

## Assessment of Liver and Kidney Functional Parameters along with Oxidative Stress and Inflammatory Biomarker in Patients with $\beta$ -Thalassemia Major

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### Abstract

**Background:** Thalassemias are the most common inherited blood disorders caused by some mutations which can reduce the synthesis of globin chains. Iron overload and its organ deposition are responsible for functional abnormalities and tissue injury in patients who affected by  $\beta$ -thalassemia major. The aim of this case-control study was evaluation of hematological parameters, oxidative stress and some serum liver and kidney risk factors which play crucial role for early prediction and prevention of patients to end-stage tissue failure and mortality.

**Materials and Methods:** the present study consisted of Fifty young adult subjects with  $\beta$ -thalassemia major ( $\beta$ -TM) (aged<18 years) and same number age and sex- matched healthy subjects as control group. Hematological and biochemical laboratory parameters included Urea, Creatinine, Uric Acid, Aspartate Aminotransferase (AST), Alanine transaminase (ALT), Alkaline phosphatase (ALP) (pars azmoon kit), oxidative stress biomarker PAB, giving a redox index (chemically), and serum high-sensitivity C-reactive protein (hs-CRP) were evaluated.

**Results:** Urea, Creatinine and Uric Acid were significantly decreased in patients group ( $P<0.001$ ); in spite of, serum ferritin, liver biomarkers AST, ALT, ALP and risk factor biomarker PAB were statistically increased in patients versus control group ( $P<0.001$ ), whereas hs-CRP ( $P>0.05$ ) was not significantly difference in study groups. Exception hs-CRP and PAB ( $P>0.05$ ), liver risk factors had a positive correlation with ferritin and serum Urea, Creatinine and Uric Acid tests had negative meaningful with hematological parameters ( $P<0.001$ ). Likewise, PAB with AST showed a positive correlation ( $P<0.001$ ) and irreversibly with urea and creatinine ( $P<0.001$ ). We did not find a slight correlation between hs-CRP in the company to hematological and biochemical laboratory finding ( $P>0.05$ ).

**Conclusion:** Higher level of risk factors PAB values and key liver enzyme profiles are able to involve in the prognostic pathological consequences in patients with  $\beta$ -thalassemia major. Even so, they contribute toward the gradual development of tissue injuries.

**Key Words:** Beta-Thalassemia Major, Kidney, Liver, Inflammatory, Oxidative Stress

### Introduction

Alpha ( $\alpha$ ) and Beta ( $\beta$ ) thalassemia are the most common inherited single gene worldwide hemoglobin disorders, characterized by impaired or decreased rate of production in one or more

hemoglobin chains. The prevalence of thalassemia gene is about 3% all around the world and World Health Organization (WHO) estimates that at least 6.5% of the world populations are carries of different inherited disorders of hemoglobin(1, 2). It

occurs commonly in Mediterranean region, African-Americans, Chinese, Syria, India and Iran. Approximately, 15000 people are known as thalassemic and concerning 3,000,000 people are carrying thalassemia gene in Iran (3-5). Beta-thalassemia which was first described by Cooley and Lee, represents a group of recessively inherited hemoglobin disorders which impaired to production of beta globin chains, leading to a relative excess of alpha globin chains(4).  $\beta$ -Thalassemia major ( $\beta$ -TM), with both impaired  $\beta$ -globin genes refers to patients who suffer from profound anemia because imbalance of globin-chain synthesis. Subsequently; require regular blood transfusions for survival (6). Frequent blood transfusions are inevitably associated with iron overload and also peripheral hemolysis, increased intestinal iron absorption and ineffective erythropoiesis which cause iron accumulation in the reticulo-endothelial system (RES), as well as, enhanced generation of reactive oxygen species (ROS) and chronic oxidative stress(7-9). Furthermore, in patients with  $\beta$ -thalassemia major, the iron saturation in the RES that lead to organ toxicity and subsequent organ dysfunction is associated with considerable morbidity and mortality (7, 10). Iron has a catalytic role to produce powerful reactive oxidant species (ROS) and free radicals which lead to oxidative damage. Therefore evaluation of oxidative stress can be useful in protecting  $\beta$ -thalassemia patients from more serious complications of the disease followed by iron deposition in different parts of body, notably in heart, liver, kidney and endocrine glands (6, 11). Tissue damage occurs due to oxidative stress, and accumulation of iron in the body (25). With the regards to past studies, renal and liver diseases have not been major issues in patients with  $\beta$ -TM because survival was limited by severe cardiac iron loading from chronic transfusion therapy leading to premature early death and simply patients did not live long enough to develop

