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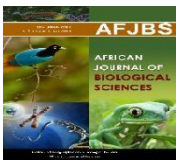

ResearchPaper OpenAccess

The correlation between HBBP1 gene mutation, imbalance of zinc and copper levels, and iron overload in pediatric Egyptian β-thalassemia patients

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Abstract:Background: β-thalassemia is a group of inherited blood disorders marked by decreased (β+) or absent (β0) production of the beta globin chain of hemoglobin tetramer. It causes three progressively worse hematological and clinical diseases.

Patients and Methods: The study involved 45 cases of β-thalassemia, with 24 males and 21 females. The participants' average age was 8.3 ± 3.2. There were 40 patients with major thalassemia and five with intermediate thalassemia. A control group of 15 cases, comprising 9 men and 6 females, had a mean age of 8.8 ± 3.4 years. HB, iron, ferritin, copper, and zinc levels were evaluated in all instances. The rs2071348 SNP for the HBBP1 gene mutation was genotyped using the Thermo Scientific ARKTIK Thermal Cycler at the SIGMA SCIENTIFIC genetic laboratory.

Results: Individuals with β-thalassemia had markedly elevated levels of iron, ferritin, and copper in comparison to the control group. β-thalassemia cases had markedly reduced amounts of hemoglobin and zinc in comparison to the control group. Significant differences were observed in the HBBP1 genotyping and alleles between individuals with β-thalassemia and the control group. There was a substantial difference in the distribution of homogeneous and heterogeneous HBBP1 genotyping between β-thalassemia cases and the control group. In β-thalassemia cases, the CC genotype was more common (84.4%) compared to the control group (46.7%). No AA genotyping was present in the control group.

Conclusion: The C/C and A/A genotypes were more common and linked in both β-TM and β-TI patients compared to healthy individuals. β-thalassemia cases showed significantly elevated levels of iron, ferritin, and copper in comparison to the control group (P-value < 0.001). β-thalassemia cases had markedly reduced hemoglobin and zinc levels in comparison to the control group (P-value < 0.001).

Keywords:*HBBP1 gene, zinc, β-thalassemia, copper, iron overload*

Introduction

A group of diseases called β-thalassemia are characterized by an imbalance between the production of α-globin chains and the levels of adult hemoglobin (HbA). This imbalance is caused by inadequate or absent β-globin chain synthesis**. [1]** Beta-thalassemia major (β-TM) It is caused by mutations in the β-globin gene and results in an imbalance of the globin chains due to an absence or decrease in β-globin chain synthesis. It is a hereditary hemolytic anemia that demands a regimen of blood transfusions for therapy. **[2]** In order to keep their hemoglobin levels normal and to prevent inefficient erythropoiesis, people with beta-TM require frequent blood transfusions frequently. Frequent red blood cell transfusions can cause iron excess, which can lead to iron precipitation in most important organs**. [3]**

Iron overload, a typical consequence of thalassemia syndromes, has the potential to cause organ damage and an increased risk of mortality**. [4]** Most of the iron that the body accumulates is in the form of ferritin. The organism secretes ferritin into the plasma in minute quantities. In an inflammatory-free body, the larger the overall iron stores, the higher the content of serum or plasma ferritin. Age and sex determine the normal ferritin concentrations. During the first two months of life, concentrations are high; later in infancy, they diminish**. [5]**

Copper is one of the trace elements that contribute to iron metabolism. Enzymes that regulate iron metabolism contain copper and zinc; intracellular copper may cause red blood cell hemolysis. In addition to its antioxidant function, copper can also operate as a pro-oxidant. As a pro-oxidant, copper amplifies the destructive power of free radicals**. [6]**

Factors that affect the serum copper concentrations in thalassemia major patients include the amount of dietary copper intake, intestinal uptake of copper, iron accumulation, renal function, copper-to-zinc ratio, and administration of Desferal, among others**. [7]**

In addition to being an essential metal for proper immune system function, zinc also plays a role in regulating hepcidin synthesis, a key factor in iron absorption. Zinc regulates the quantity and activity of immune cells, such as T and B cells, macrophages, dendritic cells, mast cells, and neutrophils; it has an impact on both adaptive and innate immunity**. [8]**

