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Haptoglobin gene polymorphism and oxidative stress effects on ovarian reserve in Beta-Thalassemia major women

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ABSTRACT

Objective: to study the impact of haptoglobin gene polymorphism on iron overload, oxidative stress and anti-mullerian hormone in BTM women in Egypt.

Methods: case-control study. 47 BTM women, aged between 16-26 years and 47 age-matched regularly menstruating women as control. Haptoglobin Hp1/2 gene polymorphism by PCR, Hemoglobin electrophoresis, serum haptoglobin, ferritin, malondialdehyde MDA, FSH, LH, estrogen and ant-mullerian hormone levels were all analyzed.

Results: serum ferritin level and Serum MDA, showed a significant higher level in thalassemic group, p<0.001. Significant lower levels of LH, E2 and AMH were found in thalassemic group compared to the control p<0.001. A significant difference (p<0.05) between both groups as regarding distribution of haptoglobin gene polymorphism was detected. In thalassemic group, Hp2-2 was the most frequent. (odds ratio 2.616 at 95% CI, 1.134-6.033). A significantly lower AMH level in patients with Hp2-2 gene polymorphism.

Conclusion: beta thalassemic patients are at great risk of oxidative stress. Haptoglobin gene polymorphism has a great impact on the extent of oxidative stress in these patients. BTM patients have lower serum AMH levels than the age -matched control. AMH significantly negatively correlated with serum ferritin and MDA. This was more evident in patients with HP2-2 genotype.

Keywords: thalassemia, haptoglobin gene polymorphism, oxidative stress, ovarian reserve, MDA, anti-mullerian hormone.

SOMMARIO

Obiettivo: studiare l'impatto di aptoglobina polimorfismo del gene sul sovraccarico di ferro, lo stress ossidativo e anti-mullerian ormone in beta-thalassemia major de donne in Egitto.

Metodi: caso-controllo in studio terziario centro medico. 46 beta-major di donne di eta compresa tra i 16-26 anni sono stati inclusi nello studio. 47 pari età sani durante il periodo mestruale regolarmente le donne e servitor come controllo. Aptoglobina Hp1/2 polimorfismo del gene mediante PCR, emoglobina elettroforesi, siero aptoglobina, ferritina,la malondialdeide MDA livello, FSH, LH, estrogeni e anti-mullerian i livelli ormonali: sono stati analizzati tutti.

Risultati: livelli siericidi di ferritina sierica e MDA, ha monstrato un livello significantivamente superior nel gruppo thalassemia, p<0.001. Abbassamento significativo dei livelli di LH, E2 e AMH sono stati trovati in thalassemia gruppo rispetto al controllo p<0.001. Una differenza significativa p<0.05 fra i due gruppi per quanto riguarda la distribuzione di aptoglobina polimorfismo del gene e stata rilevata. In thalassemic grappo, Hp 2-2 e stato il piu frequente e seguita da Hp 2-1 quindi Hp 1-1 (odds ratio 2.616 al 95% CI 1.134-6.033). Un significativamente inferior livello di AMH in pazienti con Hp 2-2 poliorfismo del gene.

Conclusione: o pazienti Beta thalassemi sono a grande rischio di stress ossidativo. Aptoglobina poliformismo del gene ha un grande impatto sulla misura di stress ossidative in questi pazienti. BTM pazienti hanno inferiore AMH nel siero livelli rispetto a quelli dell'eta-adattato per il controllo. AMH significativamente correlate negativament con la ferritina sierica e MDA. Questo e stato pui evidente nei pazienti con Hp2-2 genotipo.

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INTRODUCTION

Beta thalassemia major β-TM is the most common genetically-determined chronic haemolytic anaemia in Egypt, as in many other Mediterranean countries. It has been estimated that 1000 children out of 1.5 million live births are born annually with β-TM, with a carrier rate of 9-10%[1]. β -thalassemia occurs when there is a quantitative reduction of β globin chains that are usually structurally normal. They are caused by mutations that affect the β globin locus and are extremely heterogeneous. These genetic defects lead to a variable reduction in β globin output ranging from a minimal deficit (mild β+ thalassemia alleles) to complete absence (β0 thalassemia)[2].

