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Oxidative Stress Damage and Risk of Atherosclerosis in Beta-Thalassemia Patients

Mohsen Rageb Tolba[1], Nabil Abbas Ahmed Soliman[2], Ghada yousef El-Kamah[3],

HalaBassyouni El-Tabie [4], Sawsan MohammedAbd Al-Hamid[5], Amira Ibrahim EL-Shehaby[6]

[1,2]Zagazig University Faculty of sciences, Department of Zoology

[3,4]National Research Center, Department of Clinical genetics

[5,6]National Research Center, Department of Molecular genetics and enzymes

Abstract: **Evaluation of the effect of the antioxidants lack on the level of oxidative stress and to find out if there is any increased susceptibility to atherogenesis.**

Patients and methods: Twenty Egyptian patients with β-thalassemia were recruited from the outpatient clinic of Clinical Genetics Department, National Research Centre and twenty healthy subjects as a control group.This study was approved by the Ethics Committee.

Results: In beta-Thalassaemia patients had a significant decrease in the mean of Paraoxonase1, Arylesterase, Reduced Glutathione and Catalase ($p<0.01$) activities along with a significant increased malondialdehyde (MDA) ($p<0.01$) level. Cholesterol, HDL-cholesterol, LDL-cholesterol levels were found to be significantly lower ($p<0.01$, $p<0.05$ and $p<0.01$ respectively), while the triglyceride level was found to be significantly higher ($p<0.01$) in patients with Beta-Thalassemia major than in the control group.

Conclusion: Beta-Thalassaemia patients are mainly exposed to higher oxidative stress of reactive oxygen intermediates due to iron overload hence, they had decreased antioxidants level (Paraoxonase 1, arylesterase , reduced glutathione and catalase) along with hypertriglyceridaemia, hypocholesterolemia ,low HDL-cholesterol levels and also increased malondialdehyde level. so that all these factors contributing to the development of atherosclerosis.

Keywords: **Beta-Thalassaemia- Oxidative stress- Atherosclerosis –Paraoxonase 1- Arylesterase- Reduced glutathione-Catalase- Malondialdehyde .**

1. INTRODUCTION

Beta Thalassemia:

β-thalassemia is a genetic disorder caused by mutations in the human hemoglobin beta (*HBB*) gene. It is a common inherited disease extending from the Mediterranean area through the Middle East to Southeast Asia. Patients homozygous with β-thalassemia mutations have severe anemia and usually require frequent transfusions and iron chelation. (Cavazzana-Calvo et al., 2010 and Xie et al., 2014).β-thalassemia is interrelated with profound anemia characterized by extreme pallor, jaundice, irritability, decreased activity or increased somnolence. hepatosplenomegaly, expanded bone marrow, siderosis, cardiomegaly, impaired erythropoiesis, hemolysis in peripheral circulation and deposition of excess iron in the tissue (Eliezer et al., 2011; Bhagat et al., 2012 and Tangvarasittichai et al., 2012).

Beta thalassemia exists in different forms depending upon the beta globin chains deficit. The most severe form is beta thalassemia major which occurs as a result of inheritance of two beta globin chain mutations either in homozygous or compound heterozygous states. Patients with beta thalassemia major need repeated blood transfusions for survival due to

severe anemia. The beta globin chain deficit for beta-thalassemia trait (minor) is 50%, while that for beta-thalassemia major is 100% and 50–80% for beta-thalassemia intermediate **(Elizabeth and Ann., 2010 and Shazia et al., 2012)**. Transfusion therapy in β-thalassemia major patients requires adequate iron chelation treatments to avoid its progressive accumulation in several organs, evoking subsequent tissue damage and, eventually, death. Although lifelong blood transfusions combined with adequate chelation therapy have significantly improved the survival of β-thalassemia major patients, cardiac complications remain the main cause of mortality in both β-thalassemia major and intermedia **(Modell et al ., 2000; Aessopos et al., 2004 ; 2005 and Stoyanova et al., 2012)**. In addition, arterial and venous thromboembolic events in β-thalassemia major patients have been reported **(Panigrahi and Agarwal., 2007 and Stoyanova et al., 2012)**. Several pathogenic factors contribute to these complications, including, adhesion of thalassemic erythrocytes in microvessels**(Hovav et al., 1999 and Stoyanova et al., 2012)**.

