

RESEARCH ARTICLE

## Biochemical and Hematological Study with the Appreciation of some Immunological Parameters in Thalassemia Patients at Kerbala Province

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### Abstract

Thalassemia is a genetic disorder occurs as a result of the imbalance in the construction of haemoglobin chains cause haemolytic anaemia. This study was aimed to evaluate the serum level of immunological parameters (Transforming growth factor beta1, Interleukin-23) and hematological parameters in patients with alpha and beta thalassemia at Kerbala province. And was conducted at Kerbala Children's Hospital / Thalassemia Department / Kerbala Governorate during the period from November 2017 to May 2018. Seventy patients involved 48 beta thalassemia patients and 22 alpha thalassemia patients with age ranged 10-20 years old and ten matched healthy controls were enrolled in the study. Result exposed non-significant increase ( $p>0.05$ ) in IL-23 and TGF- $\beta$  in thalassemia patients compared with control group. In  $\beta$ -thalassemia patients the results of the current study indicated that non-significant positive correlation between IL-23 with WBC, there was non-significant positive correlation between TGF- $\beta$ 1 with WBC and PLT. As for  $\beta$ -thalassemia patients the present study showed non-significant positive correlation between IL-23 with Hb and WBC, and there was non-significant positive correlation between TGF- $\beta$ 1 with WBC. The study showed changes in the immunological parameters in patients with thalassemia of both types, as the serum level of both IL-23 and TGF- $\beta$ 1 were raised in addition to changes in hematological and Biochemical parameters.

**Keywords:**  $\alpha$ -thalassemia,  $\beta$ -thalassemia, TGF- $\beta$ 1, IL-23, hematological parameters, liver function, kidney function.

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## INTRODUCTION

Thalassemia is common genetic disorder, especially in the Mediterranean, patients with major thalassemia suffer from haemolytic anemia since the early years of life in addition to weakness in a number of organs that the most common homozygous cases it is considered severe and fatal unless blood transfusion begin at an early stage<sup>1</sup>. The main reason of thalassemia is the abnormalities in the synthesis of globin chains and the imbalance between them, in  $\beta$ -thalassemia this cause large amount of unpaired alpha globin chain that lead to the destruction of erythroid, the expansion of erythroid marrow appears on the growth of face bone<sup>2</sup>. Types of thalassemia are vary according to the type of infected globin genes<sup>3</sup>. Alpha thalassemia occurs due to the lack or absence of alpha-globin chain on chromosome 16<sup>4</sup>, beta thalassemia occurs because of the deficiency or absence of beta chain synthesis due to a localized mutation or deletion in the beta-globin gene on chromosome 11<sup>5</sup>.

The pathogenic factors of thalassemia are hemolysis, ineffective erythropoiesis and the elevation in the absorption of iron, the defect in erythrocytes and precursor of erythroid that result from the first two factors are eliminated by phagocytosis of monocyte and macrophage which are subjected to hyperplasia and become hyperactive, and because of this hyper activity there will be defects in the phagocytosis of microorganisms<sup>6</sup>.

There are many disorders in the immune system of  $\beta$ -thalassemia patients including cell mediated immunity<sup>7</sup> and both functional, quantitative and other components of the immunity<sup>8</sup>, involving immunoglobulins increasing, complement system with lower activity and decline in granulocyte phagocytosis and the opsonisation<sup>9</sup>.

Iron and proteins that bind to it have an effect on immunity as it modifies the immune system properties. An increase in the amount of the iron has harmful effects on the patient's immunity<sup>10</sup>. Iron accumulation effects are involving: modified the distribution of lymphocyte in various immune system compartments' and alteration in the subsets of T-lymphocyte<sup>11</sup>. Iron accumulation also works on accelerating the aging of patients' immunity due to the formation of reactive oxygen

radicals that cause peroxidative injury to the tissue, where it effects the function of T cell and decrease antigen response<sup>12</sup>, prevent division of the cell, shorting the length of the telomere and decline level in the co-stimulatory receptors that stimulate the response of T cell proliferative<sup>13</sup>. In addition, The toxicity of iron is responsible of deficiency of immunity in beta thalassemia patients, these abnormalities include: disturbance in the activity of Neutrophils and Macrophages in their chemotaxis and phagocytosis, lower the activity of the Natural killer cells, alteration in the responses of cytokines, impaired in the proliferative response of lymphocytes to antigens and mitogens and alteration in the function of T and B lymphocytes<sup>13</sup>.

