.0	0.118	0.731
B	11	22.0	6	24.0	0.038	0.845
O	16	32.0	10	40.0	0.471	0.493
AB	5	10.0	1	4.0	0.815	$^{FE}p=0.657$
Coombs' test						
Negative	42	84.0	25	100.0	4.478^*	0.046^*
Positive	8	16.0	0	0.0		

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

Hb Electrophoresis : (Table 6),(Figures 30,31)

Hb A values in thalassemic group ranged from (5.0 – 24.0 %) with a mean of (Mean ± SD 11.44 ± 4.70 %),while that of control ranged from (96.0 – 98.0%) with a mean of (Mean ± SD 96.98± 0.73%). Analysis of variance showed statistically significant difference between cases and controls (P=<0.001).

HbF in thalassemic group ranged from (73.0 – 94.0%) with a mean of (Mean ± SD 85.92 ± 5.62%). In normal control it ranged from (0.50 – 1.0%) with a mean of (Mean ± SD 0.82 ± 0.24%) which is of significant difference (P=<0.001).

HbA2 values in thalassemic group ranged from (1.0 – 6.0%) with mean of (Mean ± SD 2.64 ± 1.48%) while in control it ranged from (1.0 – 3.0%) with a mean of Mean ± SD (2.12 ± 0.67%) which did not reach statistical significant (P=0.348) .

Table (6):Comparison between the studied groups according to Hb electrophoresis.

Item	Cases (n=50)	Control (n=25)	Z	P
HbA %				
Min. – Max.	5.0 – 24.0	96.0 – 98.0		
Mean ± SD.	11.44 ± 4.70	96.98 ± 0.73	7.042*	<0.001*
Median	11.0	97.0		
HbF %				
Min. – Max.	73.0 – 94.0	0.50 – 1.0		
Mean ± SD.	85.92 ± 5.62	0.82 ± 0.24	7.071*	<0.001*
Median	86.0	1.0		
HbA2%				
Min. – Max.	1.0 – 6.0	1.0 – 3.0		
Mean ± SD.	2.64 ± 1.48	2.12 ± 0.67	0.939	0.348
Median	2.0	2.0		

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

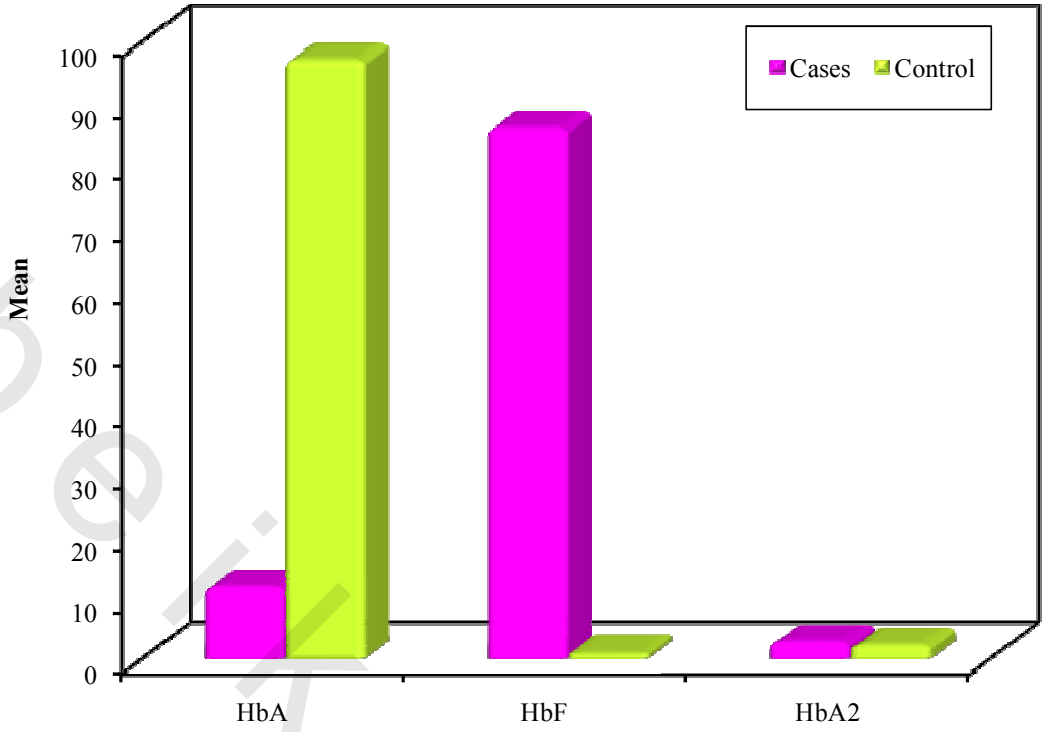

Figure (30): Comparison between the studied groups according to Hb electrophoresis

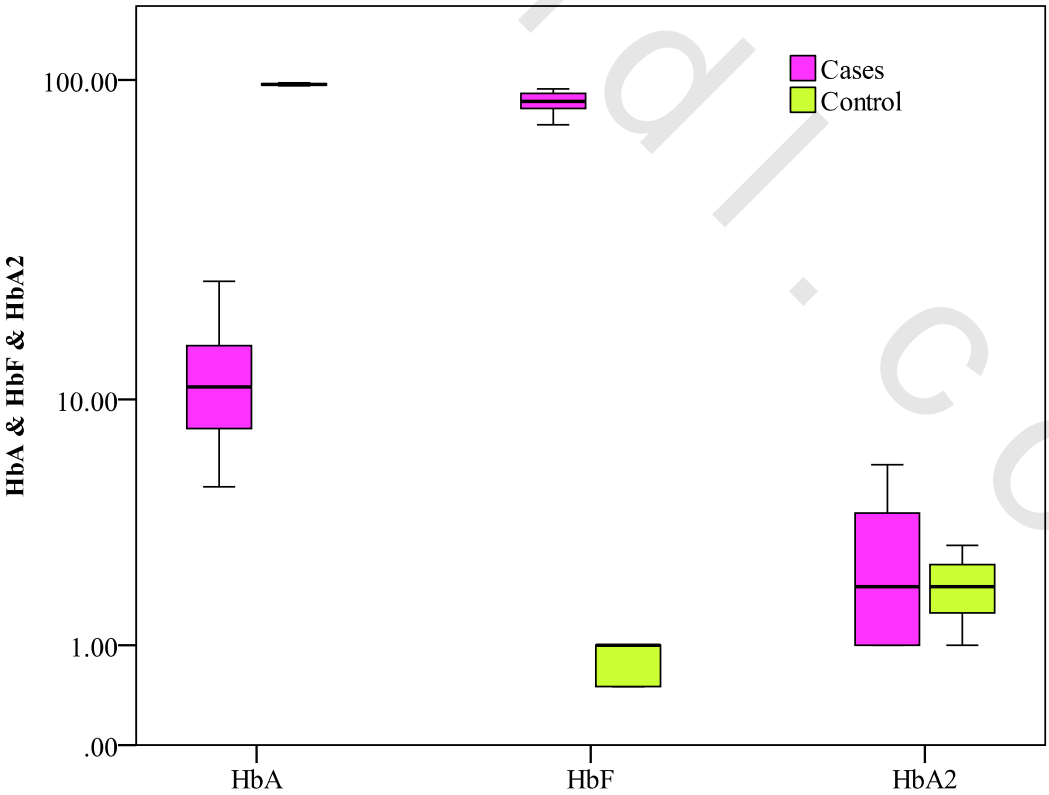

Figure (31): Comparison between the studied groups according to Hb electrophoresis.

Iron profile : (Table 7),(Figure 32-34)

As regards iron profile, the results revealed that the mean serum iron was significantly higher in patients with thalassemia than the control (Mean ± SD 42.30±9.81 and 15.53±2.83 µmol/l respectively), (P 0.001)

As regards TS, it was significantly higher in thalassemic group than control with a mean of (Mean ± SD 106.66±33.28 and 30.88±7.42 % respectively).While TIBC was significantly higher in the control group than in thalassemic patients with a mean of (Mean ± SD 51.34±5.11 and 41.72±7.87 µmol/l),(P 0.001) .

The mean serum ferritin level was significantly higher in patients with thalassemia than the controls (Mean ± SD 3287.22±2180.96 and 95.04±46.40 µg/l), (P 0.001).

Table (7):Comparison between the two studied groups according to iron profile.