Oxidative stress:

Oxidative stress is defined as the interruption of balance between oxidants and reductants within the body due to the excess production of peroxides and free radicals. This imbalance will cause damage to cellular components and tissues in the body leading to oxidative stress. In patients with beta thalassemia major where frequent blood transfusions are required due to severe anemia**(Pavlova et al., 2007; Ghone et al., 2008 and Shazia et al., 2012),** reactive oxygen species are generated in increased amounts in thalassemic red cells. Conditions such as rupture of erythrocytes, iron overload, and depletion of antioxidants in tissues promote oxidative stress. This implies the possible alteration of redox status in thalassemic patients, which may adversely affect their health. Specific treatments for thalassemia are employed based on many factors such as age of the patients and severity of the disease. The symptoms vary from relatively mild anemia to life-threateninganaemia **(Phumala et al., 2003; Kassab-Chekir et al., 2003 ; Das et al., 2004 and Jetawattana., 2005)** appear after about 2-4 months of age **(Ghone et al., 2008 and Bhagat et al., 2012)**.

The production of free radicals associated with excessive iron-loading is increased in these patients **(Canatan et al., 2001and Arıca et al ., 2012)**. Several lines of evidence support a role for oxidative stress and inflammation in atherogenesis. Epidemiological studies suggest that low levels of antioxidants are associated with increased risk for cardiovascular disease (CVD)**(Harris et al., 2002 ;Jialal and Devaraj .,2003)**.

During the past years many scientific evidences have raised the adverse effect of abnormal blood lipid levels, like total-cholesterol and other lipids and lipoproteins on health causing atherosclerotic disease **(Wilson., 1988** ; **Ginsberg., 1994** ;**Gotto ., 1994 and Ferdaus et al., 2010) .** In recent years, the relationship between the increase in blood lipid levels and atherosclerotic diseases was shown in the performed researches **(Ginsberg., 1994;Gotto., 1994** ; **Daniels et al ., 2008 and Arıca et al ., 2012).**

2. SUBJECTS AND METHODS

The present research was carried out in the department of Molecular Genentics and Enzymes department and Clinical Genetics department, National Research Centre. Study group is consisted of 20 thalassemic patients , their age range between (6 months to 30 years) participated in this study and 20 healthy volunteers. All patients were transfusion dependant. Exclusion criteria included evidence of concurrent infection, hospitalization or receiving blood transfusion for at least one month prior to the start of the study or during the sampling period. The blood samples were obtained following an overnight fasting state and collected into empty tubes and immediately one hundred µl of the blood sample was hemolysed by addition of 0.9 ml bidistilled water for reduced glutathione estimation and the remaining samples were centrifuged at 3000 rpm for 10 min for paraoxonase1, arylesterase, catalase, malondialdehyde and lipid profile determination and all samples were stored at -70 ^{o} C until analysis. All oxidative stress parameters measured by spectrophotometer (Shimadzu UV-1601, Japan). The estimation of paraoxonase1 activity was done according to the method of**Menys et al., 2006**,arylesterase b**y Kuo et al., 1995** method, reduced glutathione by **Samuel et al., 2010** method**,** catalase by **Carter et al., 2004** method, Malondialdehyde as an indicator of lipid peroxidation according to the method of**Mihara and Uchiyama.,1978 .**Cholesterol was determined by the enzymatic method as described by **Thomas ., (1998)** .High density lipoprotein-cholesterol (HDL-C) and Low density lipoprotein-cholesterol (LDL-C) were determined according to method described by **Gordon et al., (1977)**.Triglycerides were determined by the method of **Schettler andNÜssel.,(1975).**

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Descriptive results were reported as mean (standard deviation). A P value of <0.05 was considered as statistically significant.

3. RESULTS

Beta thalassemiapatients had (90%)positive family history and (10%) patients had negativefamily history (figure.1).

Significant decrease was found inparaoxonase1, arylesterase, and catalase activities and reduced glutathione level in beta-thalassemia patients compared to control group (P<0.01in all parameters). While theMDA level in patients was significantly higher in beta-thalassemia patients than in control group (P<0.01). (table.1)and(figure. 2).

Significant decrease was found in cholesterol, high density lipoproteins, cholesterol and low density lipoproteins cholesterol in beta-thalassemia patients compared to control group (P<0.01, P<0.05, P<0.01respectivel).While Triglycerides levels in beta-thalassemia patients were significantly higher than in control group (P<0.01). (table.2)and (figure.3).