conditions linked to these two organ dysfunction.(12) Liver is the primary organ of iron storage has a large capacity to produce proteins. It is the only tissue for synthesis of transferrin and ferritin. Free ferrous iron is highly toxic and normally is protein-bound within the liver. With continued transfusions, iron eventually accumulates in parenchymal cells (hepatocytes). Moreover, iron catalyzes the production of free radicals which have been implicated in the lipid peroxidation, hepatotoxicity and increasing the risk of liver injury with hepatocytes, synthetic dysfunction, fibrosis, and eventually cirrhosis (13-16). In most  $\beta$ -TM patients, remarkable increase in renal tissue iron content and oxidative stress which contribute to lipid peroxidation and functional abnormalities in tubular cells may lead to tissue injury and kidney dysfunction(12). Ancient studies have been demonstrated that nevertheless renal dysfunction in these patients is not fully understood and seems to be multi factorial, there is a correlation between markers of kidney abnormalities and severity of anemia in TM patients(17). A number of studies have been reported that mean values of creatinine clearance and glomerular filtration rate (GFR) were higher than normal in patients with  $\beta$ -TM (17, 18). Patients with thalassemia are known to have severe cardiomyopathy, reticuloendothelial, and other major systems dysfunction (19, 20), but renal involvement has received little attention. Therefore, the present study was designed to detect early prediction from risk of liver and renal involvement in b-thalassemia patients and to correlate the findings with laboratory parameters.

## **Materials and Methods**

### **Participant Population**

This case-control study was carried out on fifty  $\beta$ -TM subjects (17.6 years old) with transfusion-dependent thalassemia registered in the Thalassemia and Hemoglobinopathy Research Center

Tehran University of Medical Sciences, Tehran, Iran, in 2015 based on complete blood count and hemoglobin electrophoresis, also the same number healthy control population (17.5 years old) with proven healthy history by complete clinical and laboratory examination was recruited from our center, as well which were matched to patients in sex and age. All subjects were informed about the study protocol and written consents were obtained from all participants. Patients received packed cell every month. They received iron chelator together with regular transfusion like Desferal or Deferiprone. Participants taking vitamin supplements, anti-inflammatory drugs, those who had diabetes, myocardial infarction (MI), acute infection, liver and kidney disease, or any acute illness and smokers were excluded from the study.

#### **Blood Sample Collection**

At the beginning of the study blood from patients was collected just before the transfusion. Five milliliter venous blood sample were collected in aseptic conditions from each subjects and were collected in plain and EDTA glass tubes. Blood samples were obtained to complete blood count (CBC) by automatic hematology analyzer (Sysmex KX21; Sysmex, Kobe, Japan). Meanwhile, Serum was separated by centrifugation at 2500 rpm for 15 minutes at room temperature and divided into several aliquots and was kept at  $-80^{\circ}\text{C}$  until it was analyzed.

#### **Laboratory analyses**

##### **Measurement of hematological markers**

By the way of vast comprehensive hematological tests, we determined number of red blood cells (RBC), hemoglobin (Hb), hemotocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were measured using an automated cell counter (Sysmex NE-1500). Finally Serum ferritin levels were

assessed by immunoassay analyzer (Elecsys, Roche, Germany).

#### **Biochemical variables**

##### **Liver Function Tests**

Alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) activities were determined by using kinetic colorimetric methods (Randox).