The bone marrow-specific duplicated pseudogene HBBP1 (η-globin) has been linked to β-thalassemia. Erythropoiesis cannot occur without HBBP1. HBBP1 is a mutation-intolerant protein that is not variable. An important regulator of erythropoiesis, TAL1 mRNA, is upregulated and stabilized when HBBP1 competitively binds the RNA-binding protein (RBP), HNRNPA1. The HBBP1/TAL1 interaction is specific to humans, according to comparative research. The relationship between HBBP1 and TAL1 helps β-thalassemia patients experience less severe symptoms**. [9]** Both alternative transcription and post-transcriptional splicing contribute to the two-consensus regulatory RNAs encoded by the HBBP1 gene. The HBBP1 gene is part of the B globin cluster and has the most regulatory interactions with active and open chromatin. Its transcripts are found in at least 251 different types of human cells and tissues. The HBBP1 gene is not a mutation-intolerant nonsense gene but rather a highly functional and intelligently integrated part of the human genome**. [10]**

Materials and Methods:

A case-control study was designed to assess 60 Egyptians of both sexes, divided into two groups.

45 patients diagnosed with β thalassemia major and intermediate who need blood transfusions. And 15 healthy people as the control group. At the Damanhur Medical National Institute in Behera, Egypt, thalassemia patients participated in this study. In May 2022, the General Organization for Teaching Hospitals, and Institutes' (GOTHI) Ethics Committee granted clearance for the research under an approval certificate (HD000157).

Biochemical analyses of blood

We centrifuged the samples 45 minutes after collecting blood from eligible individuals. We stored the EDTA samples at 2–8 °C until we analyzed them for HBBP1 gene mutation using the Thermo Scientific ARKTIK Thermal Cycler at the SIGMA SCIENTIFIC genetic laboratory, as shown in **Figure 1.**

At the Damanhur National Medical Institute laboratory, the CELL-DYN Sapphire analyzer was used to measure hemoglobin. We centrifuged plain tube samples and stored the serum at -20 °C. We used spectrophotometric assays with a fully automated Mindray instrument to measure iron, ferritin, copper, and zinc. This instrument indicates that the appropriate ranges for iron, ferritin, zinc, and copper are 37–145 mg/dl, 7–140 mg/dl, 49.5–99.7 mg/dl, and 75–153 mg/dl, respectively.

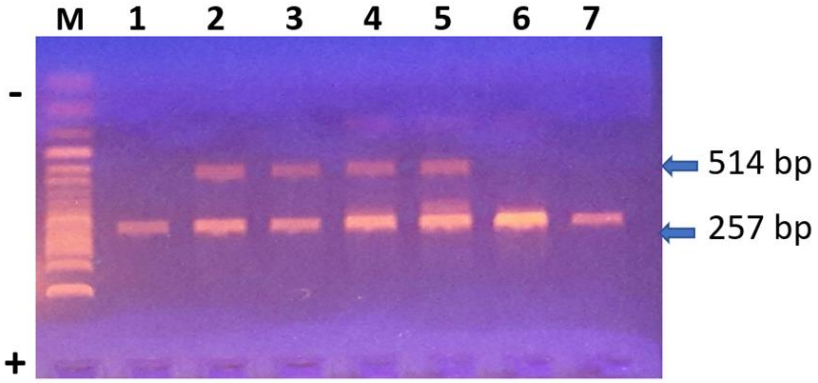

Figure 1: Genotyping analysis of the rs2071348 SNP. DNA size marker; lane 1 and 6: homozygous mutant (C/C) samples; lanes 2, 3, 4, and 5: heterozygous A/C samples; lane 7: undigested polymerase chain reaction (PCR) product (514 bp) used as control.

Results:

Table 1 summarize the main demographic and biochemical characteristics of the study population as follows: This study included **45 β-thalassemia cases**, consisting of 24 males and 21 females, and the mean value (μ±SD) of their age is 8.3 ± 3.2. Regarding type of thalassemia, there were 40 patients who have major thalassemia, and 5 patients have intermediate thalassemia.**15 cases as a control group** consisting of 9 males and 6 females, and the mean value (μ±SD) of their age is 8.8 ± 3.4. There was no statistically significant difference observed with age and sex in the two studied groups (P-value > 0.05). β-thalassemia cases had significantly higher levels of iron, ferritin, and copper compared to the control group (P-value < 0.001). However, β-thalassemia cases had significantly lower levels of hemoglobin and Zinc compared to the control group (P-value < 0.001). **Table 2** illustrates the correlation between biochemical parameters among β-thalassemia cases. The results showed that there was non-significant negative correlation between Hb values and iron, ferritin, zinc, and copper values (P-value > 0.05). iron values had a significant positive correlation with ferritin and zinc values (P-value < 0.001). zinc values had a significant positive correlation with copper values. **Table 3** summarizes the biochemical characteristics of two types of 2 types of thalassemia. There was no statistically significant difference observed with age, sex, iron, ferritin, zinc, and copper levels in the two studied groups (P-value > 0.05). and statistically significant difference in the value of hemoglobin.

