Study of Serum Immunoglobulin Levels and T lymphocyte Subsets in Children with Beta Thalassemia with Iron Overload in Egypt

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Beta thalassemia is an inherited hemoglobin disorder resulting in chronic hemolytic anemia that requires lifelong transfusion therapy. Repeated blood transfusions and RBCs hemolysis are the main causes of iron overload, which in addition to immune abnormalities, are common predisposing factors to infection in patients with thalassemia. The aim was to study serum immunoglobulin levels and T lymphocyte subsets in children with beta- thalassemia in relation to iron overload. This study was conducted on 40 children with beta thalassemia major including 24 males and 16 females with mean age of 9.22 ± 3.9 and 20 healthy children of matched age and sex as a control. All children were subjected to assessment of infection episodes, complete blood picture, Hb electrophoresis, serum iron status, T cell subsets including CD3, CD4 and CD8 using Becton Dickinson FAC Scan flow cytometer and serum immunoglobulin levels including IgM, IgA and IgG by a commercial nephelometry assay using a BN-II device. Serum ferritin and iron were significantly higher but total iron binding capacity was significantly lower in patients than controls (Mean serum ferritin was 3418.23±2950.7 in the studied patients versus 39.48±2.48 in the control group with p value of 0.00, mean serum iron was 222±56.61 in the studied patients versus 90±31.87 in the control group with *P* value of 0.00 and mean serum total iron binding capacity was 198.38±19.9in the studied patients versus 315.7±24.85 in the control group with *P* value of 0.00). CD3 and CD4 were significantly lower but CD8 was significantly higher in patients than controls. The count (mean ± SD cells/ mm^2) for $CD3^+$, $CD4^+$ and $CD8^+$ T cells in patients was 1733.25 ± 381.87, 889.67 ± 282.86 and 779.72 ± 390.63 respectively as compared to 1887 ± 390.56, 1003 ± 250.96 and 663 ± 116.71 in the control group respectively (*P* values 0.00, 0.048 and, 0.02 respectively). The mean ± SD (mg/dl) of serum immunoglobulin's G, M and A showed significant variation between patients and controls including significantly lower IgM and significantly higher IgG, and IgA in patients than controls with *P* values of 0.014, 0.049 and 0.020 for G, M, and A respectively).There were significant negative correlations between CD3, CD4, IgM and ferritin and significant positive correlations between CD8, IgG, IgA and ferritin. In conclusion; Iron overload affects humeral and cell mediated immunity in thalassemic patients therefore regular follow up for iron overload is recommended.

Beta thalassemia is hereditary blood disorder due to defect in beta globin gene with excess of free alpha globin chains which become abnormal components in maturing red blood cells (RBCs) leading to RBCs destruction by the spleen with subsequent anemia (Glanello & Origa 2010).

Patients with beta-thalassemia major require regular blood transfusions to survive (Borgna-pignatti *et al.,* 2005). The primary long term complication of chronic RBCs transfusions is iron overload with resultant parenchymal organ damage (Kontoghiorghes *et al.,* 2010). Also there is increased incidence of infections as it reduces phagocytosis by neutrophils, reduces opsonization and increases bacterial activity. It also causes alterations in T-lymphocyte subsets, with modification of their distribution in different compartments of the immune system. (Monica, 2005). β-thalassemia patients suffer from too many problems rather than severe anemia especially increased susceptibility to bacterial infections which is the second most common cause of mortality and the main cause of morbidity in patients with thalassemia (Chern *et al.,* 2007).

Susceptibility to infections in thalassemia arises from spectrum of immunological abnormalities that occurs in patients with

thalassemia due to four main causes including; the disease itself due to pathological changes which can interfere of the immune system, transfusion therapy, iron overload and chelation therapy. The benefits offered by blood transfusions come together with the disadvantages of the transfusion burden in terms of direct exposure to infectious risks and indirectly, transfusion related immunomodulation and iron overload (Ladis *et al.,* 2005). Iron overload damage derives from disequilibrium between iron oxidation and the effectiveness and availability of those systems able to counteract oxidative stress (Walker & Walker, 2000). Iron overload impairs phagocytosis and its negative effect on neutrophil functions has been clearly demonstrated (Amer & Fiban, 2005). Desferoxamine (DFO) is associated with immune abnormalities especially significant reduction of serum IgM (Tourkantoni *et al.,* 2008).