Highly significant increase was detected in atherogenic index log(TG/HDL-C) (P<0.001) and significant increase in cardiac risk ratio (TC/ HDL-C) (P<0.05) in beta- thalassemia patients compared to control group (table.3) and (figure.4).

Significant decrease was found in (Pon1/HDL) ratio(P<0.05) in beta thalassemia patients than in control group(table.4) and (figure.5).

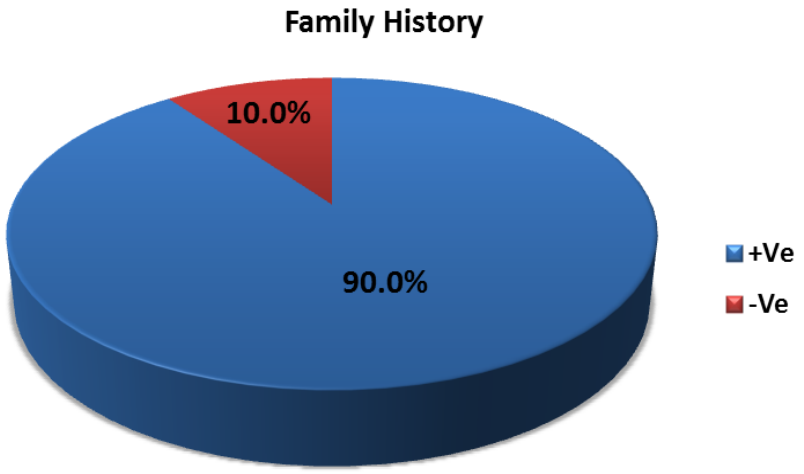

Figure.2: Family history in beta-thalassemia patients

Table. 1: comparison of PON1, ARYL, GR, CAT and MDA means between beta-thalassemia patients and control group.

Enzyme	Control (n=20)	β-Thalassemia subjects (n=20)	P value
	Mean ± S.D.	Mean ± S.D.	
PON1	101.05 ± 11.71	43.30 ± 12.41	(P**<0.01)
ARYL	54.17 ±6.78	24.04 ±5.42	(P**<0.01)
GSH	26.47 ±4.57	8.63 ± 2.63	(P**<0.01)
CAT	42.30 ±9.66	17.17 ±4.78	(P**<0.01)
MDA	4.07 ± 1.12	11.80 ±3.34	(P**<0.01)

P*significant< 0.05

P**highly significant< 0.01

PON1=Paraoxonase1, **ARYL**= Arylesterase, **GSH**=Reduced Glutathione ,**CAT**=Catalase, **MDA**=Malondialdehyde

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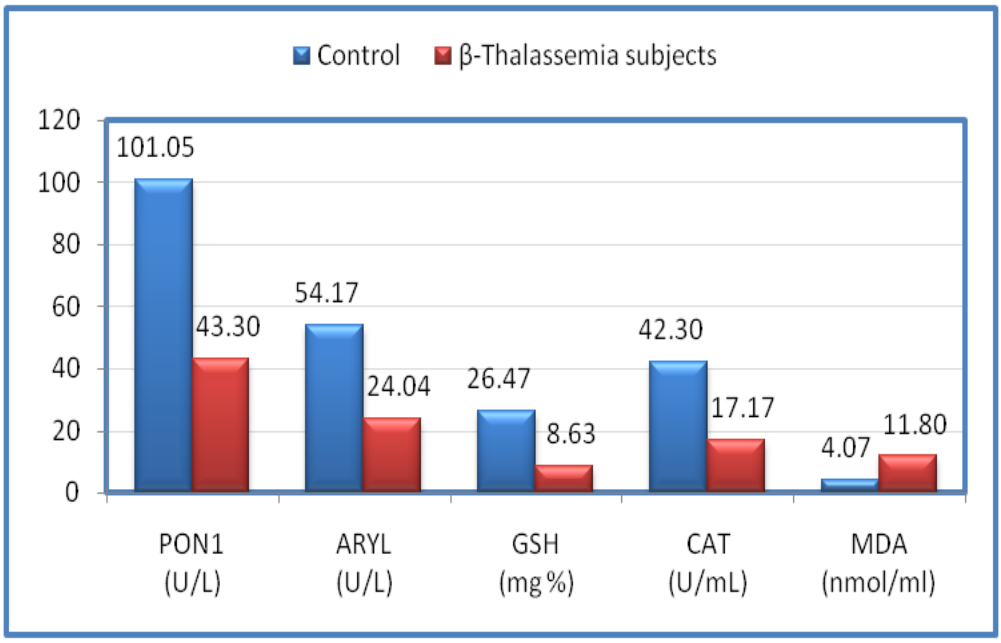

Figure.2: comparison of PON1, ARYL, GR, CAT and MDA means between beta-thalassemia patients and control group

Table.2: comparison of cholesterol, triglycerides, HDL and LDL means between beta- thalassemia patients and control group.