Table (1): demographic data, biochemical parameters, and statistical results for all cases.			
Parameters	**Control group. (no=15)**	**β-thalassemia Cases (no=45)**	**p-value**
	Mean ± SD		

Age	8.8 ± 3.4	8.3 ± 3.2	0.617
Sex	**Male =** 9 **Female =** 6	**Male =** 24 **Female =** 21	0.653
Types of thalassemia	**-**	**Intermediate =** 5 **Major =** 40	-
Hb	13.47± 1.06	7.40 ± 1.02	< 0.001
Fe	100.87 ± 26.56	175.6 ± 65.15	< 0.001
Ferritin	97.27 ± 28.5	2024.3 ± 1298.6	< 0.001
Zinc	86.47 ± 17.40	52.7 ± 14.2	< 0.001
Copper	89.27 ± 11.39	136.2 ± 30.6	< 0.001

Table (2): The Correlations between biochemical parameters among β-thalassemia cases.

		Hb	**iron**	**ferritin**	**zinc**	**copper**
Hb	**Pearson Correlation**	1	-0.149	-0.150	-0.005	-0.264
	Sig. (2-tailed)		0.329	0.324	0.976	0.080
iron	**Pearson Correlation**	-0.149	1	0.398**	0.334*	0.107
	Sig. (2-tailed)	0.329		0.007	0.025	0.484
ferritin	**Pearson Correlation**	-0.150	0.398**	1	0.144	-0.166
	Sig. (2-tailed)	0.324	0.007		0.344	0.274
zinc	**Pearson Correlation**	-0.005	0.334*	0.144	1	0.375*
	Sig. (2-tailed)	0.976	0.025	0.344		0.011
copper	**Pearson Correlation**	0-.264	0.107	-0.166	0.375*	1
	Sig. (2-tailed)	0.080	0.484	0.274	0.011	

Table (3): demographic data, biochemical parameters, genotyping frequency, and statistical results for Types of thalassemia.

parameters	**Types of thalassemia**		**P-value**
	Intermediate (no=5)	**Major (no=40)**	
	Mean ± SD		
Age	8.2 ± 2.9	8.4 ± 3.2	0.897
Sex	Male = 2 Female = 3	Male = 22 Female = 18	0.526
HBBP1 genotyping	**CC**=4 **AC**=0 **AA**=1	**CC**=33 **AC**=5 **AA**=2	0.345
Hb	8.60 ± 1.33	7.26 ± 0.89	0.004
Fe	126.20 ± 77.34	181.78 ± 61.84	0.072
Ferritin	1436.18 ± 1605.73	2097.85 ± 1272.81	0.288

Zinc	65.40 ± 17.60	53.85 ± 26.23	0.226
Copper	140.80 ± 64.96	131.82 ± 32.83	0.990

Tables 4 and 5 illustrate the distribution of HBBP1 gene genotyping and allele frequencies in the two studied groups. The Hardy-Weinberg equilibrium test was used to compare the results of observed values with expected values for the SNP locus genotypes of HBBP1 gene. The results showed that the HBBP1 sites had statistically significant differences ($P < 0.05$) among β-thalassemia cases and had no statistically significant differences ($P > 0.05$) among Control group. The distribution of HBBP1 genotyping and alleles showed statistically significant differences between β-thalassemia cases and the control group (P-value <0.001). The distribution between homogeneous and heterogeneous of HBBP1 genotyping had a significant difference between β-thalassemia cases and Control group. HBBP1 genotyping: CC was a more frequent genotype in the β-thalassemia cases (84.4%) than in control group (46.7%). In the control group, AA genotyping was completely absent. The p-value was <0.001.

HBBP1 alleles: C allele was more frequently in β-thalassemia cases (90%) than in the control group (73.3%); however, the A allele was more frequently in the control group (26.7%) than in β-thalassemia cases (10%) with a p-value <0.001.

Table (4): The Hardy-Weinberg equilibrium test for HBBP1 gene among two studied groups.

SNP	genotype	Control group	β-thalassemia cases
HBBP1	CC	7	38
	CA	8	5
	AA	0	2
	HWE	0.796	<0.001

Table (5): The distribution and association of HBBP1 gene polymorphism among two studied groups.