In β-thalassemia, the synthesis of normal α globin chains from the unaffected α globin gene continues as normal, resulting in the accumulation within the erythroid precursors of excess unmatched α globin. The free α globin chains are not able to form viable tetramers and instead precipitate in the red cell precursors in the bone marrow forming inclusion bodies. They are responsible for the extensive intramedullary destruction of the erythroid precursors and hence the ineffective erythropoiesis that underlies all β-thalassemia. Anemia in β-thalassemia thus results from a combination of ineffective erythropoiesis, peripheral hemolysis, and an overall reduction in hemoglobin synthesis[3]. The life expectancy of patients with TM has significantly increased in recent years with subsequent increase in the reproductive potential and desire to have children. However, complications are still frequent and affect the patient's quality of life[4]. β-TM female patients usually suffer from hypogonadotropic hypogonadism associated with amenorrhea, anovulation and infertility. Infertility is assumed to be mainly from iron deposition in the hypothalamic-pituitary-ovarian axis, but recent evidence attribute it to iron-induced oxidative stress[5].

Oxidative stress occurs when reactive oxygen species ROS exceeds anti-oxidant capacity. These oxygen free radicals are unstable, highly reactive and capable of initiating uncontrolled chain reactions, resulting in cellular damage[6]. In the cell membrane, lipid peroxidation begins when electrons from lipids reacts with unstable free radicals promoting a chain reaction with successive oxidation that results in lipid instability and formation of byproducts such as malondialdehyde MDA, a highly toxic molecule. Thus, serves as a reliable marker of oxidative stress[7].

Haptoglobin, Hp is an abundant acute phase plasma glycoprotein of the α 2-globulin fraction that binds free hemoglobin Hb forming a stable Hp-Hb complex cleared through endocytosis by the macrophage scavenger receptors CD 163 and inhibits the dissociation of ferric heam from globin, thus preventing oxidative damage[8]. Several Hp-genotyping methods based on conventional polymerase chain reaction PCR have been developed. Hp genotypes have different biophysical characteristics and functional efficacies that account for their distinict anti-oxidant capacities[9].

Previous research showed that Hp 1-1 is more effective than Hp 2-2 in preventing Hb-induced oxidation of plasma components. It was found that Hp 2-2-Hb complexes are cleared more slowly than Hp 1-1-Hb complexes, which may allow the Hb which is complexed with Hp 2-2 more opportunity to react with oxidizing agents [10].

Ovarian reserve refers to residual follicles that are available for procreation, recent studies have shown that anti-mullerian hormone AMH and antral follicle count are equally effective in testing ovarian reserve[11]. Moreover, it has been demonstrated that performing both together doesn't increase the predictive power[12]. Early follicle development, before secondary follicle recruitment, is largely gonadotrophin independent. AMH serum levels are not affected by dominant follicle growth during the late follicular phase of the normal menstrual cycle. This renders AMH easy to use clinically as opposed to other currently available markers of ovarian aging, such as inhibin B, estradiol (E2) and FSH, which are all menstrual cycle dependent and constitute relatively late markers of the ongoing process of primordial follicle pool depletion[13].

OBJECTIVES

To study the impact of haptoglobin gene polymorphism on iron overload and oxidative stress in beta-thalassemia major women in Egypt, and their subsequent influence on AMH level as a marker of ovarian reserve.

MATERIAL AND METHODS

After obtaining the approval of both Medical Research Institute Ethics Committee and Alexandria faculty of medicine Ethics Commitee on the study protocol, and an informed consent from all participants or their parents, 94 women were included in the study.

47 females with established β-TM followed up in the Haematology Clinic of Student Sporting Hospital, as well as the Haematology department, Medical Research Institute from September 2015-June 2016. They were previously diagnosed as having β-TM by clinical signs, hemoglobin electrophoresis and were followed up regularly in haematology clinic. All were on regular transfusion of 350-500 ml of packed RBCs at 2-4 weeks interval for more than 10years. All were adherent to iron chelation therapy using desferrioxamine DFO at a dose 20-50 mg/kg/day. Patients with apparent acute infection were excluded. The presence of an acute-phase reaction was excluded by measurement of C-reactive protein CRP. If CRP >6mg/L, they were excluded from the study. 47 healthy regularly menstruating females, of matched age served as control. None of the control were thalassemia minor and none had a history of previous blood transfusion, anemia, liver diseases, or active inflammatory conditions, previous pelvic surgery and were not taking any medication.