Enzyme	Control (n=20)	β-Thalassemia subjects (n=20)	P value
	Mean ± S.D.	Mean ± S.D.	
Cholesterol	**165.70 ± 15.90**	**109.40 ± 21.61**	**(P**<0.01)**
Triglycerides	**71.10 ± 16.35**	**136.65 ± 26.61**	**(P**<0.01)**
HDL	**46.90 ± 6.90**	**27.60 ± 7.730**	**(P*<0.05)**
LDL	**107.60 ± 17.00**	**53.14 ± 15.43**	**(P**<0.01)**

P*significant (p< 0.05)

P**highly significant (p< 0.01)

HDL= High density Lipoproteins, **LDL**= Low density Lipoproteins

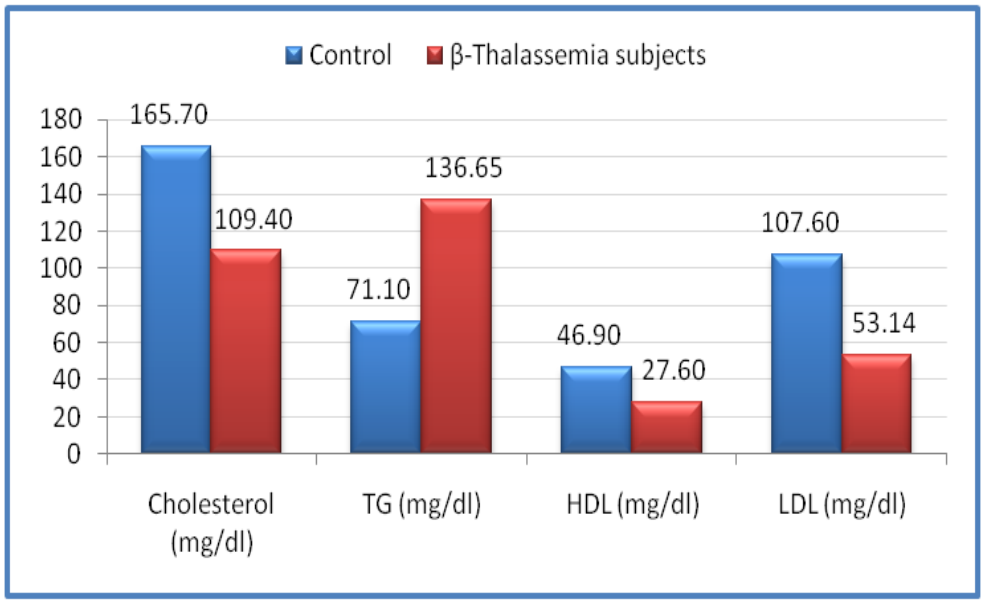

Figure.3: comparison of cholesterol, triglycerides, HDL and LDL means between beta-thalassemia patients and control group

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Table.3: Comparison of atherogenic index Cardiac risk ratio between β-Thalassemia and control group.

Parameter	Control (n=20)	β-Thalassemia subjects (n=20)	P value
	Mean ± S.D.	Mean ± S.D.	
Atherogenic index log(TG/HDL-C)	**0.23 ± 0.20**	**0.69 ± 0.15**	**$p^{**} < 0.001$**
Cardiac risk ratio1 (TC/ HDL-C)	**3.59 ± 0.56**	**4.31 ± 1.37**	**$P^{*} < 0.05$**

p^{*} is significant at ≤ 0.05 level.

p^{**} is significant at ≤ 0.01 level.