HBBP1 genotyping & alleles	Control Group N=15	β-thalassemia cases N=45	X^2	P-value	OR (95%CI)
Homozygous (CC / AA)	7 (46.7%)	40 (88.9%)	11.817	0.001	9.143 (2.309 - 36.196)
Heterozygous (AC)	8 (53.3%)	5 (11.1%)			
Homozygous CC (ref)	7 (46.7%)	38 (84.4%)	12.064	0.002	-
Heterozygous AC	8 (53.3%)	5 (11.1%)			0.218 (0.084 -0.564)
Homozygous AA	0	2 (4.4%)			0.950 (0.885 - 1.020)
A	8 (26.7%)	9 (10%)	5.140	0.023	0.306 (0.106 - 0.884)
C	22 (73.3%)	81 (90%)			

Table 6 represents ANOVA test results for compared mean values (μ±SD), and the significant difference among values was analyzed for biochemical parameters between HBBP1 genotyping. The results showed no significant difference in value of studied parameters.

Regarding the association of biochemical parameters with different HBBP1 genotyping among β-thalassemia cases, the multinominal regression was carried out taking the HBBP1 genotyping as dependent variables and biochemical parameters as independent variables. The CC genotype was considered the reference category. The logistic regression analysis was used to determine the risk associated of iron toxicity, Zinc and Copper

Dysregulation with HBBP1 gene polymorphism, the results showed there was no significant risk associated. **Table 7**.

Table (6): biochemical parameters, and statistical results for HBBP1 genotyping among β-thalassemia cases.				
Parameters	**Homozygous C/C (no=45)**	**Homozygous A/A (no=2)**	**Heterozygous A/C (no=13)**	**P-value**
	Mean (SD)			
Hb	7.4±0.9	7.4±2.5	7.4±0.39	0.999
Fe	171.6±58.6	181.1±142	201.4±68.2	0.636
Ferritin	1982.1±1110.6	2697.3±367	1932.4±580	0.658
Zinc	53.7±14.1	51±8.5	45.9±18	0.511
Copper	137.9±30.7	123.3±1.5	131.2±39.8	0.685

Table (7): the association of biochemical parameters with HBBP1 genotyping among β-thalassemia cases.						
Parameters	**AC**			**AA**		
	B	**OR (95%CI)**	**p-value**	**B**	**OR (95%CI)**	**p-value**
Hb	0.079	1.082 (90.36 - 3.17)	0.886	0.056	1.05 (0.31 -3.52)	0.928
Fe	0.013	1.01 (0.99 - 1.03)	0.138	0.001	1.0 (0.9- 1.02)	0.963
Ferritin	0.000	1.0 (0.99 - 1.001)	0.651	0.000	1.0 (0.9 -1.001)	0.519
Zinc	-0.060	0.94 (0.86 -1.02)	0.183	-0.011	0.9 (0.89 - 1.09)	0.824
Copper	0.000	0.99 (0.961 -1.02)	0.985	-0.013	0.9 (0.9 - 1.03)	0.611
The reference category is CC.						

Discussion

Genetic modifiers and environmental factors are linked to the varied phenotypic variety of beta (β)-thalassemia. Primary genetic modifiers are various β-globin mutations that mainly impact the synthesis of the β-globin chain. Secondary modifiers impact β-globin synthesis by altering the production of alpha (α)-globin or gamma (γ)-globin. Tertiary modifiers do not affect β-globin synthesis but impact the disease's consequences**. [11]** Tertiary modifiers are specific genetic variations that are selected along with β-thalassemia, which then alter the phenotype by influencing the complications. Notable consequences include iron overload, hyperbilirubinemia, and osteoporosis**. [12]** More than 600 mutations have been documented in the beta globin gene, with around 200 of them associated with beta-thalassemia to some extent**. [13]** The HBBP1 gene in the beta-globin gene cluster contains the rs2071348 mutation, which raises HbF levels and MCH, resulting in less severe thalassemia symptoms. Previous research indicates that the A/C mutation in HBBP1 leads to increased HbF levels in thalassemia and milder symptoms but does not affect the response to hydroxyl-urea**. [14]**

Our aim is to investigate the possible association of the SNP rs2071348 residing within the HBBP1 pseudogene with β-thal disease, and correlate of mutations with iron overload, zinc, and copper deregulations in beta-thalassemia patients.