Item	Cases (n = 50)	Control (n = 25)	Test of sig.	P
Serum iron (µmol/l)				
Min. – Max	22.40-64.40	10.70-23.30		
Mean ± SD.	42.30±9.81	15.53±2.83	t = 17.868*	<0.001*
Median	41.20	15.0		
TIBC(µmol/l)				
Min. – Max	33.50-64.40	42.90-62.80		
Mean ± SD.	41.72±7.87	51.34±5.11	Z = 4.856*	<0.001*
Median	38.75	50.50		
TS (%)				
Min. – Max	38.0-160.0	17.0-47.0		
Mean ± SD.	106.66±33.28	30.88±7.42	t = 15.355*	<0.001*
Median	112.0	30.0		
Serum ferritin (µg/l)				
Min. – Max	890.0-9830.0	30.0-210.0		
Mean ± SD.	3287.22±2180.96	95.04±46.40	Z = 7.025	<0.001*
Median	2705.0	82.0		

p: p value for comparing between the two studied groups
t: Student t-test
Z: Z for Mann Whitney test
*: Statistically significant at $p \leq 0.05$

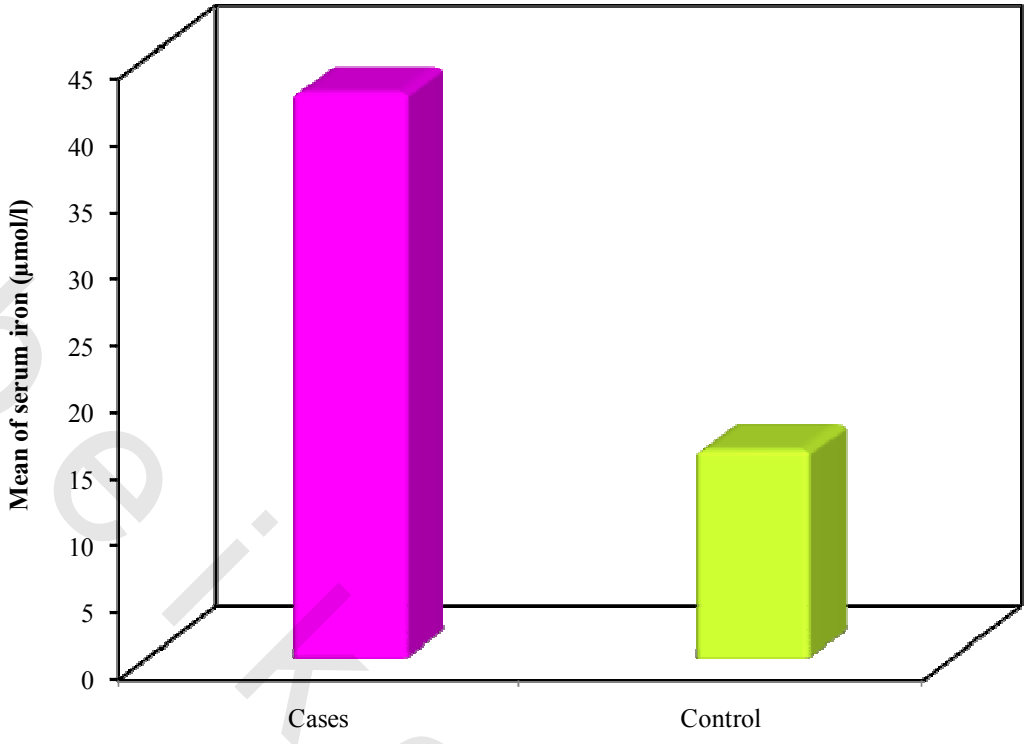

Figure (32): Comparison between the two studied groups according to serum iron (µmol/l).

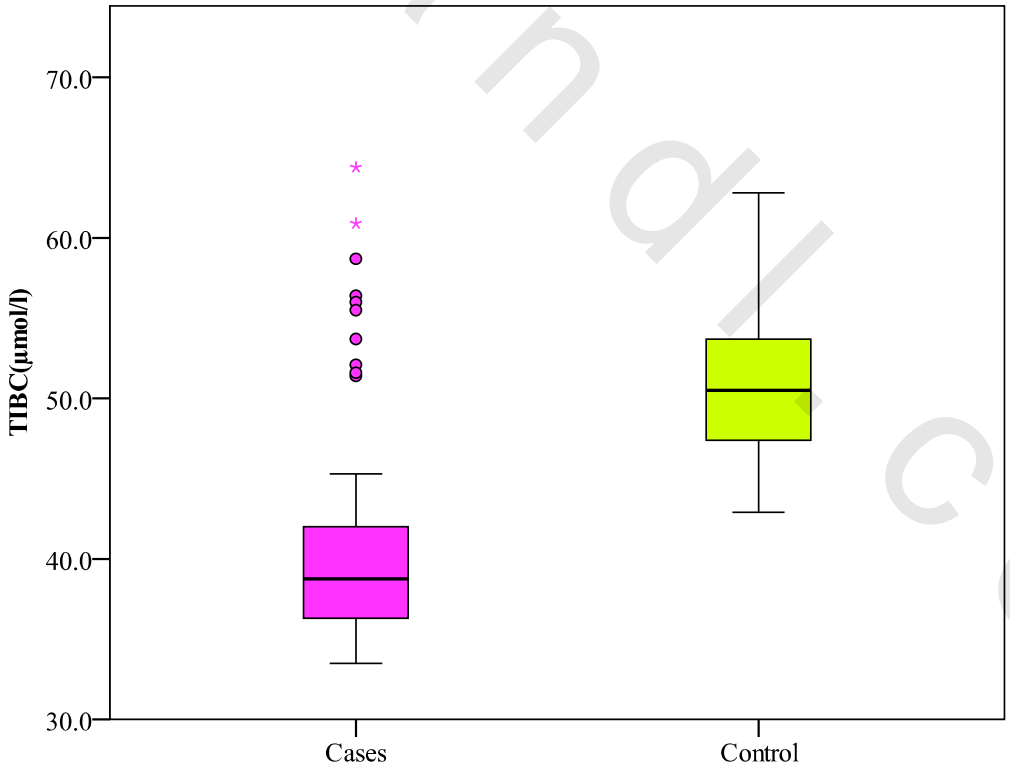

Figure (33): Comparison between the two studied groups according to TIBC (µmol/l).

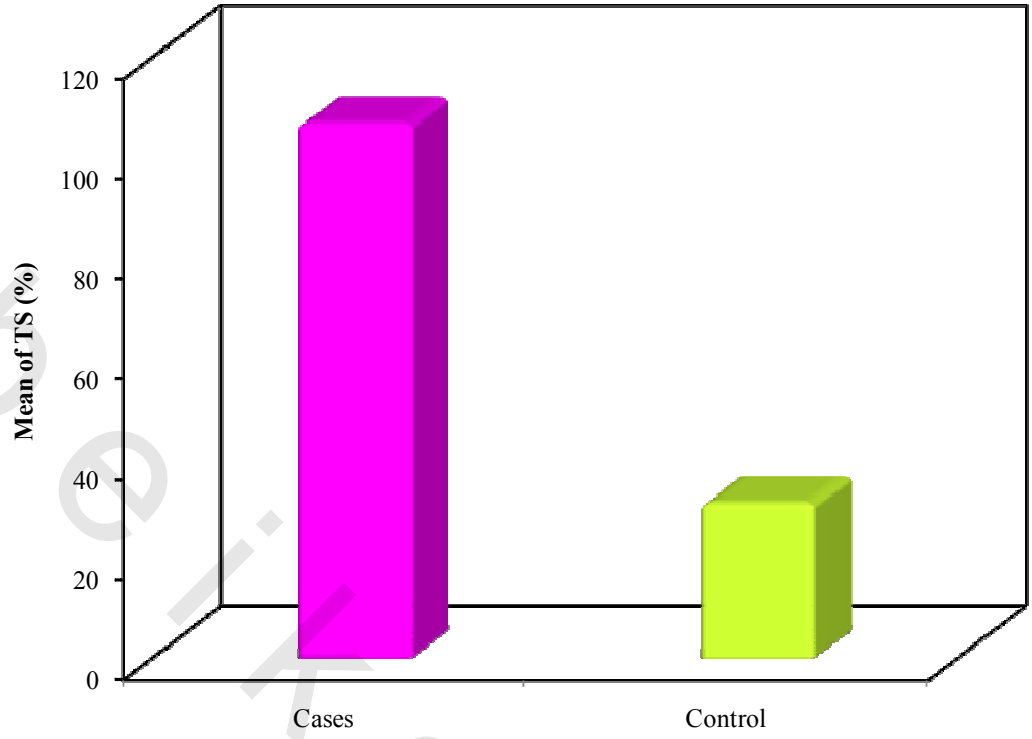

Figure (34): Comparison between the two studied groups according to TS (%).