Thalassemia patients suffer from lower state of systemic inflammatory with a high level of neutrophil, leukocyte and lymphocyte<sup>14</sup>, the high level of lymphocyte counts is due to blood transfusion that leads to constant challenge of the antigen<sup>15</sup>.

TGF- $\beta$ 1 gene is a risk agent for the decrease in bone density<sup>16</sup>. The effective erythropoiesis work on inhibited the transforming growth factor- $\beta$  (TGF- $\beta$ ) that is increased with stress of the cells<sup>17</sup>.

Aim of the study is Estimate the serum level for IL-23 and TGF- $\beta$ 1 in patients with alpha and beta thalassemia.

## METHODS

This study was organized at Thalassemia department / Kerbala Hospital for children / Kerbala Province during the period from November 2017 to May 2018. The practical part of the study was performed at the laboratories of children Hospital and Al-Hussain Hospital, the collection of the samples included 70 patients with thalassemia 33 males and 37 females whose ages ranged between 10-20 years old. The patients divided into two groups, the first group included forty eight patients with  $\beta$ -thalassemia major where the number of the female was 23 and the number of the males was 25, the second group included twenty two patients with  $\alpha$ -thalassemia where the number of the females was 14 and the number of the males was 8.

Patients who faced the following conditions were except from the current study who is suffering from: diabetes mellitus, Hepatitis,

iron deficiency anemia and patients who was taking medication (antioxidant drug and chelation therapy) before 2 to 3 days from the collection of serum. The control group contained from 10 normal individuals 6 females and 4 males with age range between 10-20 years old, who were not taking medication and had negative history of hematological disease and were free from anaemia, diabetes mellitus and liver disease. The patients were identified by physician based on history and clinical examination. Blood samples were gathered from each patient in the morning before blood transfusion by venipuncture using Ten milliliters disposable syringes. Around the arm of the patient tourniquet was applied then sterilized the skin with 70% ethyl alcohol before the collection of the blood.

The Hematologic parameters were measured by auto-analyzer device known as sysmex KX 2, Japanese; liver function test (ALT, ALP, AST) and renal function test (urea, creatinine) were estimated by the use of auto-analyzer known as Cobas Integra 400 Plus, Germany; and the measurement of ferritin were done by VIDAS, France.

#### Enzyme linked immunosorbent assay (ELISA)

IL-23 and TGF- $\beta$ 1 were measured according to sandwich-ELIZA by Elabscience, China. 100uL of standards or samples were added to each well of the plate, Biotinylated Detection Antibody in 100 $\mu$ L was added, then incubated for 45 minutes at 37°C. The solution was aspirated for 3 times. HRP Conjugate was added to each

well (100  $\mu$ L) then the plate was incubated for 30 minutes at 37°C. The solution was aspirated or decanted from each well for 5 times, Substrate Reagent was added to each well (90  $\mu$ L), then 50 $\mu$ L of Stop Solution was added to each well. The optical density (OD value) of each well was determined at once, using a micro-plate reader set 450 nm, The results were expressed in pg/mL.

#### Statistical analysis

Data was analyzed using the software statistical package for social sciences (SPSS version 18) and the results were presented as mean  $\pm$  standard error (Mean  $\pm$  E.R). One-way analysis of variation (ANOVA test) for more than two independent means was used for statistical analysis of the significance differences of the quantitative data. The Pearson's correlation coefficient test was used as appropriate for correlation between two quantitative data in different groups. P-values  $\leq$ 0.05 were considered statistically significant.

#### RESULTS

In  $\beta$ -thalassemia patients compared to the control group the statistical analysis showed non-significant decrease ( $P>0.05$ ) in MCV and MCH the result was in agreement with<sup>18</sup>. Moreover, a high significant decrease ( $P\leq 0.01$ ) in Hb, this result is in agreement with<sup>19,20</sup>. Also showed non-significant increase ( $P>0.05$ ) in PLT, this is disagreed with<sup>18</sup> and there is a significant increase ( $P\leq 0.05$ ) in ferritin, our finding are in agreement with<sup>21</sup>.