The aim of this study was to study serum immunoglobulin levels and T lymphocyte subsets in children with beta- thalassemia in relation to iron overload.

Material and Methods

This study was approved by Tanta University research ethical committee and informed written parental consent was obtained from all participants. The study was carried out on 40 children with beta thalassemia major who were attendants to Hematology unit, Pediatric Department, Tanta university hospital including 24 males and 16 females with their age ranging from 2.6–15 years and mean age value of 9.22 $\pm$ 3.9 and 20 healthy children as a control group including 11males and 9 females with their age ranging from 2.2–15 years and mean age value of 8.38 $\pm$ 4.48. The study was done in the period between December 2012 and January 2015.

Inclusion criteria

Children with β-thalassemia major with serum ferritin levels more than 1000 ng/ml and who were maintained on regular use of chelation during this study.

Exclusion criteria

Children with thalassemia with serum ferritin level less than 1000 ng/ml.

Children with thalassemia who were splenoctomized.

All patients and controls were subjected to the following:

-Complete history taking with special account on age of onset of thalassemia, frequency of blood transfusion, iron chelation therapy, history of splenectomy, and detailed history of frequency of infectious episodes.

-Through clinical examination with special account on pallor, jaundice, mongoloid faces, splenomegaly, and hepatomegaly.

Laboratory investigations

- Specimen collection and handling

Six ml of venous blood were collected using sterile needles through gentle venipuncture after sterilization of puncture site by alcohol, and collected samples were divided into; one ml in 20 μL EDTA solution for complete blood count including differential WBCs count which was done on Leishman stained peripheral blood smear with evaluation using ERMA PCE-210 N cell –counter (George-Gay & Parker, 2003), 2 ml in 20 μL EDTA solution for assay of T cell populations using Becton Dickinson FAC Scan flow cytometer (BD FACS) (Perfetto *et al.,* 2006) and serum immunoglobulins levels including IgG, IgA and IgM by commercial nephelometry assay using a BN-II device (Gonzalez-Quintela, *et al.,* 2008) and 3 ml blood in a plain tube for Hb electrophoresis (Schneider *et al.,* 1976) and serum iron status including iron, total iron binding capacity (TIBC) and ferritin (Kuvibidila *et al.,* 1994, Muntzel *et al.,* 1992 , Beard, 2001).

Assay of T cell populations

One ml of venous blood sample was withdrawn on EDTA tubes for assay of T cell populations. Evaluation of CD3, CD4 and CD8 were done by Becton Dickinson FAC Scan flow cytometer (BD FACS) (Perfetto *et al.,* 2006) using fluorescence labeled monoclonal antibodies against CD3, CD4 and CD8 which bind to those cells that express CD3, CD4 and CD8 receptors on their surface. After incubation and removal of excess unbound antibodies and fluorochrome by washing, the cells are analyzed and counted by flow cytometer using 488 nm of wave length laser excitation (Ashcroft & Lopez, 2000).

Serum immunoglobulin assay

One ml of venous blood sample was withdrawn on EDTA tubes. Serum immunoglobulins (IgG, IgA and IgM) levels were determined by a commercial nephelometry assay using a BN-II device (Dade Behring, Marburg, Germany) and expressed in mg/dl. The manufacturer indicates the following reference range: IgA 70–400 mg/dl, IgG 700–1600 mg/dl and IgM 40–230 mg/dl. In nephelometry, a light source is projected through a liquid sample within a transparent container and the nephelometry measures scatter of light as it passes through the sample, which is proportional to the concentration of the immunoglobulin in the solution (Gonzalez-Quintela *et al.,* 2008) and the result is evaluated by comparison with a standard of known concentration (Webster, 1985).

Statistical Analysis

Data were collected and analyzed using statistical package for social science (SPSS) version for windows (version 12). All Data were expressed as in terms of mean values ± SD. Comparisons of parameters among groups were made using the paired t test. Two-group comparisons were performed nonparametrically using the Mann-Whitney U test. All statistical tests were two tailed, and $P < 0.05$ was considered statistically significant.