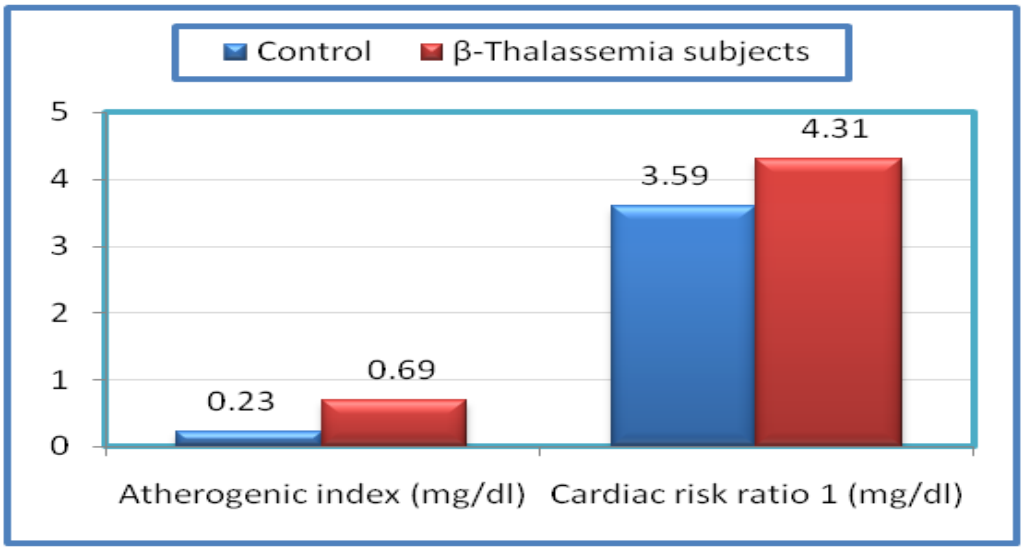

Figure.4: Comparison of atherogenic index Cardiac risk ratio between β-Thalassemia and control group

Table.4: Comparison of Pon1/HDL ratio between β-Thalassemia and control group.

Parameter	Control (n=20)	β-Thalassemia subjects (n=20)	P value
	Mean ± S.D.	Mean ± S.D.	
Pon1/HDL ratio(mmol/L)	**0.084 ± 0.022**	**0.061 ± 0.025**	**P < 0.05**

p^{*} is significant at ≤ 0.05 level.

p^{**} is significant at ≤ 0.01 level.

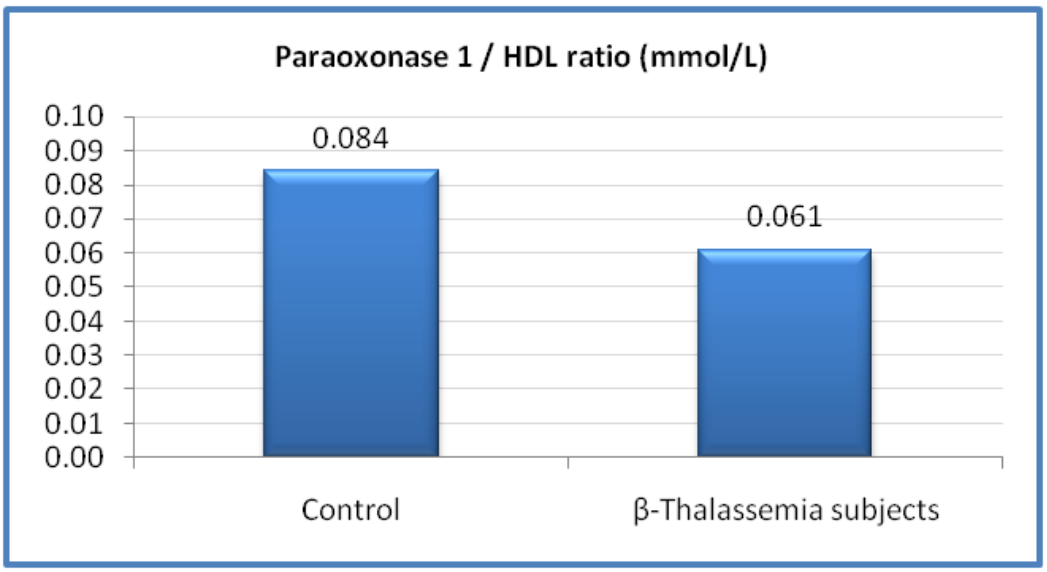

Figure.5: Comparison between mean of (Pon1/HDL) ratio in control group and β-Thalassemia patients.