**Table 1.** Hematological and Immunological parameters in patients with thalassemia

Parameter	Control Mean $\pm$ S.E	$\beta$ -Thalassemia Mean $\pm$ S.E	$\alpha$ -Thalassemia Mean $\pm$ S.E
Hbg/dl	12.64 $\pm$ 1.23	7.79 $\pm$ 0.34**	9.02 $\pm$ 0.65*
MCV fL	79.72 $\pm$ 2.91	70.25 $\pm$ 1.04•	65.35 $\pm$ 1.30**
MCH pg	27.32 $\pm$ 2.03	26.11 $\pm$ 0.53•	18.60 $\pm$ 0.61**
WBC uL	8.44 $\pm$ 0.55	29.32 $\pm$ 8.92	7.38 $\pm$ 0.40
PLT uL	383.60 $\pm$ 32.28	588.45 $\pm$ 75.18?	341.27 $\pm$ 36.10
Ferritin ng/ml	18.35 $\pm$ 4.42	3796.84 $\pm$ 523.48**	169.98 $\pm$ 39.19•
IL-23 pg/mL	33.16 $\pm$ 6.04	447.24 $\pm$ 144.89	172.72 $\pm$ 40.14
TGF- $\beta$ 1 pg/mL	1.55 $\pm$ 0.29	3.00 $\pm$ 1.05	4.00 $\pm$ 1.17

(\* ) P values  $\leq$  0.05: significantly different in comparing with control group.

\*\* P values  $\leq$  0.01: highly significant different in comparing with control group.

(•): significant different in comparing  $\alpha$ -Thalassemia and  $\alpha$ -Thalassemia groups.

In Alpha patients compared to control groups, the statistical analysis showed high significant decrease ( $P \leq 0.01$ ) in MCH and MCV, due to the lack of transfer of packed red cell (PRC) this result agreed with<sup>22</sup>. And significant decrease ( $P \leq 0.05$ ) in Hb concentration in serum of patients this result was compatible with<sup>22</sup>. Also study showed non-significant increase ( $P > 0.05$ ) in ferritin level, this result was in agreement with<sup>23</sup>.

Statistical analysis in thalassemia patients showed non-significant different increase ( $P > 0.05$ ) in WBC in both groups of thalassemia patients compared to healthy control group this result was in agreement with<sup>24</sup>. non-significant higher ( $P > 0.05$ ) in PLT, this result was disagreement with<sup>25</sup>. as presented in Table 1.

According to serum IL-23 and TGF- $\beta$ 1 levels there were non-significant differences

increases ( $P > 0.05$ ) in thalassemia patients as shown in Table 1, these results are disagreement with<sup>26</sup>.

In thalassemia Patients compared to control group, serum urea level was non-significant increase ( $P > 0.05$ ), this result was disagreement with<sup>27</sup>.

While, serum creatinine level was highly significant decrease ( $P \leq 0.01$ ), this result was agreement with<sup>28,29</sup>

Also, the levels of ALT and ALP were significantly higher ( $P \leq 0.05$ ) this result was similar to<sup>28</sup>, and the level of AST was non-significant differences increases ( $P > 0.05$ ), this agreed with<sup>30</sup> and disagreement with<sup>31</sup> as shown in Table 2. Nafady<sup>32</sup> showed significant higher levels of ALT and AST in  $\beta$ -thalassemia patients as in our results, which may indicate acute or chronic liver injury<sup>33</sup>.

**Table 2.** Kidney and Liver function tests in patients with thalassemia

Parameter	Control Mean $\pm$ S.E	$\beta$ -Thalassemia Mean $\pm$ S.E	$\alpha$ -Thalassemia Mean $\pm$ S.E
Creatinine mg/dl	0.58 $\pm$ 0.05	0.35 $\pm$ 0.03 **	0.32 $\pm$ 0.02 **
Urea mg/dl	21.40 $\pm$ 1.16	23.69 $\pm$ 1.24	21.98 $\pm$ 2.35
ALT U/L	16.02 $\pm$ 1.45	41.37 $\pm$ 5.71*	16.12 $\pm$ 1.96•
ALP U/L	125.28 $\pm$ 24.24	196.73 $\pm$ 18.12*	128.19 $\pm$ 16.49•
AST U/L	24.64 $\pm$ 2.07	44.03 $\pm$ 5.32	34.48 $\pm$ 4.40

(\* ) P values  $\leq 0.05$ : significantly different in comparing with control group.