The menstrual pattern, age at menarch, presence of amenorrhea either primary or secondary were obtained by self -reporting. Blood samples from all regularly menstruating women were taken in the early follicular phase to obtain basal levels of E2, FSH, AMH. For those having with amenorrhea or irregular mensis, blood samples were collected randomly. A cut-off value of AMH ≤ 1ng/ml was used to predict poor ovarian reserve[14].

Haptoglobin Hp level was quantitatively determined by means of immunonephelometry automated chemistry analyzer (BN ProSpec system, Siemens). Using anti sera (NAS HAPT), a liquid animal sera which produced by immunization of rabbits with highly purified human Hp. Hp in serum form immune complexes in an immunochemical reaction with specific antibodies. These complexes scatter a beam of light passed through the sample; the intensity of the scattered light is proportion of the Hp in the serum.

Serum malondialdehyde MDA, determined as thiobarbituric acid reactive substances (TBARS) according to the method of Draper and Hadley. MDA has been identified as the product of lipid peroxidation that reacts with TBA to give pink species absorbing at 532nm. The unknown MDA containing samples are first reacted with TBA at 95° C and at low PH. After a brief incubation, the samples and standards can be read spectrophotometrically. The concentration (nmol/ml) of MDA in sample was obtained from a standard curve made by preparing serial dilutions of tetramethoxypropane TMP 1,2,4,6.8.12 nmol/ml, in ethanol treating them like the sample.

Genotyping of Hp polymorphism by conventional PCR: Venous EDTA blood samples were obtained and nuclear DNA was isolated from blood using Fermentas whole blood genomic DNA isolation kit (Fermentas, EU) according to the manufacturer instructions. Genotyping of Hp was assayed by PCR based DNA amplification of a 1757-bp Hp 1 allele-specific sequence and 3481-bp Hp2 allele specific sequence using four primers set.

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

Two sets of PCR reactions were performed. After electrophoresis of the reaction products in 1% agarose gel, Hp genotyping-specific banding patterns were obtained. With primers A and B, Hp1-1 and Hp 2-2 genotypes were characterized by single bands representing the 1757 and 3481 bp products. Primers C and D were used for detecting 349 bp Hp-2 allele specific product. Serum concentrations of FSH were measured using Chemiluminescent Microparticle Immunoassay (CMIA), (ARCHITECT system, Abbott Laboratories, Abbott Park, IL, USA) expressed as mIU/ml. Serum AMH was measured by enzyme-linked immunosorbent assay (ELISA) kit (Immunotech-Beckman Coulter, Webster, TX, USA) and expressed in ng/ml.

STATISTICAL ANALYSIS

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. Qualitative data were described using number and percent. Quantitative data were described using median, range (minimum and maximum) or mean, standard deviation and significance of the obtained results was judged at the 5% level. Chi-square test, for categorical variables, to

compare between different groups. Student t-test, for normally quantitative variables, to compare between two studied groups. Mann Whitney test, for abnormally quantitative variables, to compare between two studied groups. Odd ratio (OR):used to calculate the ratio of the odds and 95% Confidence Interval of an event occurring in one risk group to the odds of it occurring in the non-risk group. Kruskall–Wallis test was performed where appropriate. Multivariate linear regression analysis for serum AMH, ferritin and MDA after log transformation to correct the heterogeneity of variance, using Wilcoxon signed rank test.

RESULTS

The study was conducted on 47 β-TM women, aged between 16-26 years, mean 21.4±2.7, a positive family history was found in 24%, a positive consanguinity between parents was found in 32%. As regards frequency of blood transfusion, 52% had regular transfusion every 2 weeks, 16% had transfusion every 3 weeks and 32% had less frequent transfusion every 4 weeks. The age at first transfusion was given ranged from 6 to 36 months of age, with mean of 12.9±7.58 months.