4. DISCUSSION

Beta-thalassemia is considered the most frequent hereditary blood disorder worldwide. β-thalassemia encompass a wide variety of clinical phenotypes ranging in severity from clinically silent heterozygous β -thalassemia to severe transfusion dependent β-thalassemia major **(Haghpanah et al., 2010 and Labib et al., 2011)**.Oxidative stress in β thalassemia could explain many complications and may have therapeutic implications **(Elsayh et al., 2013)**. β -Thalassemia is a disease of red blood cells, clinical complications in several organs result from oxidative stress induced by iron overload in these patients and atherosclerosis-related vascular complications **(Sonakul et al., 1987 and Labib et al., 2011)**. In beta thalassemia abnormally high levels of oxidative stress account for accelerated and increased destruction of erythrocytes **(Muanprasat et al, 2013)**. Oxidative stress occurs when there is an imbalance between free radical production and antioxidant capacity. This may be due to increased free radical formation in the body and/or loss of normal antioxidant defenses **(Vassalle et al., 2003 and Nabatchian et al., 2004)** that can lead to a critical failure of biological functions and ultimately cell death **(Sayre et al., 2001and Nikolova., 2012).**

β-Thalassemia patients had a similarly affected family member (90.0%). while,**El-Kamah et al., 2009**found that, Positive family history in 34.4% of thalassemia intermedia and thalassemia major. Paraoxonase1 is an antioxidant enzyme that inhibits the oxidative modifications of LDL as it can destroy active lipids in mildy oxidised LDL **(Mackness et al., 1993., Durrington et al., 2001 and Labib.,2011)** Therefore, the decrease in PON1activity together with the decrease in total antioxidant capacity(TAC) leads to an increase in lipid peroxidation and oxidative modification of lipoproteins that may lead to an increase in atherogenic risk.This study revealed that Paraoxonase1 and arylesterasewere significantly lowerin beta thalassemia patients than in the control group ($P<0.01$). The values of serum Paraoxonase1 and arylesterase in beta-thalassemia patients were 2.3 and 2.6 times respectively lower in beta-thalassemia patientsthan in healthy control group. These findings denoted that these patients suffered from the effect of increased oxidative stress.**Livrea et al. 1998 and Barrano et al., 2000 Cakmak et al., 2009** reported that BTM patients had decreased paraoxonase1and arylesterase activities.**Aydin et al., 2012** results showed that there was a highly significant decrease in serum PON1 and serum TAC levels in beta-thalassemia intermediate (BTT) compared with control group**. Selek etal., 2007 and Aydin et al., 2012** noted decreased levels of serum PON1 activity and increased oxidative stress in β-thalassaemia minor.

In the current study, Reduced glutathione (GSH), an antioxidant which prevents damage to the cellular components were measured in beta thalassemia patients. catalase (CAT), responsible for detoxification of hydrogen peroxide in the cells. Reducedglutathione level and catalase activity had highly significant decrease in beta- thalassemia patientsthan in control group. Level of reduced glutathione were 3.1 times lower in beta-thalassemia patients than in healthy control group. These results weresimilar tothose of**Ruchaneekornet al., (2010) and Attiaet al., (2011)** wherelow level of reduced glutathionewas detected ,the decrease was 3.2 times lower than in the healthy control . glutathione (GSH) is a major interacellular reducing agent which is very sensitive to oxidative pressures and has several important functions such as: protection against oxidative stress and regulation of gene expression**(Attiaet al., 2011).**The data of the present study reported a deficiency in levels of catalase, which is 2.46 times lower in beta-thalassemia patients than in healthy control. These results weresimilar tothose of**Ruchaneekornet al., (2010)** and **Attiaet al., (2011),** wherelow activity of catalasewas detected ,the decrease in activity was 2.75 times lower in beta-thalassemia patients than in the healthy control group . A possible explanation for lower red cell catalase activity found in the more severe genotype of beta thalassemia is that the greater amount of hydrogen peroxide might produce direct toxic damage to catalase **(Eaton et al., 1972., Kirkman and Gaetani ., 1984 and AL-Mudalal et al., 2005)**, the concentration of this is considerably reduced in conditions of high oxidative stress **(Consolini et al., 2001 and AL-Mudalal et al., 2005).**