##### **Renal Function Tests**

Besides, Serum urea, uric acid, and creatinine were determined for each subject by kinetic colorimetric methods with the use of commercial kits using the BT-3000 autoanalyzer machine (Biotechnical, Rome, Italy).

##### **Measurement of Inflammatory Biomarker Hs-CRP**

High-sensitivity C-reactive protein was measured in serum samples by a PEG (polyethylene glycol) enhanced immunoturbidimetry method with an Alcyon® analyzer (ABBOTT, Chicago, IL, USA).

##### **Chemicals**

Chemical solutions included Peroxidase enzyme (Applichem: 230 U/mg, A3791, 0005, Darmstadt, Germany), TMB powder (3, 3', 5, 5'-Tetramethylbenzidine, Fluka), chloramine-T, trihydrate (Applichem: A4331, Darmstadt, Germany) and hydrogen peroxide (30%) (Merck).

##### **Assessment of Serum Oxidative Stress**

In summary, this method was described by Alamdari et al(21) based on measurement of the balance between oxidants and antioxidants simultaneously by using chromogen TMB Throughout the assessment TMB supplemented with Peroxidase enzyme and chloramine-T which can be either oxidized to a color cation by oxidants or reduced to a colorless compound by antioxidants which finally provides a redox index. In order to provide standard solutions, various proportions (0–100%) of 250  $\mu\text{M}$  hydrogen peroxide as an oxidant substance, were mixed with 3 mM uric acid (in 10 mM NaOH), as antioxidants. The absorbance of samples was measured

with an enzyme-linked immunosorbent assay reader at 450 nm with reference 620 nm and the values of Prooxidant-Antioxidant Balance (PAB) are expressed in arbitrary (H.K) unit.

### **Statistical Analysis**

All data was expressed as mean  $\pm$  standard deviation (SD) or frequency as per the parameter based on testimonial. Normally distributed parametric variables between groups were performed using Student's t-test. Mann-Whitney U test and Spearman's correlation univariate correlation analysis were conducted for relationship between all parameters. Multiple linear regression analysis was performed to determine the level of association between PAB and hs-CRP vs. the independent variables (liver, kidney and ferritin). Data was analyzed using the SPSS for Windows software (version 18 software package SPSS Inc, Chicago, IL, USA). P-value less than 0.05 accepted statistically significant.

### **Results**

#### **Participants' characteristics**

An equal numbers of young adult's subjects less than 18 years old were entered in this present work. Demographic information and acquired hematologic results were summarized in Table 1. With the exception of age, gender and MCHC, other acquired hematological indices in cases were significantly different from normal subjects ( $p < 0.001$ ). Furthermore, the hematological variables of Hb, HCT and RBC in normal group were within reference range, whereas  $\beta$ -thalassemia major patients showed significant difference from normal subjects, as they were statistically lower ( $P < 0.001$ ).

#### **Liver and kidney risk parameters**

Collective data was also analyzed separately to assess the liver and kidney function organic parameters. As described in table 2, thalassemic patients exhibited high abnormal levels of AST ( $P < 0.001$ ),

ALT ( $P < 0.001$ ) and ALP ( $P < 0.05$ ) compared to normal healthy group. Data was also analyzed separately to test the kidney function organic parameters as potential sources of variation. Likewise, beta-thalassemia major subjects had significantly lower Urea, Creatinine and Uric Acid compared to normal healthy participant ( $P < 0.001$ ).

#### **Serum oxidative stress and hs-CRP concentration among different groups**

Table 3 represents that level of PAB was markedly higher in  $\beta$ -TM than healthy controls ( $P < 0.001$ ), whereas no significant differences were observed between patients and healthy counterpart with regard to serum hs-CRP concentration ( $p = 0.527$ ).