All beta-TM patients were receiving DFO chelation therapy regularly, 32% by subcutaneous infusion using electronic infusion pump over 8-12 hours, 3-5 days per week, 44% via intramuscular route and 24% via intravenous route. 47 age matched with mean 20.5±3.3, regularly menstruating females served as control. Using the definition of hypo-gonadotrophic hypogonadism as FSH and LH levels ≤ 2 mIU /ml with accompanying E2 level ≤ 20pg/ml, 21 of 47 β-TM (44.6%) suffered from it. Primary amenorrhea was found in 10/47 (21%) and secondary amenorrhea in 11/47 (23.4%). Poor ovarian reserve taking ≤1ng/ml as a cut-off level[14] was found in 6/47 patients (12.5%). None of the patients was on hormone replacement therapy and all women were virgin, eliminating the possibility of transvaginal ultrasound for antral follicular count AFC, leaving AMH as the ideal choice for ovarian reserve assessment in these patients.

Both groups were compared against several laboratory items **(table 1)**, hemoglobin level Hb was significantly lower in thalassemic group p<0.001, with mean 6.7±1.2 reflecting a range far away from the optimum target. At the same time, iron overload represented by serum ferritin level showed a significant higher level p<0.001. Serum MDA, the chosen marker of oxidative stress showed a significant higher level in thalassemic group, p<0.001. Moreover, significant lower levels of LH, E2 and AMH were found in thalassemic group compared to the control p<0.001. The data in **table 2**, shows significant difference (p<0.05) between both groups as regarding distribution of haptoglobin gene polymorphism.

Table 1.
Comparison between the two studied groups according to different parameters

	Cases (n=47)	Control (n=47)	p
Age	21.4 ± 2.7	20.5 ± 3.3	0.138
Serum Ferritin	3371.5±2220	98.0±45.7	<0.001*
HP	20.3±22.3	96.4±30.2	<0.001*
Serum MDA	3.5±1.9	0.9±0.3	<0.001*
HB	6.7 ± 1.2	13.2 ± 0.9	<0.001*
FSH	5.4±4.1	5.3±1.1	0.555
LH	2.6±1.0	6.4±2.3	<0.001*
E	31.5 ± 13.4	53.9 ± 17.7	<0.001*
AMH	1.6 ± 0.6	3.1 ± 0.5	<0.001*

Table 2.
Comparison between the two studied groups according to HP poly

	Cases (n=47)	Control (n=47)	p	OR	95% CI LL	95% CI UL
HP poly						
HP1-1	6 (12.8%)	9 (19.1%)	0.398	0.618	0.201	1.900
HP2-1	14 (29.8%)	22 (46.8%)	0.090	0.482	0.206	1.126
HP2-2	27 (57.4%)	16 (34.0%)	0.023*	2.616	1.134	6.033
Allele frequency						
HP1	26(27.7%)	40(42.6%)	0.032*	0.516	0.281	0.949
HP2	68(72.3%)	54(57.4%)		1.937	1.053	3.564

In thalassemic group, Hp2-2 was the most frequent followed by Hp2-1 then Hp1-1 (odds ratio 2.616 at 95% CI, 1.134-6.033), with allele frequency Hp-2 72.3%. In contrast to the control group, in which Hp2-1 was more frequent than Hp2-2 followed by Hp1-1, with allele frequency Hp-2 57.4%. **Table 3** illustrates a correlation between the three haptoglobin genotypes with serum haptoglobin, serum ferritin, MDA and AMH in thalassemic group.

Table 3.
Relation between HP gene polymorphism with different parameters

	HP poly HP2-2 (n= 27)	HP2-1 (n= 14)	HP1-1 (n= 6)	P
HP	7.0 (1.9 – 99.0)	14.0 (2.8 – 49.5)	27.8 (5.0 – 74.0)	0.139
Serum Ferritin	3100.0(890.0–9830.0)	2689.0(900.0–8400.0)	2900.0(1200.0–7000.0)	0.600
Serum MDA	3.5 (1.10 – 9.10)	2.75 (1.10 – 7.60)	1.85 (1.10 – 4.60)	0.040*
AMH	1.30 (0.50 – 2.30)	1.90 (1.50 – 3.20)	1.85 (1.40 – 2.60)	<0.001*

Quantitative abnormally distributed data was expressed in median (Min. - Max.) And was compared using Kruskal Wallis Test.

Hp was lowest with Hp2-2 and serum ferritin was highest in the same Hp genotype but with no statistic significant difference. A significant higher MDA p<0.05 and a significant lower AMH p<0.001 **(figure 1)** was in patients with Hp2-2 genotype.