Results

There were no statistically significant differences between patients and controls regarding age and sex.

Pallor, jaundice, splenomegaly and hepatomegaly represent the most common presenting clinical manifestations in studied patients. The most common transfusion pattern is every 4 weeks and the most common iron chelator in the studied patients was oral Deferasirox.

There were significant differences between patients and controls regarding mean infective episodes per year with higher infective episodes in patients group (Table 1).

There were significantly lower mean Hb, MCV and MCH and significantly higher reticulocyte %, WBCs, platelets counts and lymphocytes % in patients than controls but no significant differences in MCHC between patients and controls (Table 1).

There were significantly higher serum ferritin and iron levels and significantly lower TIBC in patients than controls (Table 2).

There were significantly lower CD3, CD4 and IgM and significantly higher CD8, IgG and IgA levels in patients than controls (Table 3).

There were significant negative correlations between serum ferritin and CD3, CD4 and IgM (Figure 1 and table 4) and significant positive correlation between serum ferritin and CD8, IgG and IgA (Figure 1 and table 4).

Table 1. Demographic and laboratory findings in children with Thalassemia major.

	Patients (no=40)	Controls (no=20)	*P* value
Age:			
Range	5-15	6-13	NS
Mean± SD	9.22±3.90	8.38±4.48	
Sex :			
Males /Females	17/13	12/8	NS
Infective episodes per year			
Range	5-10	2-4	0.001*
Mean ± SD	7.5±2.1	3.4±0.9	
Clinical data		No of patients (%)	
Pallor		40 (100%)	
Jaundice		40 (100%)	
Thalassemic facies		12 (30%)	
Hepatomegaly		35 (87.5%)	
Splenomegaly		16 (40%)	
Splenectomy		20 (50%)	
Transfusion frequency			
Every 2 weeks		4 (10%)	
Every 3 weeks		10 (25%)	
Every 4 weeks		26(65%)	
Chelation therapy			
Types			
SC therapy		8 (20%)	
Oral therapy		20 (50%)	
Combined oral and Sc		12 (30%)	
Regularity			
Regular chelation therapy		24(60%)	
Irregular chelation therapy		16 (40%)	

* *P*>0.05 is not Significant (NS).

Table 2. Comparison between patients and controls as regard complete blood picture.

		Patients (No=40)	Control (No=20)	**P* value
RBCs (Millions/mm^3)	Range	2.6-4.1	4.6-5.6	0.001
	Mean ±SD	3.2±0.65	5.1±0.5	
Hb (gm/dl)	Range	6.5-9	11-13.1	0.001
	Mean ±SD	7.2±0.51	12.01±0.91	
MCV(FL)	Range	61-73.2	75.6-86	0.001
	Mean ±SD	67.1±6	80.8±5.2	
MCH (pg)	Range	23.1-27.3	26.8-30.6	0.014
	Mean ±SD	25.2±2.1	28.7±1.89	
MCHC (%)	Range	27-30	28-31	NS
	Mean ±SD	28.66±1.4	29.24±2.3	
Platelets (Thousands/mm^3)	Range	264.4-699.6	280-430.5	0.020*
	Mean ±SD	482±217.6	335±74.5	
WBCs (Thousands/mm^3)	Range	5.8-75.6	8-12	0.009*
	Mean ±SD	45.15±29.54	10.22±1.88	
Lymphocyte%	Range	41.69-57.71	30.3-39.9	0.001*
	Mean ±SD	49.7±8.01	35.1±4.8	
Reticulocyte%	Range	4.2-8	0.4-1.1	0.001*
	Mean ±SD	6.12±1.5	0.84±0.22	

* *P*>0.05 is not Significant (NS).

Table 3. Comparison between patients and controls regarding serum immunoglobulin levels and T cell subsets and serum iron status.