Malondialdehyde (MDA), which is the end product of the primary reactions that lead to the significant oxidation of such polyunsaturated fatty acids in cellular membranes and, thus, serves as a reliable marker of oxidative stress **(Irmak et al., 2003 and Yildirim et al., 2011),** is generated in excess amounts in β-thalassemia.MDA is a bifunctional reagent and has been reported to crosslink several cell constituents including membrane components. A cross-linked erythrocyte membrane is expected to be rigid and this could probably explain the rigidity of thalassemic erythrocytes when compared to normal ones. Further, erythrocyte deformability is a major determinant of anemia in thalassemia **(Lang et al., 2002 andAttia et al., 2011)** . In one of the previous studies, free and total MDA was found to be higher in regularly transfused thalassemia major patients than in the thalassemia intermedia patients **(Cighetti et al., 2002 and Attia et al., 2011)**. As a result of continuous blood transfusions, the patients might be subjected to peroxidative tissue injury by the secondary iron overload. These findings might support the idea of iron overload in β-thalassemia leads to an enhanced generation of

reactive oxygen species and oxidative stress. **Das et al., (2004)** and **Ghone et al., 2008Abd El-Maksoud et al., (2009) and Elalfy et al., (2013)** reported an increase in plasma malondialdehyde (MDA) level, as measured by the thiobarbituric acid reaction substance (TBARS) methods, in β-thalassemia patients, which is an indicator of an increase in lipid peroxidation and the ongoing oxidative stress in beta-Thalassemia patients, which is a factor of increased parthenogenesis and coronary heart disease. In addition the current study measured the values of MDA in beta-thalassemia patients and found a highly significant increase in beta-Thalassemia patients than in healthy control group($P<0.01$). **Naithani et al.,(2006) and Walter et al., (2006)**, explained the significant increase in MDA in their thalassemic patients to be due to excess unpaired α -hemoglobin chains which are more prone to denaturation and oxidation. Furthermore, in **Aydin et al., (2012),** reported a highly significant increase in the level of MDA (as a marker of lipid peroxidation) in BTT subjects compared with controls. In the present study, malondialdehyde concentration was 2.9 times higher in β-thalassemia patients than in healthy control. Similarly, **Attia et al., (2011)** reported a high elevation in the malondialdehyde concentrations 3.4 times higher in β-thalassemia patients than in healthy control group.

Thalassemic patients are at much higher coronary risk than their matched controls, because of the low HDL cholesterol production, even if they are within normal values of total cholesterol (**Giardini et al., 1978 and Patne et al., 2012).**In addition, **Patne et al., (2012)** observed that total serum phospholipids, their fractions and cholesterol were significantly lower among patients with thalassemia major. Increased concentrations of triglycerides (TG) were observed in most published studies on lipid profiles of thalassemic patients.The LDL-C–lowering effect of thalassemia is a well-known entity in all forms of thalassemia syndromes **(Calandra et al., 2004 and Cakmak et al., 2009).** Increased uptake of LDL-cholesterol by the bone marrow to provide cholesterol for the increased proliferation of erythroid progenitor cells and increased production of inflammatory cytokines that reduce the hepatic secretion and increase the catabolism of LDL-cholesterol has been suggested to be responsible for the low LDL-cholesterol levels observed in thalassemia patients **(Calandra et al., 2004 and Cakmak et al., 2009)** .Increasing evidence suggests that the oxidative modification of low-density lipoprotein (LDL) is the key step in the sequence of events leading to atherogenesis-related vascular alternations **(Steinberg et al., 1989; Berliner et al., 1996 and Selek et al., 2007)** hence, In this study, we evaluated low total serum cholesterol, low HDL-cholesterol and low LDL cholesterol levels were significant lower in beta thalassemia patients than in control group with elevation of triglycerides in beta thalassemia patients, as compared to control subjects. These results were in agreement with previous reports with regarding the above altered serum lipid pattern **Goldfarb et al., (1991)**; **Maioli et al ., (1997); Hartman et al., 2002 ; Cakmak et al., (2009); Madaniet al., (2011) and Bordbar et al., (2012)Patne et al., (2012**).The decrease in LDL-C and TC levels appears to have a protective effect against atherosclerosis. However, the modification of LDL in the arterial wall, particularly by oxidation, appears to be more important than the level of LDL-C in the development of atherogenesis. This hypothesis is supported by the assumption of **Haghpanah et al., (2010),** stated that at any level of serum cholesterol, there was a wide variation in the incidence of coronary heart disease, and oxidation is crucial to the cellular uptake of LDL in the first stages of atherosclerotic plaque formation **(Aydin et al., 2012)**. Lipid abnormality has been frequently reported in thalassemia, but its pathophysiology is not totally clear **(Meral et al., 2000; Calandra et al., 2004 and Patne et al., (2012**). Different results were obtained in studies in terms of explaining the serum lipid changes observed in patients with beta-thalassemia major (B-TM). Liver damage **(Maioliet al., 1984 and Arıca et al., 2012)**, low activity of hepatic and extrahepatic lipase enzymes **(Cherchi et al., 1983 and Arıca et al., 2012),** and the quick cleaning of modified HDL and LDL (richer than triglyceride, poor, cholesterol ester) by activated monocytes and macrophages were held responsible. There are many factors for these blood lipid changes in children with B-TM such as excessive iron loading (high ferritin values), liver damage (deterioration of the ratio between AST and ALT) and hormonal disorders **(Cherchi et al., 1983; Maioliet al., 1984 ; Goldfarb et al., 1991 Papanastasious et al., 1996 and Arıca et al., 2012).**Some studies have suggested that low blood cholesterol values may occur as a result of an increase of erythropoiesis in patients with B-TM and increase of LDL uptake by macrophages and histiocytes that exist in reticuloendothelial system (RES) **(Maioliet al., 1989 &1997 and Arıca et al., 2012).** A study demonstrated that total phospholipids and its functions also decrease with the decrease of total cholesterol. In the same study it was shown that the levels of serum lipid multiple unsaturated fatty acids decreased. Those changes appear as a result of excessive iron-loading and liver damage **(Giardini et al., 2004 and Arıca et al., 2012).**