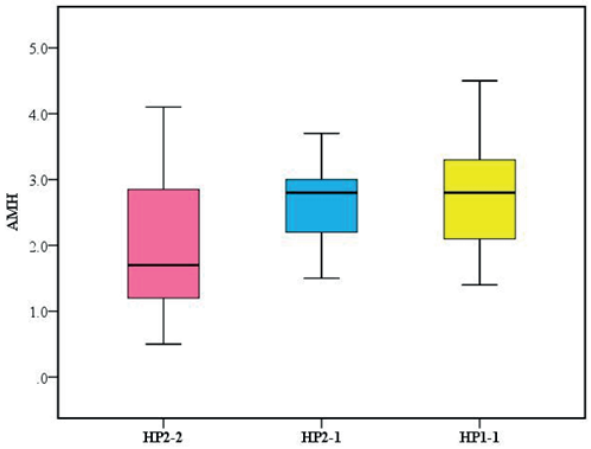

Figure 1.
Correlation between serum AMH, ferritin and MDA in cases group

Multi-variant regression analyses were done to determine the factors that correlate with AMH, serum ferritin and MDA in the thalassemic group **(table 4)**.

Table 4.
Multivariate linear regression analysis for serum AMH, ferritin & MDA

	AMH			(log) Serum Ferritin			(log) Serum MDA		
	β	SE	p Value	β	SE	p Value	β	SE	p Value
Age	-0.055	0.019	0.374	0.070	0.011	0.110	-0.074	0.008	0.278
(log) Ferritin	-0.532	0.166	<0.001*	-	-	-	0.590	0.069	<0.001*
(log) HP	0.138	0.157	0.163	-0.238	0.089	<0.001*	-0.213	0.065	0.051
(log)MDA	-0.080	0.257	0.412	0.242	0.144	<0.001*	-	-	-
(log)FSH	0.097	0.238	0.157	0.007	0.145	0.879	0.094	0.100	0.216
(log) LH	-0.016	0.380	0.878	-0.323	0.200	<0.001*	0.163	0.157	0.146
(log)E	0.139	0.004	0.094	0.004	0.002	0.950	-0.173	0.002	0.059
(log)AMH	-	-	-	-0.268	0.060	<0.001*	-0.098	0.045	0.412

When AMH was used as the dependent variable, only serum ferritin of all the explanatory variables was found to correlate significantly and inversely with serum AMH (B= -0.532, SE=0.166, p<0.001).

When serum ferritin was used as the dependent variable; haptoglobin, LH, and AMH of the explanatory variables were found to correlate significantly and inversely with serum ferritin (B= -0.238, SE=0.0.089, p<0.001), (B=-0.323, SE=0.2, p<0.001) (B= -0.268, SE=0.06, p<0.001) respectively. While MDA correlated significantly positive with serum ferritin (B= 0.242, SE=0.144, p<0.001). When MDA was used as the dependent variable, only serum ferritin of all the explanatory factors correlated significantly positively with MDA (B= 0.59, SE=0.069, p<0.001).

Finally, **figure 2**, demonstrate the significantly lower AMH level in patients with Hp2-2 gene polymorphism.