#### **Association between PAB values, hs-CRP and Ferritin concentration with lab findings**

Univariate Spearman correlation analysis was performed to evaluate the association between PAB values, Ferritin and hs-CRP concentration versus other lab parameters. As shown in Table 4, wonderfully, a significant correlation was observed between Ferritin with liver laboratory tests ( $P < 0.001$ ) Figure 1. Also, It was remarkable to consider a negative significant correlation between ferritin with kidney parameters ( $P < 0.001$ ) Figure 2. Furthermore, comparing the correlation between serum PAB with other parameters showed statistical correlation with AST ( $r = 0.320$   $p = 0.001$ ) and inversely significant correlation only with Cr ( $r = -0.319$   $p = 0.001$ ). Moreover, comparing the relationship between inflammatory markers with other values interestingly showed any significant correlation between hs-CRP to other laboratory finding. Finally, in the healthy participants, we did not find any changes between PAB values, hs-CRP concentration and all other variables (data not shown).

### Multivariate Analysis

Multiple linear regression analysis was conducted to explore the predictors of PAB and hs-CRP levels. As shown in Tables 5, we did not find any significant

independent association when PAB and hs-CRP were treated as a dependent variable. On the other hand, neither parameter correlated with PAB and hs-CRP.

Table I: Demographic and clinical characteristics of the Study Subjects

Variable	Patients	Controls (n=50)	P-value
Age	17.62±3.04	17.5±2.92	NS
Gender M/F	24/26	23/27	NS
RBC (X10 <sup>12</sup> /L)	1.52±0.51	5.17±1.24	< 0.001
Hb (g/dL)	5.91±1.38	14.95±2.55	< 0.001
HCT (%)	16.91±6.24	38.92±5.85	< 0.001
MCV (fL)	68.85±16.35	86.73±16.03	< 0.001
MCH (pg)	20.9±4.04	30.94±5.92	< 0.001
MCHC (g/dL)	28.48±7.62	29.31±5.43	NS
Ferritin (mg/dL)	1313.52±673.31	212.5±154.5	<0.001

RBC: Number of red blood cells; Hb: Hemoglobin; Hct: Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration.

Values represent means ± SD. Comparisons were made using Student's t test between groups. Significance was defined as  $P < 0.05$ . NS (Not significant).

Table II: Comparison between serum levels of kidney and renal laboratory tests in patients and controls

Variable	Patients	Controls (n=50)	P-value
<b>kidney function tests</b>			
AST(IU/L)	64.43 ± 19.02	21 ± 3.57	<0.001
ALT(IU/L)	31.76 ± 9.34	20.36 ± 6.84	<0.001
ALP(IU/L)	174.46 ± 47.02	144.02 ± 81.8	<0.05*
<b>Renal function tests</b>			
Urea (mg/dl)	19.25 ± 4.29	30.36 ± 6.08	<0.001
Creatinine (mg/dl)	0.93 ± 0.12	2.77 ± 1.01	<0.001
Uric Acid (mg/dl)	5.33 ± 0.73	7.09 ± 1.16	<0.001

AST: Aspartate Aminotransferase, ALT: Alanine transaminase, ALP: Alkaline phosphatase

Values represent means ± SD. Comparisons were made using Student's t test between groups.

Significance was defined as  $P < 0.05$ .

Table III: Comparison between serum Levels of PAB and hs-CRP in study population

Variable	Patients	Controls (n=50)	P-value
PAB(H.K)	63.59 $\pm$ 15.31	50.35 $\pm$ 11.96	<0.001*
hs-CRP(mg/L)	5.47 $\pm$ 2.48	5.18 $\pm$ 1.88	0.527

PAB: Prooxidant-antioxidant balance; hs-CRP: High-sensitivity C-reactive protein.

Values represent means  $\pm$  SD. Comparisons were made using Student's t-test between groups. Significance was defined as  $P < 0.05$ .

Table IV: Correlations between kidney and liver risk factors with PAB,hs-CRP and ferritin in patients.