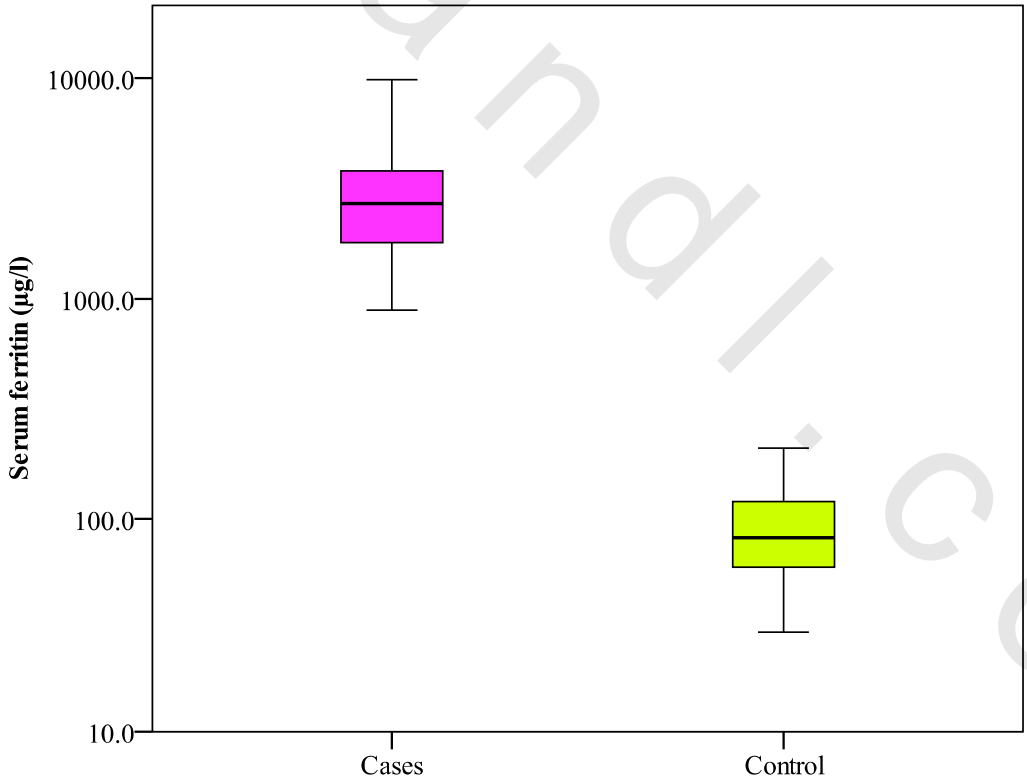

Figure (35): Comparison between the two studied groups according to serum ferritin (µg/l).

Serum ferritin levels in thalassemic patients : (Table 8)

Only two patients (4%) had serum ferritin levels of less than 1000µg/l, twenty patients (40%) had serum ferritin levels between 1000 – 2500, µg/l. While twenty eight patients (56%) had values more than 2500 µg/l.

Table (8) Serum ferritin levels in thalassemic patients.

Serum Ferritin levels in Patients with Beta Thalassemia Major		
Levels	**Number of patients**	
< 1000 µg/l	2	4%
1000 – 2500 µg/l	20	40%
>2500 µg/l	28	56%

Laboratory findings: (Table 9), (Figures 36-39)

The mean C-reactive protein was significantly higher (Mean ± SD 3.39±0.83 mg/l) in thalassemic group than the control group (Mean ± SD 2.47±0.83 mg/l) although all cases got levels in the normal range (P 0.001).

As regards liver function, serum ALT was significantly higher in thalassemic group, compared to the controls (Mean ± SD 52.24±19.16 and 14.32±4.78 u/l respectively) (P 0.001).

The results of the present study showed that the mean serum haptoglobin level was significantly lower in patients with BT than in the controls (Mean ± SD 21.74±23.53 and 96.84±30.19 mg/dl respectively), (P <0.001).

Moreover, the mean serum MDA level was significantly higher in thalassemic group compared with the control group (Mean ± SD 3.47±1.90 and 0.90±0.28 nmol/l respectively),(P <0.001).

Table (9): Comparison between the two studied groups according to laboratory profile.

Item	Cases (n = 50)	Control (n = 25)	Test of sig.	P
CRP (mg/l)				
Min. – Max	1.90-4.80	1.2-4.30		
Mean ± SD.	3.39±0.83	2.47±0.83	t = 4.507*	<0.001*
Median	3.45	2.50		
ALT (u/l)				
Min. – Max	26.0-120.0	8.0-25.0		
Mean ± SD.	52.24±19.16	14.32±4.78	Z = 7.029*	<0.001*
Median	47.50	14.0		
Serum haptoglobin (mg/dl)				
Min. – Max	1.90-99.0	48.50-158.0		
Mean ± SD.	21.74±23.53	96.84±30.19	Z = 6.592	<0.001*
Median	10.20	90.0		
Serum MDA (nmol/l)				
Min. – Max	1.10-9.10	0.20-1.30		
Mean ± SD.	3.47±1.90	0.90±0.28	Z = 6.679	<0.001*
Median	3.25	0.80		

p: p value for comparing between the two studied groups
t: Student t-test
Z: Z for Mann Whitney test
*: Statistically significant at $p \leq 0.05$

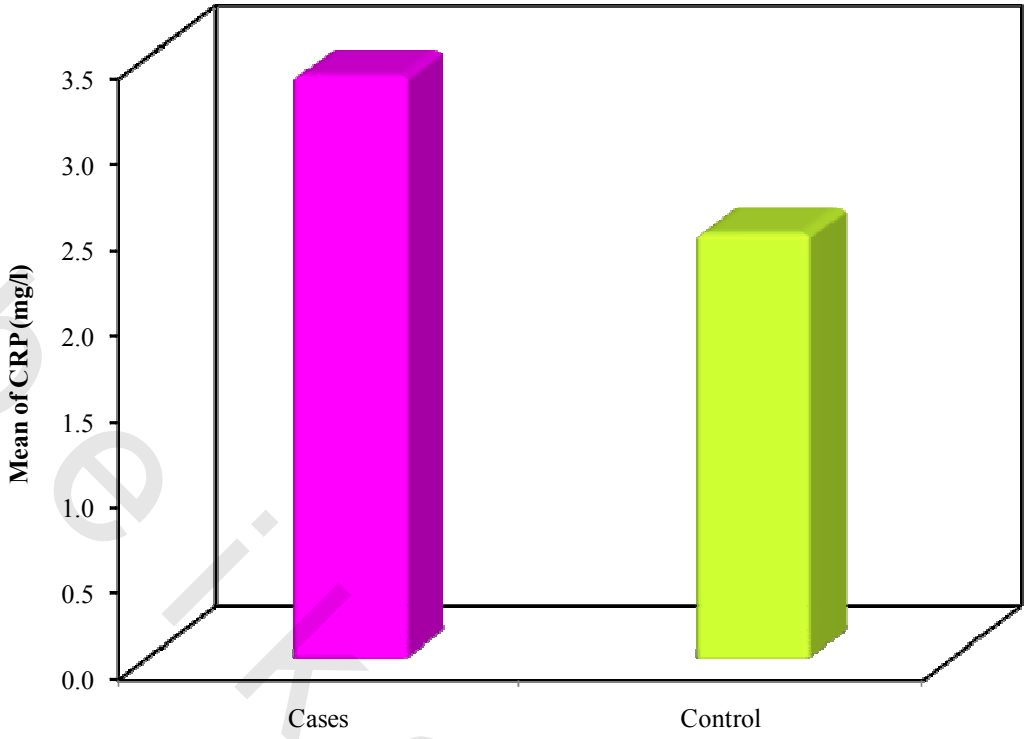

Figure (36): Comparison between the two studied groups according to CRP (mg/l).

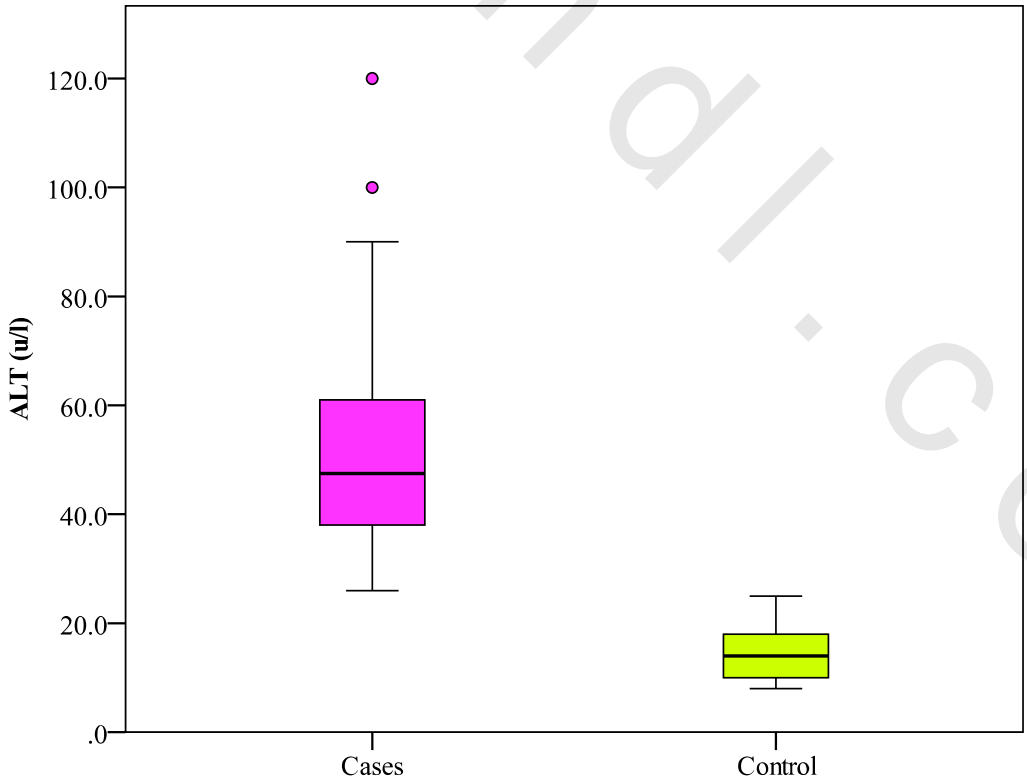

Figure (37): Comparison between the two studied groups according to ALT (u/l)