Our findings indicated no significant variations in age and sex between the control and patient groups. Regarding the several kinds of thalassemia Hemoglobin levels drop with the severity of thalassemia, but

serum iron and ferritin levels increase with the degree of thalassemia. Individuals with β-thalassemia exhibit a deficiency or diminished production of β-globin chains, resulting in decreased hemoglobin levels in red blood cells and ultimately causing anemia**. [15]** Standard treatment for β-thalassemia major (BTM) involves frequent blood transfusions leading to iron accumulation, while β-thalassemia intermedia (BTI) is a milder kind of anemia compared to BTM. Accumulation of excessive iron in essential organs can lead to reduced organ function and higher rates of illness and death. Iron overload in patients with transfusion-dependent thalassemia (TDT) primarily arises from transfusions. Iron overload can arise in cases of non-transfusion-dependent thalassemia (NTDT) due to increased absorption in the intestines, even when infrequent transfusions are given**. [16]** This excess iron accumulation is causing peroxidative damage by elevating the generation of reactive oxygen species in the red blood cells, resulting in oxidative stress. Oxidative stress in beta thalassemia can lead to growth failure and several issues in the liver, cardiovascular system, endocrine system, and nervous system**. [17]**

Various haemoglobinopathies are influenced by the combined impact of trace elements, vitamins, and growth hormones. Trace metals such as copper and zinc are crucial in reducing oxidative stress in humans**. [18]** Zinc is a vital element for humans, serving as a cofactor for over 300 enzymes and playing a crucial role in human growth and development. **[19]**

Some data indicate that people with beta thalassemia major may experience zinc shortage, which could contribute to delayed maturation in these patients**. [20]**

Zinc deficiency can result in several clinical problems such as growth retardation, hypogonadism, osteoporosis, osteopenia, immunologic abnormalities, and recurrent infections. Tabatabei and colleagues found that 84.8% of patients with thalassemia major had a zinc deficit. The patients' zinc insufficiency was attributed to inadequate dietary zinc intake**. [21]** Mahyar A. et al. demonstrated a statistically significant difference in serum zinc concentration levels between thalassemic patients (37±1.9mg/dl) and the control group (51±1.8). Zinc supplements were suggested for individuals with thalassemia. Other studies, such as Al-Samarrai et al, reported that hypozincemia in thalassemic individuals is caused by hypozincemia resulting from the hemolysis of red blood cells**. [22]** Hashemi Poor and colleagues showed that the zinc levels in the hair of thalassemic patients (112.7±53.11 ppm) were reduced compared to the control group (149.6±72.21 ppm). They proposed that malnutrition and insufficient zinc consumption are the causes of zinc insufficiency. They recommend the use of a zinc supplement**. [23]**

Mehdizadeh et al. found that the average serum zinc level was notably elevated in the thalassemic group. Zinc deficiency is uncommon in thalassemia. A study by Reshadat et al. revealed that 77% of thalassemic patients have normal serum zinc levels, whereas the remaining individuals have levels above normal**. [24]**Kosarian et colleagues found that serum zinc levels in major thalassemic patients and the control group were normal, indicating that these patients do not have zinc deficiency. **[25]** Another study indicated that 65% of children with thalassemia have hypozincemia. Possible causes of zinc deficiency in these patients include inadequate zinc intake in daily meals, impaired urinary absorption of zinc, kidney dysfunction, excessive urinary secretion of zinc, disruption in zinc metabolism, and elevated levels of zinc excretion in sweat**. [26]**

Studies have demonstrated an elevated serum copper content in people with thalassemia major**. [27]** Al-Samarrai et al determined that hemochromatosis is the main cause of hypercupremia, a common consequence of thalassemia. Other findings have indicated a decrease in the serum level of copper. **[28]** Kassab-Chekir's investigation found no alteration in the serum copper levels**. [29]**

The rs2071348 A/C polymorphism, situated in the intergenic region between the HBPB and the HBD genes within the HBB cluster, exhibits the most significant correlation with HbF levels in a group of 618 Thai patients with β-thalassemia/HbE**. [30]** Additional research conducted on Indonesian patients demonstrated an association between rs2071348 and HbF levels. The C allele of rs2071348 was shown to be more prevalent in mildly afflicted β-thalassemia/HbE patients compared to highly affected people, indicating its potential use in predicting disease severity**. [31]** In a prior study, it was discovered that out of 37 individuals with β-thalassemia major, 31 patients (83.8%) had wild C/C genotypes, 5 patients (13.5%) had heterozygous