	Urea	Crea	UA	AST	ALT	ALP	hs-CRP	PAB	Ferritin
Urea	----								
Creatinine	r=0.545 p=0.000	----							
Uric Acid	r=0.539 p=0.000	r=0.573 p=0.000	----						
AST	r=-.616 p=0.000	r=-.663 p=0.000	r=-.549 p=0.000	----					
ALT	r=-.434 p=0.000	r=-.444 p=0.000	r=-0.426 p=0.000	r=0.571 p=0.000	----				
ALP	r=-.146 p=0.155	r=-.102 p=0.316	r=-.0207 p=0.441	r=0.264 p=0.009	r=-.013 p=0.899	----			
CRP	r=-.053 p=0.607	r=-.034 p=0.737	r=0.147 p=0.152	r=0.097 p=0.345	r=-.009 p=0.930	r=0.084 p=0.412	---		
PAB	r=-.321 p=0.201	r=-.319 p=0.001	r=-.0326 p=0.101	r=0.320 p=0.001	r=0.25 p=0.312	r=0.212 p=0.336	r=0.359 p=0.830	---	
Ferritin	r=-.509 p=0.000	r=-.532 p=0.000	r=-0.42 p=0.000	r=0.686 p=0.000	r=0.526 p=0.000	r=0.170 p=0.010	r=0.103 p=0.412	r=0.241 p=0.449	---

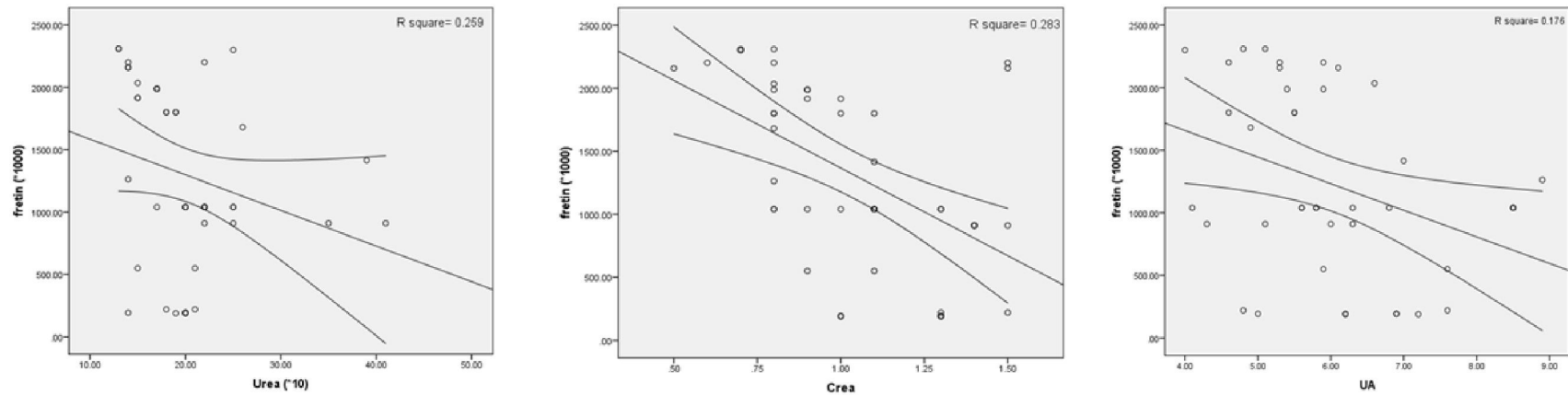
AST: Aspartate Aminotransferase , ALT: Alanine transaminase, ALP: Alkaline phosphatase

Values represent means  $\pm$  SD. Comparisons were made using Student's t-test between groups. Significance was defined as  $P < 0.05$ .