(\*\*) P values  $\leq 0.01$ : highly significant different in comparing with control group.

(•): significant different in comparing  $\beta$ -Thalassemia and  $\alpha$ -Thalassemia groups.

**Table 3.** Correlation between study parameters in  $\alpha$ -thalassemia and  $\beta$ -thalassemia patients.

Correlation between study Parameters in alpha thalassemia patients ( No.=22)					
		Ferritin ng/ml	Hb g/dl	WBC uL	PLT uL
IL_23	r	-0.246	-0.191	0.009	-0.322
pg/mL	p	0.466	0.573	0.979	0.334
TGF	r	-0.016	-0.126	0.298	0.324
pg/mL	p	0.962	0.712	0.373	0.331
Correlation between study Parameters in beta thalassemia patients ( No.=48)					
		Ferritin ng/ml	Hb g/dl	WBC uL	PLT uL
IL_23	r	-0.314	0.067	0.466	-0.062
pg/mL	p	0.22	0.798	0.06	0.812
TGf	r	-0.262	-0.027	0.013	-0.123
pg/mL	p	0.217	0.9	0.952	0.568

In  $\alpha$ -thalassemia and  $\beta$ -thalassemia patients the results of the current study indicated that there was non-significant negative correlation between IL-23 with Hb and ferritin. Also there was non-significant negative correlation between TGF- $\beta$ 1 with ferritin, as presented in Table 3.

## DISCUSSION

Depending on the type of thalassemia, the change in the hematological parameters occurs. Low levels of mean corpuscular hemoglobin (MCH) < 27 pg or mean corpuscular volume (MCV) < 78 fl considered as indicator of thalassemia<sup>34,24</sup>.

The liver is the main iron store, where 70% of the total iron content in the body is stored in the liver<sup>35</sup>. The previous study of Bilto and Assaf<sup>36</sup> showed that there is an increase in the amount of iron and ferritin in the serum of Iraqi thalassemia patients, accumulation of iron leads to increase absorption of iron in the intestine, the main cause of oxidative damage to organelles of the cell and erythrocytes in thalassemia patients is due to increase in iron overload<sup>37</sup>. And that damage is due to susceptible of thalassemia patients' erythrocytes to auto-oxidant in comparison to the control group<sup>38</sup>. This susceptible is due to high content of polyunsaturated fatty acid in the membrane of erythrocytes and the high concentration of hemoglobin and oxygen in cell which is the main source of ROS<sup>39</sup>, and is also because the inability of erythrocytes to synthesis damage components of cells<sup>40</sup>.

Antioxidants that present in the erythrocytes routinely make them resistance to oxidative damage, but for patients suffering from thalassemia there are important factors responsible for abnormalities of physiologic and function: life of red blood cells is short, turnover of iron is rapid and excessive deposition of iron in tissues, this accumulation of iron considered very harmful in patients because low solubility of iron and it is ability in stimulating the formation of toxic oxidants<sup>41,42</sup>.

Moreover, the presence of the free iron in the blood and tissues is due to chronic blood transfusion, which leads to accumulation of iron that may exceed the ability of detoxification of ferritin<sup>43</sup>.

White blood cells is higher in Beta thalassemia patients than in controls, the increase

number in the red blood cells that is immature leads to observe a high number of white blood cells as a result of the error in cell counter which is identify as WBCs<sup>34</sup>. Also inflammations and abnormalities that affected thalassemia patients led to an increase in the level of white blood cells which considered an indicator of infection with viruses or microbes<sup>44</sup>.

Platelets levels were high in thalassemia patients and that may be due to splenctomized in some patients<sup>45</sup>. The high levels of ferritin in serum occur as a result of iron accumulation, the increasing in iron is due to chronic blood transfusion or hemolysis of erythrocytes<sup>46</sup>.

TGF- $\beta$ 1 its effect on immunity and the status of inflammation<sup>47</sup>, TGF- $\beta$ 1 produced by phagocytes cells or cells that are infected, its important was manifested in the differentiation and activation of T-regulatory cells<sup>48</sup>. Through levels of both pro-inflammatory cytokines IL-23 and immunosuppressive cytokines TGF- $\beta$ 1 in the serum, lymphocytes functional status is determined in patients with thalassemia, in compration with control group<sup>26</sup>. The increasing in the level of both indicates an inflammatory state and this is mean suppression the immune response of T-cell<sup>26,49</sup>.