	Patients (No=40)	Controls (No=20)	*P* value
IgG (mg/dl)			
Range	782-1990	850-1350	0.014*
Mean ±SD	1345.3±325	1135±177	
IgA (mg/dl)			
Range	56.9-497	85-150	0.020*
Mean ±SD	198.1±56.9	114.5±20.3	
IgM (mg/dl)			
Range	26.6-292	90-208	0.049*
Mean ±SD	119.3±68.1	158.6±41.5	
CD3 (cells/mm^3)			
Range	1100-3000	1150-3250	0.00*
Mean ± SD	1733.25 ± 381.87	1887 ± 390.56	
CD4(cells/mm^3)			
Range	400-1400	720-1700	0.048*
Mean ± SD	889.67±282.86	1003±250.96	
CD8 (cells/mm^3)			
Range	190-1560	180-870	0.02*
Mean ± SD	779.72±390.63	663±116.71	
Ferritin (ng/ml)			
Range	1039-10467	35-43	0.00*
Mean ± SD	3418.23±2950.7	39.48±2.48	
Iron (μg/dl)			
Range	145-311	50-130	0.00*
Mean ± SD	222±56.61	90 ±31.87	
Iron binding capacity (μg/dl)			
Range	170-231	274-350	0.00*
Mean ± SD	198.38±19.9	315.7±24.85	

**P*<0.05 is significant.

Table 4. Correlation between serum immunoglobulins levels and serum ferritin.

	Ferritin	
	r.	**P* value
IgG	0.253	0.001
IgA	0.536	0.001
IgM	0.417	0.002

**P*<0.05 is significant.

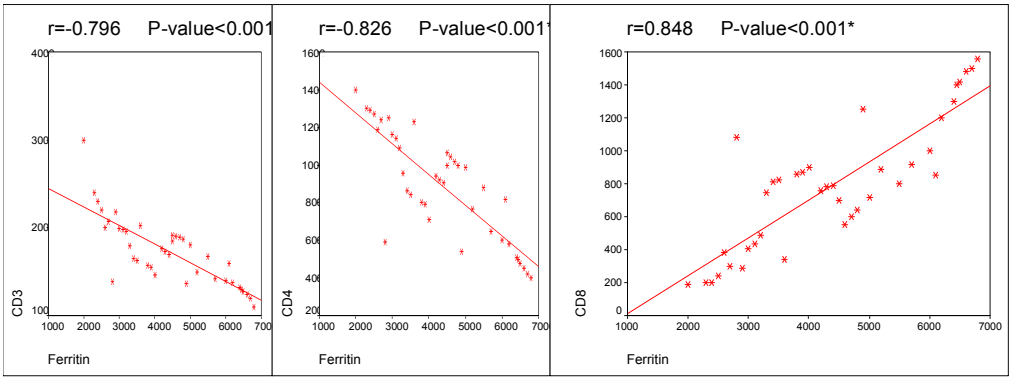

Figure 1. Correlation between ferritin and CD3 (left), CD4 (middle) and CD8 (right). There is negative correlation with CD3 and CD4 and positive correlation with CD8.

Discussion

Beta thalassemias are hereditary blood disorders (Crielaard & Rivella, 2014; Giardina & Forget, 2008) caused by reduced (beta +) or absent (beta 0) chains synthesis of Hb. This basic defect results in imbalanced globin chain synthesis, as decrease of beta globin chains causes early destruction of the red blood cells and anemia (Muncie & Campbell, 2009).

Thalassemia major usually requires regular blood transfusions which lead to continuous alloantigenic stimulation, autoimmune hemolysis, T and B lymphocyte changes and modification of monocyte macrophage functions. Iron overload has influence on cell mediated immunity that plays a major role in host defense against intracellular pathogens. Interferon gamma secreted by THI cells, activate macrophages to produce reactive oxygen species and enzymes to kill phagocytosed pathogens. Iron laden macrophages lose ability to kill intracellular pathogens (Danesh & Mir-Ahmadian, 1999).

In this study immunoglobulin panels including IgG, IgM and IgA and T lymphocyte subsets including CD3, CD4 and CD8 and were analysed in 40 children with beta thalassemia major compared with 20 healthy children as a control group.

In this study; pallor and jaundice, splenomegaly and hepatomegaly represented the most common presenting clinical manifestations. These data were in agreement with Galanello & Origa, 2010 who concluded that these clinical findings were due to chronic hemolysis, extramedullry erythropoiesis and iron overload.