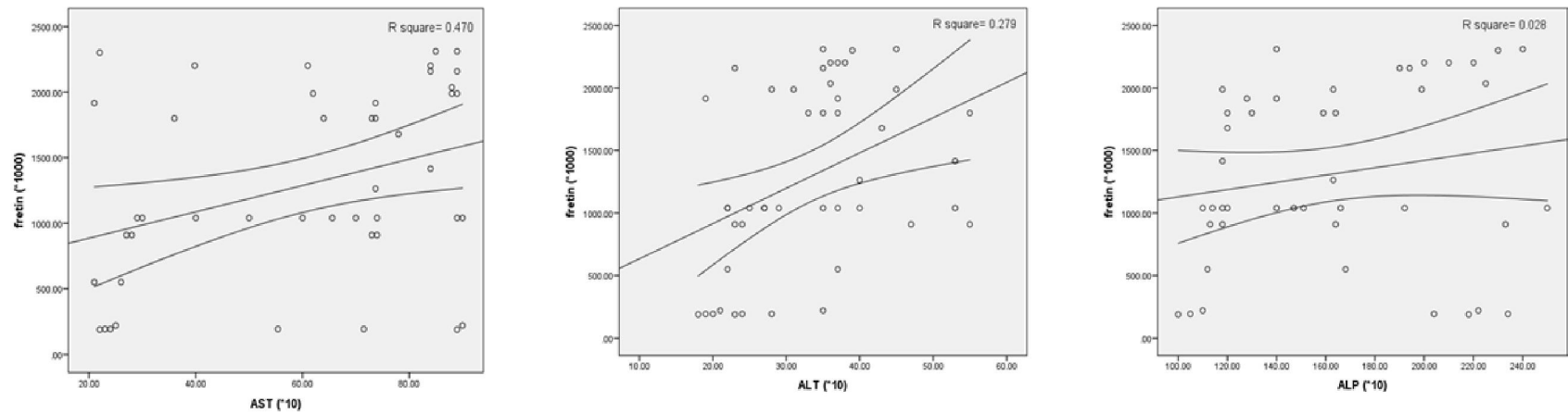
Table V: Multiple linear regression analysis of PAB and hs-CRP (dependent variables) versus liver, kidney and ferritin independent variables

	Variables	Beta(b)	SEb	p-value
hs-CRP	Urea	-.010	.043	NS
	Crea	-.104	.271	NS
	UA	.360	.231	NS
	AST	.007	.029	NS
	ALT	.005	.049	NS
	ALP	-.002	.003	NS
	Ratio	.222	.295	NS
	fretin	-0.0003	.001	NS
PAB	Urea	.287	.278	NS
	Crea	.256	1.738	NS
	UA	.120	1.499	NS
	AST	.228	.191	NS
	ALT	.424	.315	NS
	ALP	.019	.023	NS
	Ratio	1.429	1.920	NS
	fretin	-.004	.004	NS

b: Regression coefficient; SEb: Standard error of b. Significance was defined as  $P < 0.05$ .



**Figure 1.** Scatter plot shown ferritin correlated significantly with Urea, Creatinine and Uric Acid values.



**Figure 2.** Scatter plot shown Ferritin correlated significantly with AST, ALT and ALP variables.

## **Discussion**

Thalassemia disorders are the commonest single-gene hemoglobin disorders that more than 90 million people affected by this inherited defect throughout the world like Middle Eastern countries (22). Patients with Beta thalassemia major usually suffer from profound anemia that necessitates regular blood transfusion to survive. These patients usually experience iron overload as consequence of recurrent transfusion and ineffective erythropoiesis. Several major factors are responsible for functional abnormalities found in  $\beta$ -TM which include shortened red cell life span, rapid iron turnover and tissue deposition of excess iron. Furthermore, in beta thalassemia major, repeated blood transfusions are inevitably lead to multiple organ dysfunctions namely heart, liver and kidney(23, 24). Specifically, results of this study point to several significant finding: (I) except MCHC, other hematological parameters as well as, the levels of pro-oxidant-antioxidant balance in patients presenting with  $\beta$ -thalassemia major were significantly higher when compared with healthy control subjects, (II) hs-CRP concentration did not show any variations in  $\beta$ -tathalassemia compared to healthy participant. (III) surprisingly, renal function tests unlike liver parameters significantly were decreased in patients compared to healthy controls (IV) high levels of PAB in thalassemic patients was significantly correlated with AST and conversely correlated with serum creatinine, while ferritin was found to be correlated with serum liver and kidney biochemical laboratory variables, and finally, Spearman's univariate analysis showed that kidney functional tests irreversibly correlated with only ALT and AST activity. In present study, as described in table 3 and in accordance with AsmaKassab-Chekir et al and Ghahremanlu E(12, 25) findings, indicate that in young adult b-thalassemia patients the levels of oxidative stress were