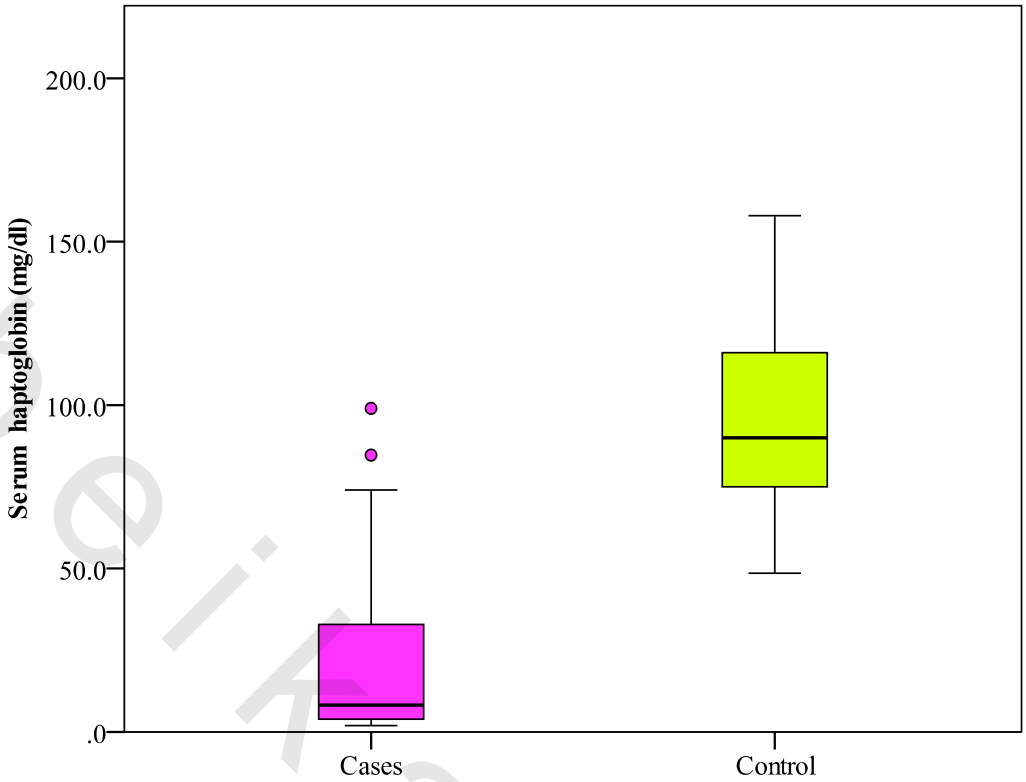

Figure (38): Comparison between the two studied groups according to serum haptoglobin (mg/dl).

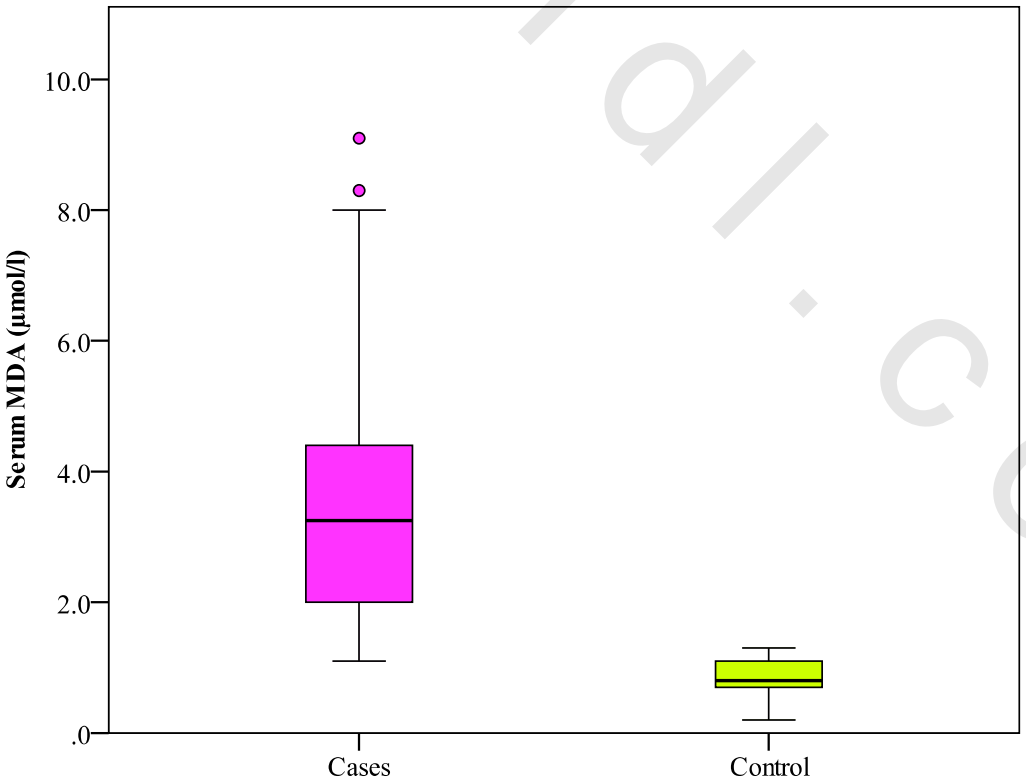

Figure (39): Comparison between the two studied groups according to serum MDA (nmol/l).

Haptoglobin polymorphism:(Table 10),(Figures 40 and 41)

The data in table 10 showed no significant differences between thalassemic and control groups concerning the distribution of haptoglobin genotype.

In spite of the higher incidence of Hp 2-2 (56%) compared with 2-1 (30%) and 1-1 (14%) in thalassemic group with allele frequency (71% and 58% respectively), the control group showed more prevalence of Hp2-1(44%)followed by Hp2-2(36%) then Hp1-1(20%) with allele frequency (58% and 42%).

Table (10):Comparison between the two studied groups according to haptoglobin Polymorphism.

Item	Cases (n = 50)		Control (n = 25)		χ^2	P	OR	95%CI	
	No.	%	No.	%				LL	UL
Haptoglobin									
Hp1-1$^{(R)}$	7	14.0	5	20.0	0.446	0.519	1.000	-	-
Hp2-1	15	30.0	11	44.0	1.442	0.230	0.974	0.243	3.897
Hp2-2	28	56.0	9	36.0	2.667	0.102	2.222	0.564	8.759
Hp 0-0	0	0.0	0	0.0	-	-	-	-	
Allele frequency									
Hp1$^{(R)}$	29	29.0	21	42.0	2.535	0.111	1.000	-	-
Hp2	71	71.0	29	58.0			1.773	0.873	3.601

χ^2: Chi square test

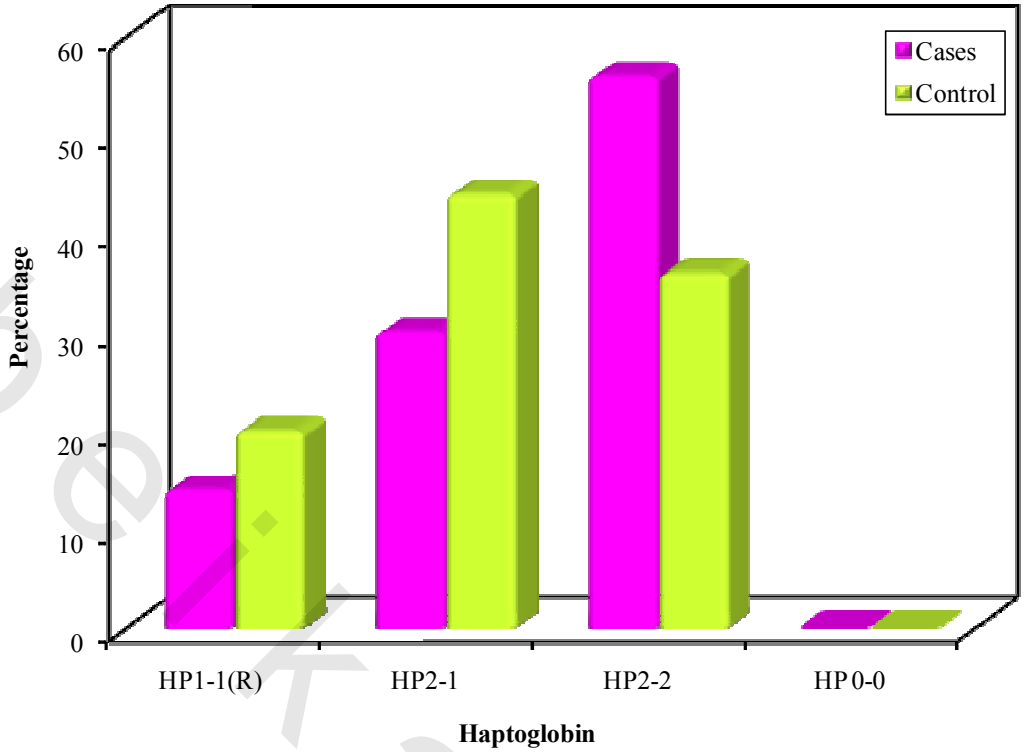

Figure (40): Comparison between the two studied groups according to haptoglobin.