In the present study, the most common transfusion pattern in the studied thalassemic patients is every 4 weeks. This correlated with the guidelines for transfusion therapy by Giardina & Forget 2008 that administrated 10 - 15 ml/kg of red blood cells every 2 - 4 weeks as β-thalassemia major is a transfusion-dependent anemia requiring life blood transfusions to stay alive.

In this study there was significantly lower Hb, MCV, MCH and significantly higher reticulocytes, WBCs and platelets counts in patients than controls. This is in agreement with Galanello and Origa 2010 who found the same results. This finding correlates with the nature of thalassemia as chronic hemolytic anemia with hemolysis and hyperactive bone marrow unless hypersplenism has developed. (Hershko, 2010)

In the present work, there was significant higher level of serum ferritin, serum iron and lower total iron binding capacity in patients than controls. This is in agreement with Hershko, 2010 and Ghone *et al.,* 2008 who demonstrated that iron overload is the main outcome of multiple blood transfusions and inappropriately increased iron absorption due to ineffective erythropoiesis.

The present study revealed significant increase in the frequency of infectious episodes in β-thalassemic patients compared with controls. This is in agreement with Farmakis *et al.,* 2003, Egarit, 2014 who concluded that infection is a common complication in β-thalassemia and this has been thought to be partly due to immunological abnormalities including increased immunoglobulin production, deficient activity of the complement system, decreased opsonization and phagocytosis and abnormalities in the cell-mediated immune response.

In this work there was significantly lower CD3 and CD4 and significantly higher CD8 in patients compared with controls. This was in accordance with Vento *et al.,*2006 who found lower $CD4^+$ and higher $CD8^+$ and Gharagozloo *et al.,* 2009 who found

significantly higher $CD8^+$ in thalassemia patients compared with controls.

This is not in agreement with Kadam *et al.,* 2014 who found that iron overload in thalassemic children led to decline in CD4 and CD8 levels, Al Awadhi, *et al.,*2010 who found comparable levels of T-cell markers between patients and controls and they concluded that high iron levels in thalassemic patients have a more significant effect on functions and activity of T cells rather than cell number and percentage and Ahmadiafshar 2012 who studied immunologic markers including CD8, CD4 [T-lymphocyte], CD19 [B-lymphocyte], and CD56 [NK cell] in thirty patients with β-thalassemia under 18 years. They did not found any abnormality in cellular and humeral system. However, mean CD56 level in thalassemia group were significantly lower than control group and mean CD4 in thalassemia patients with spelenectomy was significantly lower than patients without splenectomy.

In the current study there were significantly lower IgM in studied patients compared with controls. This finding is in accordance with Taurkantni *et al.,* 2008 who found significantly lower IgM in thalassemic patients on blood transfusion but this is not in agreement with Amin et al 2005 who found higher IgM in thalassemic patients due to repeated exposure to antigen from repeated blood transfusion and infections which stimulate production of IgM and Ghaffari *et al.,* 2011 who found normal level of IgM in thalassemic patients in comparison with controls due to better control of infections and serious filtration of transfused packed cells resulting in decreasing the chance of repeated exposure to antigens.

In this study there were significantly higher IgA and IgG levels in patients than controls. This finding is in accordance with Amin *et al.,* 2005 and Farkmakis *et al.,* 2003 who found increased levels of IgG and IgA due to repeated exposure to antigens due to repeated transfusions and infections which stimulate IgG but not in agreement with Ghaffari *et al.,* 2011 who found normal level of IgG and significantly higher levels of IgA due to better control of infections.

In this study there was significant positive correlation between serum ferritin and IgG and IgA and significant negative correlation between ferritin and IgM. This is not in agreement with Amin *et al.,* 2005 who found no significant correlation between serum ferritin and all immunoglobulins and they attributed the abnormality in the immune system to defects in the complement system or splenectomy.