significantly higher than normal controls. The significant increase of serum ferritin in cases indicated an existing iron overload in our patients. A rise in iron indices may be due to erythrocyte hyperhemolysis or/and to chronic blood transfusion that similar results were found by Haj Khelil et al(26). Emerging laboratory data suggest that enhanced oxidative stress is a high risk for organ injury than normal group. High oxidative stress in thalassemia patients is one of the most important factors causing cell injury and organ dysfunction (27-29). Similar to Livrea MA et al, In our study, important elevated aspartate and alanine aminotransferase than controls were possibly due to cytolysis syndrome and to hepatic necro-inflammatory mechanisms(30). Accompanied with finding, Haj Khelil et al(26) and Soliman, A et al(31) Parallel to our present work described that AST and ALT levels were correlated significantly with serum ferritin concentrations ( $r = 0.72$  and  $0.47$  respectively,  $p < 0.001$ ). A research on 104 patients with beta thalassemia major showed a significant correlation between serum ferritin levels and SGOT, SGPT levels. Abnormal liver function represented by elevated levels of SGOT, SGPT and serum alkaline phosphatase which was observed more frequently in patients with iron overload than in patients with a lower level of iron (32). As well as, a study in Pakistan showed that 47% of their patients had an increased alkaline phosphatase, which might be attributable to the liver disease (33). Further, serum ALP activity, another marker of tissue injury, was increased in  $\beta$ -TM provided information on the association of cholestasis syndrome that it is indicative of liver dysfunction and leakage of liver metabolites (3, 31). We also, analyzed the kidney serum markers uric acid, creatinine and urea. The main findings indicated that mean serum levels of all three parameters were significantly lower in patients than controls, similar to



AsmaKassab-Chekiretalva(12). This result is confusing but can be partially explained by the low muscle mass of our young study population. By contrast, Aldudak et al(34), did not find any remarkable difference concerning blood urea and creatinine in the patient. Evidence indicates that increased oxidative stress and inflammation may mediate most of the effects of risk factors on the kidney(35). We suggested that oxidative stress may cause inflammation and harms to kidneys in future. There is evidence that oxidative stress and inflammation are features of chronic kidney disease (36, 37). As well as, we also evaluated hs-CRP to determine the important inflammation that may exist between participants. hs-CRP is an acute phase protein, which is synthesized in response to tissue damage. Its production is stimulated mainly by interleukin-6(IL-6)(38). The important elevation of hs-CRP is a major inflammatory marker(39) and little is known about the role of acute phase proteins in hemoglobinopathies. Interestingly, the levels of C-reactive biomarker was less increased in patients comparison to control, but are not statically significant with healthy subjects in response to elevated of Ferritin and PAB value. Similar to our study, many investigators reported the reduction of the hs-CRP levels in patients compared to healthy subject(5). Patrick B. Walter (3), reported that hs-CRP concentration in healthy volunteers is lower than Thalassaemia patients. But Kanavakietal (40, 41) mentioned that CRP was elevated in thalassemia participant, implying a chronic inflammatory state presents in these patients. Arinzon confirms that levels of CRP were significantly higher in pneumonia disease with short term mortality and positively correlated with rate of death(42).

## Conclusion

Clearly, significantly higher levels of an indicator of oxidative stress in patients

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with thalassemia compared with healthy individuals needing therapy to prevent endothelial dysfunction and development of damage to other tissue and organs. Measurement of Urea, Crea, UA, AST, ALT, ALP and inflammatory biomarker hs-CRP may be useful marker for inflammation and useful diagnostic factor to prevent injury and its development to other tissues and organs. Longer-term prospective studies with a more careful assessment of the time course of the appearance of PAB oxidative stress marker as well as liver and kidney biochemical laboratory parameters and hs-CRP relative to the development of clinical events was required which could improve patients' quality of life.

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## Conflict of interest statement

None Declared.

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