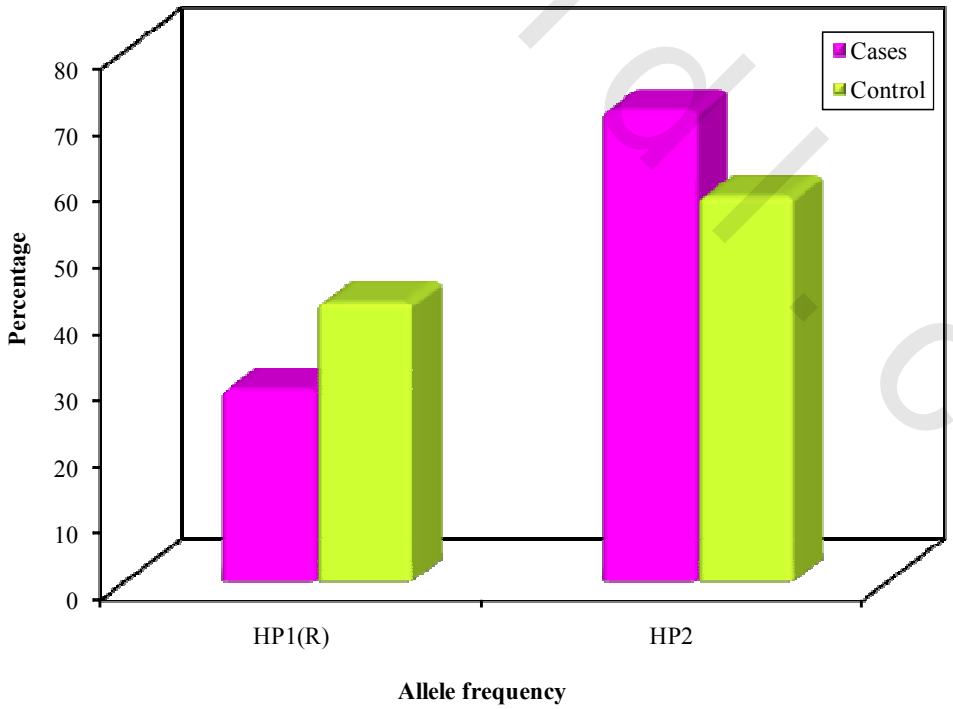

Figure (41): Comparison between the two studied groups according to allele frequency.

Table 11 showed no significant relation between haptoglobin genotype and levels of iron status in thalassemic patients. However mean serum ferritin was higher in thalassemic patients with Hp2-2genotype than in those with Hp1-1 and Hp2-1, but did not reach the significant value, (Mean ± SD 3502.8±2314.6, 3028.7±2093.5 and 3005.4±2055.7 respectively).

Table (11): Relation between haptoglobin genotype with iron profile in thalassemic group.

Item	Haptoglobin genotype			P
	Hp1-1 (n=7)	Hp2-1 (n=15)	Hp2-2 (n=28)	
Serum iron (µmol/l)				
Min. – Max	29.80 – 59.0	22.40 – 60.0	25.0 – 64.40	
Mean ± SD.	41.60 ± 11.22	40.84 ± 9.60	43.26 ± 9.82	0.736
Median	41.50	40.80	41.20	
TIBC (µmol/l)				
Min. – Max	34.50 – 60.90	33.50 – 64.40	34.40 – 56.40	
Mean ± SD.	45.70 ± 12.12	42.35 ± 8.98	40.39 ± 5.63	0.969
Median	37.60	38.70	39.70	
TS (%)				
Min. – Max	50.0 – 157.0	38.0 – 154.0	48.0 – 160.0	
Mean ± SD.	102.0 ± 45.03	102.3 ± 34.2	110.1 ± 30.4	0.714
Median	115.0	108.0	114.50	
Serum ferritin (µg/l)				
Min. – Max	1200 – 7000	900 – 8400	890 – 9830	
Mean ± SD.	3028.7±2093.5	3005.4±2055.7	3502.8±2314.6	0.608
Median	2400.0	2600.0	3091.5	

p: p value for F test (ANOVA) test.

Table 12- showed the relation between the mean values of serum MDA (nmol/l), Serum haptoglobin (mg/dl) among different haptoglobin genotypes in thalassemic patients. As regard MDA, it's mean serum level (Mean ± SD were 2.31 ± 1.27 for Hp1-1, 3.17 ± 1.80 for Hp2-1 and 3.92 ± 1.97 for Hp 2-2).The mean serum levels of MDA were significantly higher in patients with Hp2-2 genotype as compared to those with Hp1-1 genotype ($p \leq 0.05$). (Figure 42)

It also shows significant relation between total concentration of serum haptoglobin and the haptoglobin gene polymorphism, the mean serum haptoglobin levels were significantly lower in Hp2-2 genotype (Mean ± SD 18.65 ± 23.59, compared with that of Hp1-1 genotype (Mean ±SD 41.76 ± 30.17) ($P \leq 0.05$) . (Figure 43)

Table (12): Relation between haptoglobin genotype with serum MDA and Serum haptoglobin in thalassemic group.

Item	Haptoglobin genotype			P
	Hp1-1 (n=7)	Hp2-1 (n=15)	Hp2-2 (n=28)	
Serum MDA (nmol/l)				
Min. – Max	1.10 – 4.60	1.10 -7.60	1.10 – 9.10	
Mean ± SD.	2.31 ± 1.27	3.17 ± 1.80	3.92 ± 1.97	≤ 0.05
Median	1.90	2.70	3.45	
Sig. bet. Grps. #	1-1 with 2-2*			
Serum haptoglobin (mg/dl)				
Min. – Max	5.0 – 84.0	2.80 – 49.50	1.90 – 99.0	
Mean ± SD.	41.76 ± 30.17	18.17 ± 15.51	18.65 ± 23.59	≤ 0.05
Median	29.60	12.0	7.0	
Sig. bet. Grps. #	1-1 with 2-2*			

p: p value for Kruskal Wallis test
#: Pair-wise comparison was done using Mann Whitney test
*: Statistically significant at $p \leq 0.05$.

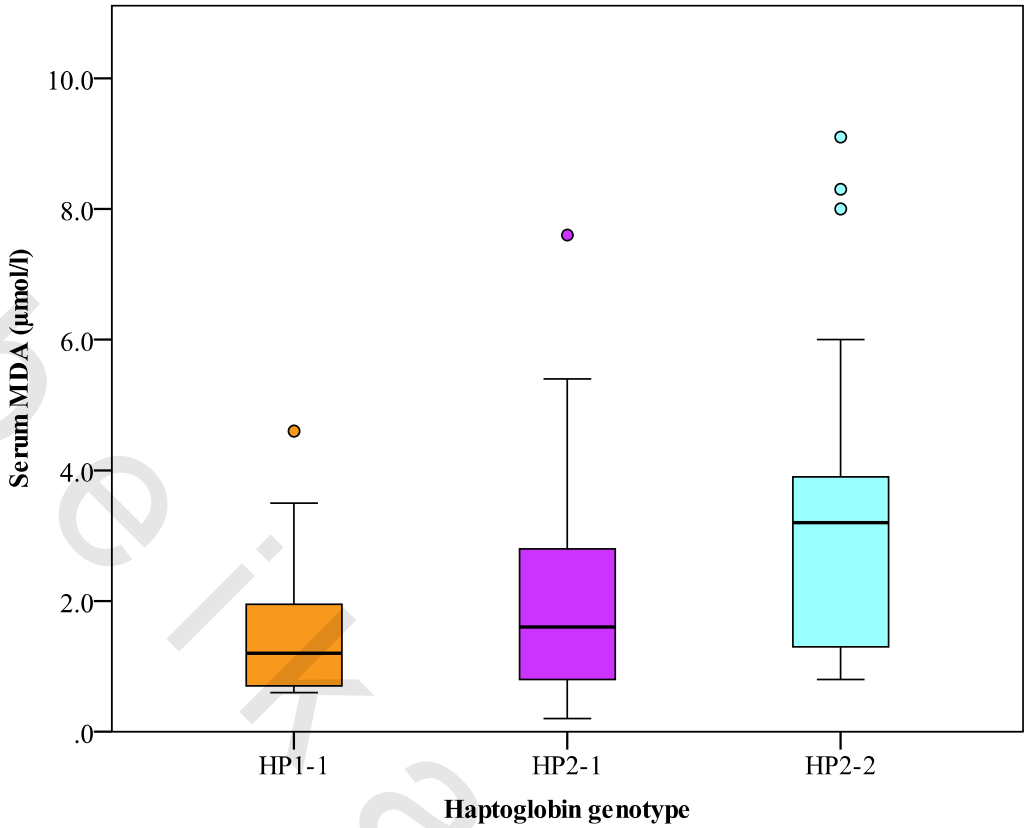

Figure (42): Relation between haptoglobin genotype with serum MDA in thalassemic group.