Brewer et al., (2003) and Patne et al., (2012) observed that the HDL cholesterol in thalassemic patients had very low values. Studies suggest that risk for myocardial infarction is high when HDL cholesterol is low. Also they highlight the importance of total-to-HDL cholesterol ratio for the evaluation of blood lipids and the prevention of atherosclerotic disease. It has also been reported that the total cholesterol-to-HDL cholesterol ratio predicts coronary heart disease risk

regardless of the absolute LDL- and HDL-cholesterol. According to **Groveret al., (1999)**, the ratio of LDL-c/HDL-c or TC/HDL-c is the best related predictor of future cardiovascular events. Later TG/HDL-c was shown to be a more accurate predictor of heart diseases **(Gotto ., 1998)** . Atherogenic index is the ratio calculated as log (TG/HDL-c). Existence of hypertriglyceridemia will increase the activity of hepatic lipase (HL) which results in the increase of HDL-c catabolism (degradation of HDL-c). Each degradation of 1mg HDL-c will correlate with 2% increase in the risk coronary heart disease (CHD) **(Dobiasova, 2004 and Susanti et al., 2010).**Hence, this study calculated Atherogenic index (TG/HDL-c) was highly significant increased and cardiac risk ratio (TC/HDL-c) was significantly higher in beta thalassemia patients than control group.

This study revealed that, reduction in paraoxonase1 activity was due to HDL decrease, we standardized the enzyme activity for HDL concentration, (PON/ HDL) which was significantly lower in beta thalassemia patients as compared to control group these results were in agreement with**Aviram et al., (2000); Labib et al., (2011) and Aydin et al., (2012),**who found that PON1 is mostly responsible for the antioxidant activity of HDL, to inhibit HDL oxidation and to preserve the anti-atherogenic function of HDL.

5. CONCLUSION

Patients with β-thalassemia are mainly exposed to higher oxidative stress of reactive oxygen intermediates due to iron overload. These productsoxidize various erythrocyte components including membrane lipid with excess production of malondialdehyde (MDA), a product of lipid peroxidation (which is a factor of increased atherogenesis and cornary heart diseases) MDA was found in β-thalassemia patients to be significantly increased with increasing depletion of antioxidants such as (paraoxonase-1, arylesterase, reduced glutathione and catalase). These antioxidants play an important role in the defense mechanism that protects the body from free radical or promoting their decomposition,especially decreased serum PON1 (the only predictor for an increase in atherosclerosis and coronary heart disease).

On the other hand, the current study revealed that thalassaemia patients had hypertriglyceridaemia, hypocholesterolemia and low HDL -cholesterol levels. All these pathophysiological factors contributing to the development of atherosclerosis.

If these laboratory findings can be translated into clinical applications, we may have a truly powerful means of blocking atherosclerosis development and progression in beta thalassemia subjects.

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