Liver and Kidney parameters are useful indicators of inflammation and can also be used to diagnose damaged organs and tissues<sup>28</sup>. In clinical medicine, Creatinine considered important parameter to detect upon the function of kidney<sup>50</sup>, study of cimcek<sup>51</sup> has been shown that the high level of creatinine indicates that capacity of kidney functions is low. Patients with a high degree of anemia and increased in the oxidation lead to abnormalities of renal tubular, the severity of these disorder is related to degree of anemia, but these imbalance occur at a lower rate in patients who take iron chelating treatment and transfused continuously<sup>52</sup>, same observation in  $\beta$ -thalassemia patients but with less severe than  $\beta$ -thalassemia patients and that is obvious through lower level of ferritin in serum, anemia and growth failure also in lower degree than  $\beta$ -thalassemia patients<sup>53</sup>. Previous study of Adil<sup>54</sup> showed the same result of our finding that patients had an increase in the ALP, which might be due to damage of the liver.

In general, elevated of these parameters (ALT, ALP and AST) are considered as indicator of

liver damage and also impaired of liver function<sup>55</sup>. Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is considered an inflammatory cytokine has a part in enhancing the proliferation of fibroblast and the accumulation of matrix in tissues, TGF- $\beta$ 1 is excreted by antigen stimulated T-cells<sup>56</sup>, CD4+ CD25+ regulatory T-cells (Treg) that are occur naturally are mainly produce TGF- $\beta$ 1, in thalassemia patients the high level of TGF- $\beta$ 1 is act on increase the frequencies of CD4+ CD25+ Treg and suppression of T-cell immunity<sup>57</sup>, TGF- $\beta$ 1 is promote differentiation T-cells that are produce IL-17 that have a link with IL-23 which are contributed to several autoimmune and inflammatory disease<sup>58</sup>.

In thalassemia patients precipitation of iron in the epithelial cells and reticuloendothelial may influence the production and regulation of TGF- $\beta$ 1 and therefore effect on its level in the circulation, in children with thalassemia major the presence of TGF- $\beta$ 1 in the circulation is considered Not a good sign for fibrotic responses unlike other fibrotic diseases<sup>59</sup>, patients with fibrotic disease have higher TGF- $\beta$ 1 level in their alveolar fluid and their circulation<sup>60</sup>, some observations in  $\beta$ -thalassemia patients with multi-transfused are the activated of T lymphocytes. Though T-cell suppression TGF- $\beta$ 1. These may have implications of the immune system for the associations between premature aging and repeated immune activation this result the immune resources exhaustion<sup>61</sup>, moreover, chronic immune activation and blood transfusion might induce Treg cells, that act on the suppression of effector functions of T-cell. at the end this profile of cytokine can be used clinically as a related marker for appreciating the severity of the disease, an index in following the disease and therapeutic intervention<sup>18</sup>. immune suppression is mediates by TGF- $\beta$ 1 to limit immunopathogenesis related with persistent infections and chronic inflammation. subsequently high production of IL-17 and TGF- $\beta$ 1 might participate to iron metabolism abnormalities and probably because of overstimulation of Th17. In fact, the deposition of iron in the reticuloendothelial system like epithelial cells and macrophages may affect the regulation of Th17 responses in patients with thalassemia and result increase in its cytokines levels in the circulation. moreover, blood transfusions in multiple times may cause

the immune system is under constant alloantigen stimulation in  $\beta$ -thalassemia patients, in spite of the immune responses that are suppressed because of iron overload<sup>62</sup>.

Level of TGF- $\beta$ 1 in the serum does not depend on ferritin , meaning that there is no correlation between them, where the results of some previous studies such as<sup>18</sup> showed that The level of TGF- $\beta$ 1 is significantly higher in all patients whether the level of ferritin is low or high.

IL-17 and IL-23 Represents the interface between cell-mediated immunity and inflammatory response in the condition of infectious diseases and cancer, Inflammation reactions are a first line of Immune response of the host against pathogens<sup>63</sup>, As was explained in other studies that agreed with our study there was no relationship between IL-21 and ferritin where the levels of IL-21 did not differ in patients with low ferritin compared to patients with high ferritin, the study also showed IL-21 did not associated with the level of Hb<sup>18</sup>

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