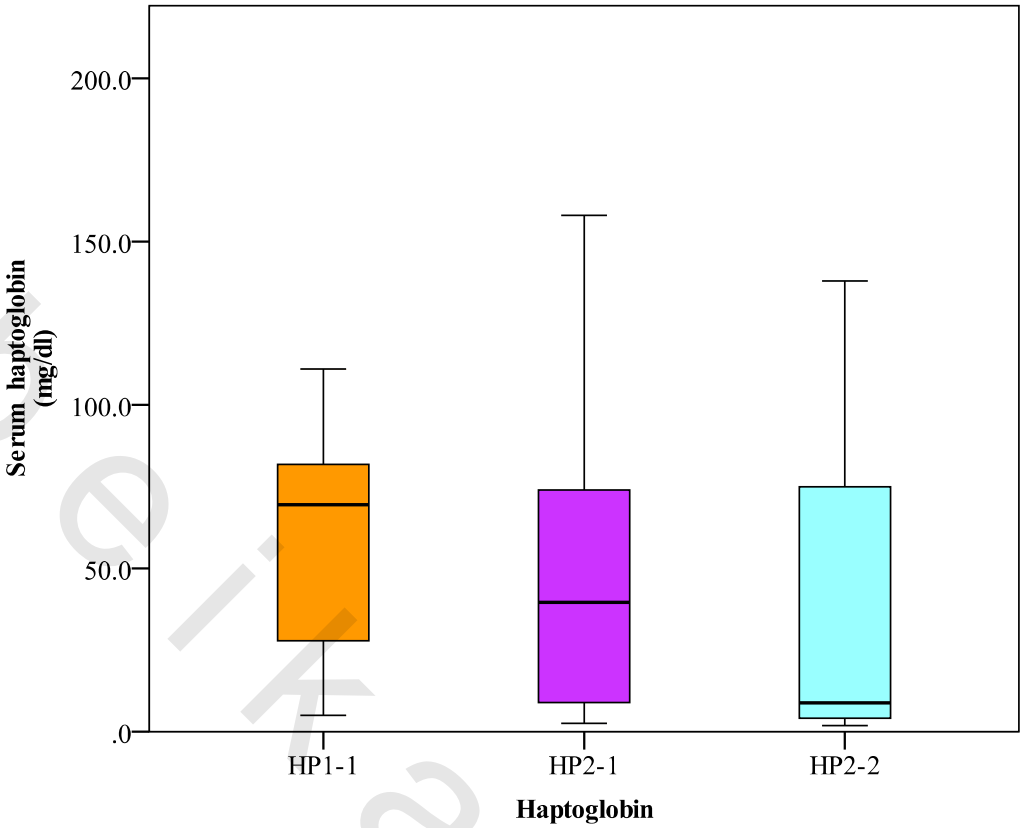

Figure (43): Rrelation between haptoglobin genotype with serum haptoglobin in thalassemic group.

Table (13) showed the relation between haptoglobin polymorphism and HCV infection. There was no significant relation between haptoglobin gene polymorphism and hepatitis C infection.

Table (13) Relation between haptoglobin Polymorphism with hepatitis C.

Item	Haptoglobin						χ^2	^{MC}p
	Hp1-1 (n=7)		Hp2-1 (n=15)		Hp2-2 (n=28)			
	No.	%	No.	%	No.	%		
Hepatitis C								
Negative	6	85.7	10	66.7	18	64.3	1.199	0.634
Positive	1	14.3	5	33.3	10	35.7		

χ^2: value for Chi square
MC: Monte Carlo test

Table (14) showed a positive relation between positive Coombs' test and the increase in the reticulocytes count (P 0.001).(Figure 44)

While serum haptoglobin was negatively correlated with Coombs' test being lower with positive test (P < 0.017).(Figure 44)

Table (14): Relation between Coombs' test with Retic count and serum haptoglobin in thalassemic group.

Item	Coombs' test		Z	P
	Negative(n=42)	Positive(n=8)		
Retic count ($x10^9$/l)				
Min. – Max.	54.0 – 238.0	188.0 – 364.0		
Mean ± SD.	100.05±36.46	253.88 ±53.93	4.3688*	<0.001*
Median	90.0	249.50		
Serum haptoglobin (mg/dl)				
Min. – Max.	1.90 – 99.0	2.10 – 7.40		
Mean ± SD.	23.66 ± 24.69	4.66 ± 1.89	2.395*	0.017*
Median	11.80	4.55		

Z: Z for Mann Whitney test

*: Statistically significant at p ≤ 0.05

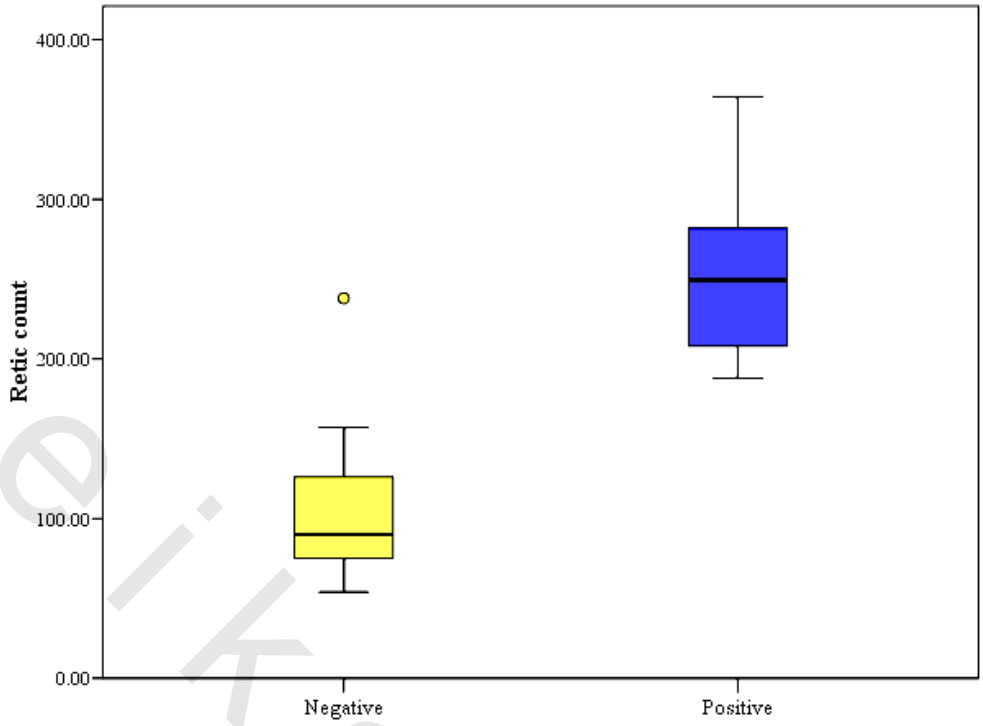

Figure (44): Relation between Coombs']=-\tests with retic count in thalassemic group.

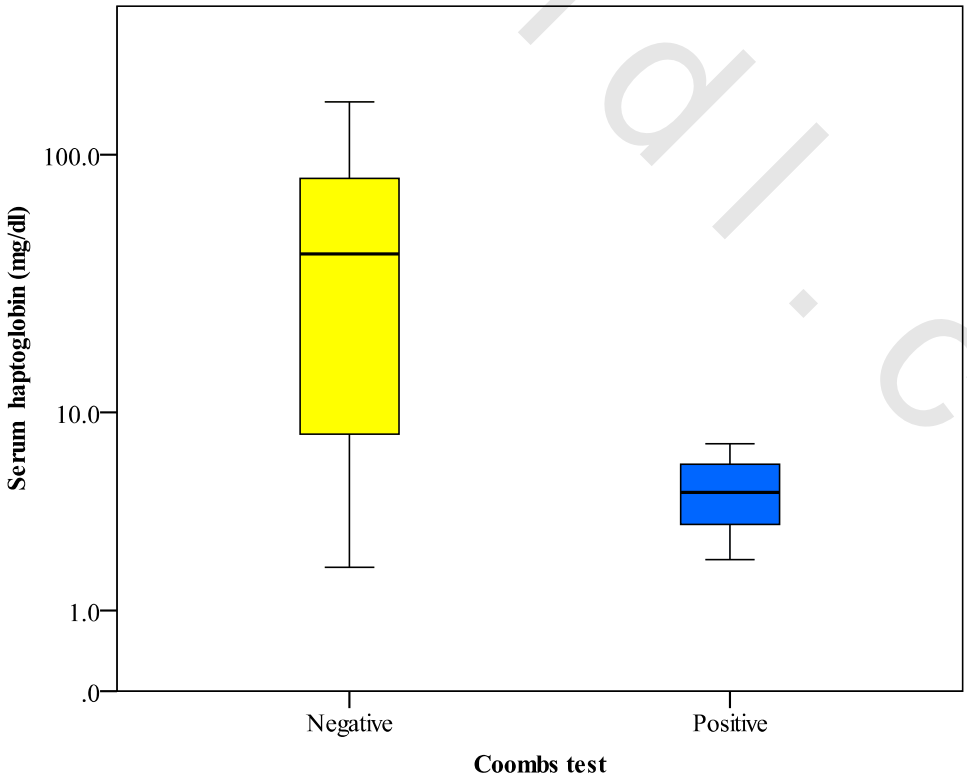

Figure (45): Relation between Coombs' tests with serum haptoglobin in thalassemic group.