C/T genotypes, and only 1 patient (2.7%) had homozygous T/T genotype**. [32]** 28 out of 206 β-thalassemia patients with homozygote IVSII-1 mutation did not show polymorphism (C/C), while 178 individuals did, with 44 being heterozygous (21.3%) and 134 being homozygous (65%). In Iran, 35 individuals (68.6%) were identified as heterozygous (C/T) and 16 individuals (31.4%) were homozygous (C/C) out of a total of 51 patients**. [33]**

Previous investigations indicated that there were weak statistically significant connections between the BCL11A SNP rs766432 and HbF levels in β-thalassemia/HbE patients. Rs766432 is associated with HbF levels in different populations with β-hemoglobinopathies, but studies comparing patients with high and low HbF levels did not find a significant correlation between HbF and rs766432. The lack of a notable correlation between rs766432 and HbF levels could be due to the low prevalence of this variant, with a minor C-allele frequency of 1.4% in the group. BCL11A is a regulator that controls the transition from γ globin to β-globin gene expression**. [34]** BCL11A collaborates with other repressor factors to create a repressor complex, which leads to the suppression of the γ globin gene in mature erythroid cells. The rs766432 SNP is situated in an erythroid-specific enhancer region of the BCL11A gene and is expected to impact the expression of BCL11A. **[35]**

Our investigation found variations in the number of homozygous and heterozygous mutants in β-thalassemia cases, but no changes were seen in the control group. The CC allele was overexpressed in 84.4% of β-thalassemia cases compared to 46.7% in the control group, while the AA allele was completely missing in the control group. In β-thalassemia cases, the C allele was more common (90%) compared to the control group (73.3%), while the A allele was more prevalent in the control group (26.7%) than in β-thalassemia cases (10%).

We noticed an association difference between biochemical parameters and HBBP1 mutants' alleles. We found that there was overexpression of CC and AC alleles and under expression of the AA allele in relation to Hb, Fe, and Ferritin levels. However, no variations were seen in Zinc and Copper levels.

The other studies conducted the first molecular analysis of the s gene, Hepcidin, a liver-produced hormone that regulates iron hemostasis in the body in response to hypoxia, anemia, and iron stores. The research focused on examining the connection between hepcidin promoter gene (HAMP) variants c.- 582A > G, c.-153C > T, and c.-443C > T, and iron overload in β-thalassemia major patients undergoing regular transfusion and iron-chelating therapy. **[36]** The previous study indicated that individuals with the GG genotype of the c.-582A > G variation have a notable level of myocardial iron overload. The CT genotype of the c.-443C > T mutation is marginally associated with ferritin levels. Since all patients had two copies of the normal allele (c.-153C), statistical analysis could not be conducted. **[37]** Homozygous patients with the G allele had significantly higher iron accumulation ($p = 0.02$). Additionally, serum ferritin levels were assessed, revealing that all patients with the GG genotype had ferritin levels exceeding 1000 ng/ml. However, no significant association was observed between this SNP and serum ferritin levels ($p = 0.12$). The second genotype identified was c.-153C > T, with a minor allele frequency that is very low in the population. Island et al first detected it in a patient with significant iron excess. The substitution in a BMP-responsive region lowered baseline hepcidin gene expression by decreasing its response to BMPs and IL-6, as established. **[38]**

Conclusion

In both TM and TI patients, the C/C and A/A genotypes were more common and linked than in healthy individuals. β-thalassemia cases showed significantly elevated levels of iron, ferritin, and copper in comparison to the control group (P-value < 0.001). β-thalassemia cases had markedly reduced hemoglobin and zinc levels in comparison to the control group (P-value < 0.001).

Statements and Declarations

Ethics approval and consent to participate: The approval was taken from the Ethical Committee at Damanhur Medical National Institute in Behera, Egypt, thalassemia patients participated in this prospective cross-sectional research. In May 2022, the General Organization for Teaching Hospitals, and Institutes' (GOTHI) Ethics Committee granted clearance for the research under approval certificate (HD000157).

Consent for publication: Not applicable.

Availability of data and materials: All data generated or analyzed during this study are included in this article.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: A.B, D.A, A.A, S.A & W.M were responsible for the conception and design of the study, collecting and entering data, literature search, methodology, laboratory investigations, statistical analysis of data and writing the results, the original draft, preparation, and editing of the final manuscript. A.B, H.A, S.A, SMB and D.A were responsible for the interpretation of the results. All authors revised and approved the final version of the manuscript.

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