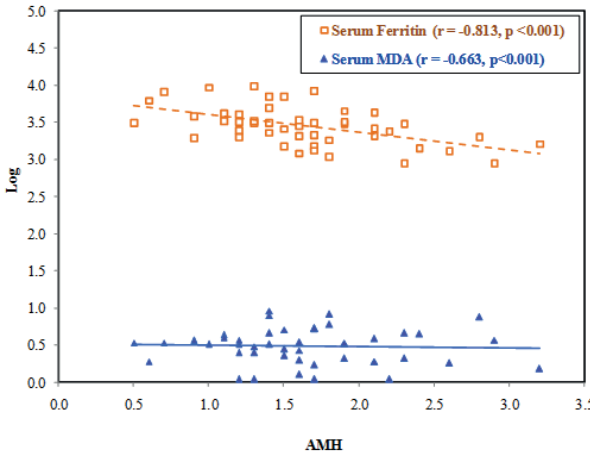

Figure 2.
Relation between HP gene polymorphism with AMH

DISCUSSION

In the past; reproductive and sexual health were considered irrelevant to β-TM patients as they rarely survived beyond adolescence. Improved medical treatment for these patients introduced in the late 1970s, involving regular optimum red blood cell transfusion and daily subcutaneous iron chelation therapy, has significantly decreased patient morbidity and increased survival beyond adolescence into middle age. As a result, patient care has expanded to include encouraging these patients to aspire to the same sexual, and reproductive goals as their peers[15]. According to the current evidence-based knowledge, a significant prooxidants/antioxidants imbalance with subsequent increased oxidative stress exists in patients with β-TM, which is mainly caused by tissue injury due to overproduction of free radicals by secondary iron overload, alteration in serum trace elements, and alteration in antioxidant enzymes level[16].

In the present study, a discrepancy in the prevalence of Hp genotypes in β-TM patients versus control was found. While Hp2-2 was more common in thalassemic patients, Hp2-1 predominated in the control group. The variable capacity of the three Hp phenotypes to prevent

oxidative stress induced by free Hb could be related to differences in their affinity for Hb. The protective effect is more marked for Hp1-1 than Hp2-1 and Hp2-2. Studies have demonstrated that internalization of Hb-Hp complex by CD 163 receptors is more potent for Hb-Hp 2-2 complex than for that bound to Hp1-1 or Hp 2-1. Hence, it was proposed that individuals with Hp2-2 phenotype are under greater oxidative stress[17]. We also demonstrated, very high ferritin levels in the β-TM. Furthermore, the study showed that HP2-2 was associated with the highest serum ferritin.

In this study, a significant higher level of MDA, a marker of lipid peroxidation and oxidative stress was found in BTM group versus control ($p<0.001$) reflecting a state of significant oxidative stress in patient group. More importantly, is the highest level of MDA significantly found in patients with Hp2-2 phenotype as compared with the other phenotypes ($p=0.005$). Our results support the finding of Blum et al[18] who reported that the Hp-2 protein is associated with increased generation of oxidative active iron, while Hp-1 protein is associated with increased production of antioxidant cytokine interleukin IL-10 in diabetic mice with myocardial infarction. They stated that Hp-1 phenotype has an antioxidant and anti-inflammatory properties.

In a similar study evaluating the reproductive capacity in thalassemic women, the study concluded that AMH served as such a better marker than AFC, which in many cases is lower despite a normal AMH. Thalassemia women had a considerably lower ovarian volume compared with reported normal controls (1.25 ± 1.2 cm3 vs 6-6.6 cm3)[19] also representing impaired ovarian reserve, probably a result of a halt in follicle maturation because of lack of gonadotropin stimulation and direct iron toxicity to ovarian tissue[20].

Meanwhile, the previous studies concluded that labile, potentially toxic, iron plays a major role in causing reproductive tissue damage,[21,22] suggesting a role for "free" iron in the pathogenesis of impaired fertility in β-TM women, our study demonstrated a significant correlation between the high ferritin level, with high MDA oxidative stress marker and low AMH levels. Moreover, β-TM women with Hp2-2 genotype found to be the most affected from the resulting higher oxidative stress. Accurate ovarian reserve testing may motivate some women to start a family at an earlier age (or alternatively apply fertility preservation by means of oocyte freezing) or alternatively reassure others that postponing childbearing will not interfere with her chances to achieve a pregnancy later on [13]. This matches a recent study which concluded that patients receiving chronic transfusion and heavy metal overload are at high risk of being associated with impaired gonadal function. Furthermore, elective cryopreservation of ovarian tissue or oocytes should possibly be discussed to preserve future fertility[23].

Beta thalassemic major patients are at great risk of oxidative stress. Haptoglobin gene polymorphism has a great impact on the extent of oxidative stress in these patients. β-TM patients have lower serum AMH levels than the age – matched healthy regularly menstruating control. AMH significantly negatively correlated with serum ferritin and MDA, marker of oxidative stress. This affection was more evident in patients with HP2-2 genotype. Thus, concluding that the higher the oxidative stress is, the more impaired ovarian function might be existed in β-TM women.

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None

DISCLOSURE

The results of this manuscript have not been distorted by research funding or conflicts of interest.

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