Splenectomy:

Table (15) showed the relation between splenectomy, age and sex. There was a positive correlation between age and splenectomy (P 0.001), on the other hand no correlation was found between sex and splenectomy.

Table (15): Relation between splenectomy and demographic data.

Item	Splenectomy				Test of sig.	P
	Non Splenectomized (n=28)		Splenectomized (n=22)			
	No.	%	No.	%		
Sex						
Males	11	39.3	11	50.0	χ^2= 0.574	0.449
Females	17	60.7	11	50.0		
Age(years)						
Min. – Max	12.0 - 20.0		12.0 - 20.0			
Mean ± SD.	15.57 ± 2.39		17.41 ± 2.65		Z= 2.563*	0.010*
Median	15.0		18.0			

p: p value for comparing between negative and positive splenectomy
χ^2: Chi square test
Z: Z for Mann Whitney test
*: Statistically significant at $p \leq 0.05$

Table (16) showed that neither blood groups, Coombs' test nor HCV have significant correlation with splenectomy.

Table (16): Relation between splenectomy and ABO Blood groups, Coombs' test and Hepatitis C.

Item	Splenectomy				χ^2	^{MC}p
	Non Splenectomized (n=28)		Splenectomized (n=22)			
	No.	%	No.	%		
ABO Blood groups						
A	14	50.0	4	18.2	5.939	0.101
B	4	14.3	7	31.8		
O	8	28.6	8	36.4		
AB	2	7.1	3	13.6		
Coombs' test						
Direct	3	75.0	4	100.0	1.143	1.000
Indirect	1	25.0	0	0.0		
Hepatitis C						
Negative	22	78.6	12	54.5	3.268	0.071
Positive	6	21.4	10	45.0		

χ^2: value for Chi square
MC: Monte Carlo test

Table (17) showed the impact of splenectomy on blood indices

A significantly lower PCV was found in non splenectomized patients compared to splenectomized patients (P=0.033). While a significantly high MCV in splenectomized patients (P=0.008) was detected, as well as significant high platelets count in splenectomized patients (P=0.008).

Table (17):Impact of splenectomy on CBC.

Item	Splenectomy		Test of sig.	P
	Non Splenectomized (n=28)	Splenectomized (n=22)		
Hb (gm/dl)				
Min. – Max	3.50 - 8.40	5.10 - 10.0		
Mean ± SD.	6.52 ± 1.18	6.91 ± 1.25	t =1.120	0.268
Median	6.55	6.65		
PCV (%)				
Min. – Max	9.90 - 25.80	16.60 - 30.50		
Mean ± SD.	20.14 ± 3.79	22.52 ± 3.80	t= 2.201*	0.033*
Median	20.05	22.60		
MCV (fl)				
Min. – Max	53.40 - 82.40	54.80 - 88.20		
Mean ± SD.	69.90 ± 8.77	76.33 ± 7.25	t=2.771*	0.008*
Median	72.85	77.75		
MCH (pg)				
Min. – Max	16.50 - 27.50	16.30 - 27.70		
Mean ± SD.	22.72 ± 3.07	23.62 ± 3.33	t= 0.998	0.323
Median	23.10	24.20		
MCHC (gm/dl)				
Min. – Max	29.10 - 38.10	23.70 - 34.70		
Mean ± SD.	32.89 ± 1.74	30.70 ± 3.31	t=2.798*	0.009*
Median	32.90	31.0		
RBCS($x10^{12}$/l)				
Min. – Max	1.35 - 4.50	1.60 - 4.20		
Mean ± SD.	2.95 ± 0.76	2.92 ± 0.64	t= 0.113	0.910
Median	2.86	2.85		
Retic count ($x10^9$/l)				
Min. – Max.	60.0 – 364.0	54.0 – 274.0		
Mean ± SD.	120.14 ±70.69	130.41±69.0	Z= 0.635	0.525
Median	96.0	102.0		
WBC ($x10^9$/l)				
Min. – Max	3.84 - 9.73	4.10 - 8.90		
Mean ± SD.	6.76 ± 1.73	6.49 ± 1.58	Z= 0.498	0.618
Median	6.55	6.25		
NLR				
Min. – Max.	0.48 – 2.60	0.68 – 3.20		
Mean ± SD.	1.44 ± 0.56	1.61 ± 0.77	t=0.860	0.395
Median	1.35	1.46		
Plts ($x10^9$/l)				
Min. – Max	133.0 - 916.0	193.0 - 1056.0		
Mean ± SD.	352.0 ± 189.56	591.27 ± 287.39	Z=2.639	0.008*
Median	296.0	732.0		

p: p value for comparing between negative and positive splenectomy
t: Student t-test Z: Z for Mann Whitney test *: Statistically significant at $p \leq 0.05$

Table 18 showed that there was no significant correlation between splenectomy and iron status.

Table (18): Impact of splenectomy on iron profile.

Item	Splenectomy		Test of sig.	P
	Non Splenectomized (n=28)	Splenectomized (n=22)		
Serum iron (µmol/l)				
Min. – Max	22.40 - 64.40	31.50 - 61.0		
Mean ± SD.	40.37 ± 9.77	44.75 ± 9.51	t= 1.593	0.118
Median	40.50	42.10		
TIBC(µmol/l)				
Min. – Max	34.10 - 64.40	33.50 - 58.70		
Mean ± SD.	42.31 ± 8.43	40.97 ± 7.22	Z= 0.518	0.604
Median	39.70	38.15		
TS (%)				
Min. – Max	38.0 - 160.0	56.0 - 157.0		
Mean ± SD.	101.11 ± 33.62	113.73 ± 32.21	t= 1.342	0.186
Median	110.0	115.0		
Serum ferritin (µg/l)				
Min. – Max	890.0 - 9830.0	1521.0 - 9249.0		
Mean ± SD.	2955.8 ± 2239.9	3709.1 ± 2077.3	Z= 1.730	0.084
Median	2350.0	3091.50		

p: p value for comparing between negative and positive splenectomy
t: Student t-test
Z: Z for Mann Whitney test
*: Statistically significant at $p \leq 0.05$

Table 19 showed that, splenectomy not affect CRP, ALT, Serum Hp and serum MDA.

Table (19): Impact of splenectomy on laboratory profile.

Item	Splenectomy		Test of sig.	P
	Non Splenectomized (n=28)	Splenectomized (n=22)		
CRP (mg/l)				
Min. – Max	1.90 - 4.80	1.90 - 4.80		
Mean ± SD.	3.43 ± 0.77	3.34 ± 0.93	t= 0.351	0.727
Median	3.55	3.15		
ALT (u/l)				
Min. – Max	28.0 - 120.0	CRP, ALT, 26.0 - 84.0		
Mean ± SD.	53.39 ± 21.27	50.77 ± 16.44	Z = 0.323	0.747
Median	48.0	45.50		
Serum haptoglobin (mg/dl)				
Min. – Max	1.90 - 74.0	2.10 - 99.0		
Mean ± SD.	16.26 ± 18.86	26.17 ± 28.13	Z =1.505	0.132
Median	6.0	11.80S		
Serum MDA (nmol/l)				
Min. – Max	1.10 - 9.10	1.10 - 8.0		
Mean ± SD.	3.51 ± 2.01	3.42 ± 1.79	Z = 0.147	0.883
Median	3.40	3.20		

p: p value for comparing between negative and positive splenectomy
t: Student t-test
Z: Z for Mann Whitney test
*: Statistically significant at $p \leq 0.05$

Table 20 showed that Hp polymorphism not correlated with splenectomy.

Table (20): Relation between splenectomy and haptoglobin polymorphism.