In this study there was significant negative correlation between serum ferritin and CD3 and CD4, and significant positive correlation with CD8. This is in agreement with Gharagozloo *et al.,* 2009 who found the same results and explained this by iron overload which generates oxygen-free radicals and causes peroxidative tissue injury. Oxidative injury is a major factor of accelerated ageing of immune system resulting in gradual decline in responsiveness to antigens, abnormal T cell functions with block in cell division, shortening of telomere length and decrease in costimulatory receptors which has pivotal role in providing the stimulation required for a full proliferative T cell response (Kadam *et al.,* 2014).

Variation in results between this study and the previous studies may be explained by clinical heterogeneity among thalassemia patients', frequency of blood transfusion, spelenctomy, body iron status, iron chelation which were proposed as the responsible factors for alteration of immunoglobulins and T lymphocyte subset in thalassemia.

In conclusion, iron overload can affects humeral and cell mediated immunity in thalassemic patients with reduction of IgM,

CD3 and CD4 and elevation of CD8, IgG, and IgA.

It is recommended that regular follow up of thalassemic patients for detection of iron overload as it can affects humeral and cell mediated immunity.

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References

1. Ahmadiafshar A, Ghadipasha A, Mousavinasab N. (2012). Immunologic Evaluation in Patients with β-Thalassemia Major. Arch Dis Child; 97, Issue suppl 2: A217.
2. Al-Awadhi AM, Alfadhli SM, Al-Khaldi D, Borhama M, Borusly M. (2010). Investigation of the distribution of lymphocyte subsets and zinc levels in multitransfused β-thalassemia major patients. Int J Lab Hematol; 32(2):191-6.
3. Amer J, Fiban E. (2005). Chronic oxidative stress reduces the respiratory burst response of neutrophils from β-thalassemia patients. Br J Haematol; 129(3):435-441.
4. Amin A, Jalali S, Amin R. (2005). Evaluation of serum levels immunoglobulin and complement factors in β-thalassemia major patients in southern iron. Iran J Immunol; 2(4): 220-225.
5. Ashcroft RG, Lopez PA. (2000). Commercial high speed machines open new opportunities in high throughput flow cytometry (HTFC). J Immunol Methods; 243(1-2):13-24.
6. Beard JL. (2001). Iron Biology in immune function, muscle metabolism and neuronal functioning. J Nutr; 131(2S-2):568S- 580S.
7. Borgna-pignatti C, Capellini MD, De Stefano P. (2005). Survival and complications in thalassemia. Ann N Y Acad Sci; 1054:40-47.
8. Chern JP, Su S, Lin KH Chang SH, Lu MY, Jou ST, Lin DT, Ho WL, Lin KS. (2007). Survival, mortality, and complications in patients with β-thalassemia major in northern Taiwan. Pediatr Blood cancer; 48(5): 550-554.
9. Crielaard, B.J; Rivella, S. (2014). β-Thalassemia and Polycythemia vera: Targeting chronic stress erythropoiesis. Int J Biochem Cell Biol. 51:89-92.
10. Danesh A, Mir-Ahmadian M. (1999). Evaluation of T lymphocyte subsets in children with beta thalassemia major. Acta Medica Iranica; 37(2): 73-77.
11. Egarit N, Surada L, Suthat F, Kovit P. (2014). Phenotypic Characterization of Circulating CD4/CD8 T-lymphocytes in β-Thalassemia Patients. Asian Pac J Allergy Immunol; 32(3):261-9.
12. Farmakis D, Giakoumis A, Polymeropoulos E, Aessopos A. (2003). Pathogenetic aspects of immune deficiency associated with beta thalassemia. Med Sci Monit; 9(1):RA19-22.
13. George-Gay B, Parker K. (2003). Understanding the Complete Blood Count with Differential. J Perianesth Nurs; 18(2):96-114.
14. Ghaffari J, Vahidshahi K, Kosaryan M, Soltantooyeh Z, Mohamadi M. (2011). Humoral immune system state in ß thalassemia major. Med Glas (Zenica); 8(2):192-6.
15. Gharagozloo M, Bagherpour B, Tahanian M Oreizy F, Amirghofran Z, Sadeghi HM, Hourfar H, Moayedi B. (2009). Premature senescence of T lymphocytes from patients with β thalassemia major. Immunol Lett; 122(1):84-8.
16. Ghone RA, Kumbar KM, Suryakar AN, Katkam RV, Joshi NG. (2008). Oxidative stress and disturbance in antioxidant balance in beta thalassemia major. Indian J Clin Biochem; 23(4):337-40.
17. Giardina PJ, Forget BG. (2009). Thalassemia Syndromes. In: Hoffman R, Benz EJ, Shattil SJ, et al, eds. Hematology Basic Principles and Practice. 5th ed. Philadelphia, PA: Churchill Livingstone; 535-563.
18. Glanello, R, Origa R. (2010). Beta thalassemia. Orphanet J Rare Dis; 5: 5-11.
19. Gonzalez-Quintela A, Alende R, Gude F, Campos J, Rey J, Meijide L M, Fernandez-Merino C, Vidal C. (2008). Serum levels of immunoglobulins (IgG, IgA, IgM) in a general adult population and their relationship with alcohol consumption, smoking and common metabolic abnormalities. Clin Exp Immunol; 151(1):42-50.