Hp phenotype	Splenectomy				χ^2	P
	Non Splenectomized (n=28)		Splenectomized (n=22)			
	No.	%	No.	%		
Hp1-1	4	14.3	3	13.6	0.062	$^{MC}p=1.000$
Hp2-1	8	28.6	7	31.8		
Hp2-2	16	57.1	12	54.5		
Hp0-0	0	0.0	0	0.0		

χ^2: value for Chi square
MC: Monte Carlo test

Table 21 showed a positive significant correlation between NLR and both CRP and ALT.

Table (21): Correlation between NLR with CRP and ALT in cases group

Item	NLR	
	r_s	P
CRP	0.323*	0.022
ALT	0.332*	0.018

r_s: Spearman coefficient
*: Statistically significant at $p \leq 0.05$

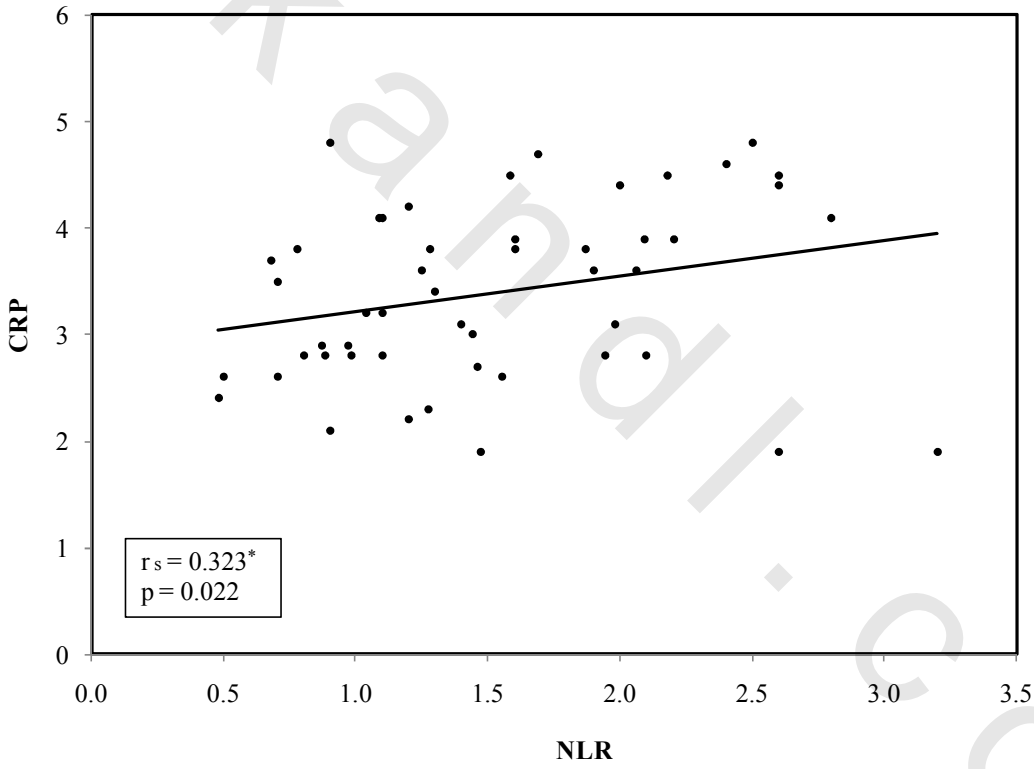

Figure (46): Correlation between NLR with CRP in cases group.

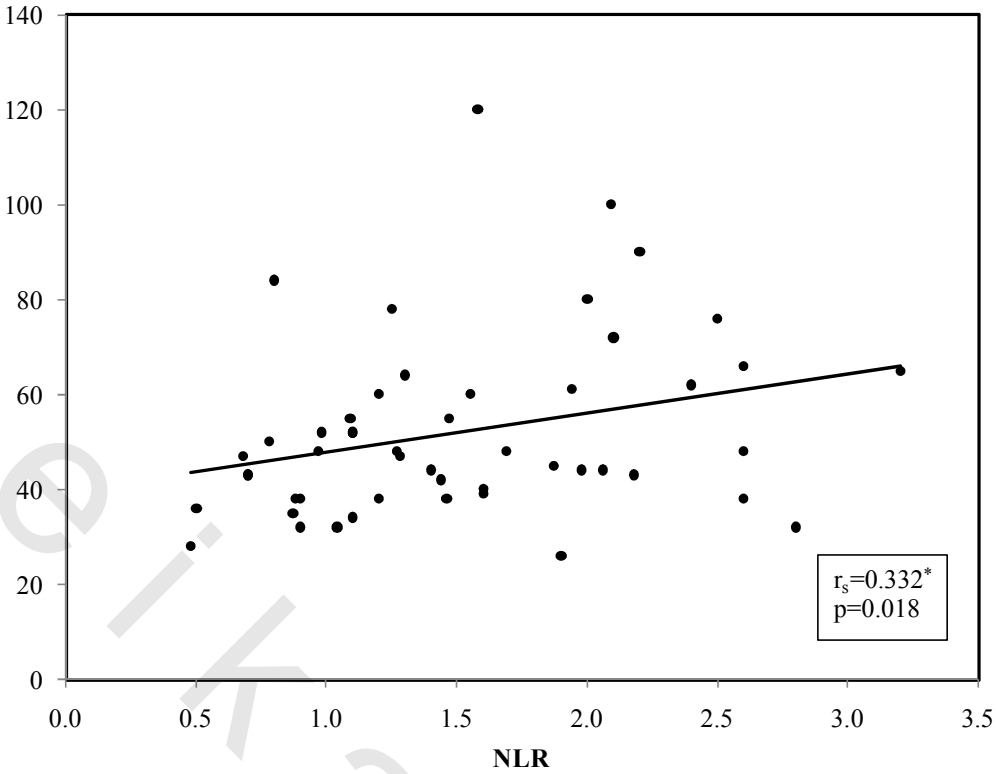

Figure (47): Correlation between NLR with ALT in cases group.

Table 22 showed a significant positive correlation between CRP and serum ferritin.

Table (22):Correlation between CRP and Serum ferritin in cases group

Item	Serum ferritin (µg/l)	
	R	**P**
CRP	0.342*	0.015

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

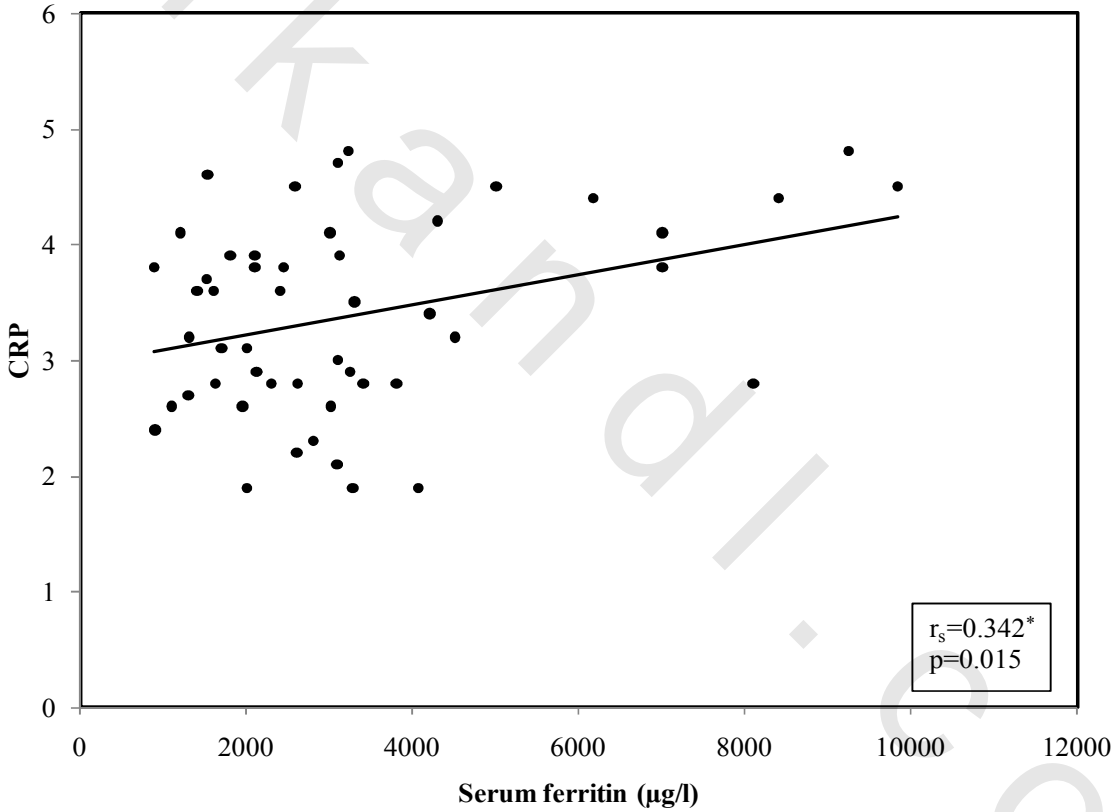

Figure (48): Correlation between CRP and serum ferritin in cases group.