20. Hershko C. (2010). Pathogenesis and management of iron toxicity in thalassemia. Ann N Y Acad Sci; 1202: 1–9.

21. Kadam PP, Manglani MV, Sharma SM, Sharma RA, Setia MS. (2014). Immunoglobulin levels and CD4 / CD8 counts in β — Thalassemia major. Indian pediatrics; 51 (12): 1000-1002.

22. Kontoghiorghes GJ, Kolnagou A, Skiada A, Petrikkos G. (2010). The role of iron and chelators on infections in iron overload and non-iron loaded conditions: Prospects for the design of new antimicrobial therapies. Hemoglobin; 34(3):227-39.

23. Kuvibidila S, Yu L, Warrier RP, Ode D, Mbele V. (1994). Usefulness of serum ferritin levels in the assessment of iron status in non-pregnant Zairean women of childbearing age. J Trop Med Hyg; 97(3):171-9.

24. Ladis V, Chouliaras G, Berdousi H, Kanavakis E, Kattamis C. (2005). Longitudinal study and causes of death in patients with thalassemia major in Greece. Ann N Y Acad Sci; 1054:445-450.

25. Monica C. (2005). Strategy of blood safety in Africa region. In: Tito A & Lewis SM (eds). District laboratory practice in tropical countries, 2nd edition. Cambridge low price Egyptian edition, chapter 4, P. 268-378.

26. Muncie HL, Campbell J. (2009). Alpha and beta thalassemia. Am Fam Physician; 80(4):339-44.

27. Muntzel M, Hannedouche T, Lacour B, Drüeke T. (1992). Effect of erythropoietin on hematocrit and blood pressure in normotensive and hypertensive rats. J Am Soc Nephrol; 3(2):182-7.

28. Perfetto SP, Chattopadhyay PK, Lamoreaux L, Nguyen R, Ambrozak D, Koup RA, Roederer M. (2006). Amine reactive dyes: an effective tool to discriminate live and dead cells in polychromatic flow cytometry. J Immunol Methods; 313(1-2): 199-208.

29. Schneider RG, Hightower B, Hosty TS Ryder H, Tomlin G, Atkins R, Brimhall B, Jones RT. (1976). Abnormal hemoglobins in a quarter million people. Blood; 84(5): 629-37.

30. Tourkantoni N, Athanassiou-Metaxa M, Zafiriou D, Tzimouli V, Economou M, Taparkou A, Perifanis V, Kanakoudi-Tsakalidou F. (2008). Immune and neural of thalassemic patients receiving deferiprone or combined deferiprone and deferoxamine chelation treatment. Haemoglobin; 32(1-2):35-40.

31. Vento S, Cainelli F, Cesario F. (2006). Infections and thalassemia. Lancet Infect Dis; 6(4):226-33.

32. Walker EM, Walker SM. (2000). Effects of iron over load on immune system. Ann Clin Lab Sci; 30: 354-465.

33. Webster AD. (1985). Laboratory Investigation of primary deficiency of the lymphoid system. Byron H Waksman. (ed), In Clinics in Immunology and Allergy. Vol. 5, 3rd edition. Philadelphia, WB Saunders Company, pp 447-468.