

Impact of Haptoglobin Gene Polymorphism on Phenotypic Variability in β -thalassemia Patients: Relation to Iron Overload and Oxidative Stress

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Background and study aim:

Thalassemia syndromes are genetic disorders in globin chain production. There is excessive synthesis of reactive oxygen species in thalassemia which leads to oxidative stress. Excessive production of malondialdehyde (MDA) provides evidence that thalassemic erythrocytes contain a significant amount of membrane-bound iron. Inflammatory cytokines trigger the production of haptoglobin (Hp) in the liver. It is characterized by molecular heterogeneity brought on by genetic polymorphism. The aim of the work was to study the impact of haptoglobin gene polymorphism on phenotypic variability in thalassemia patients and its relation to iron overload and oxidative stress.

Patients and Methods: This study was carried out on 50 β -thalassemia major patients in addition to 25 healthy age and sex-matched individuals as control. All patients and control were subjected to:

history taking, physical examination, Laboratory investigations, Iron profile, Serum ferritin, Serum haptoglobin (Hp), Serum malondialdehyde (MDA), and Haptoglobin gene polymorphism by (PCR).

Results: The study revealed significant decrease in serum haptoglobin and significant increase in MDA and ferritin level in thalassemic patients compared to the control group. Higher incidence of Hp2-2 in thalassemic group while control group showed more prevalence of Hp2-1. Serum level of MDA was significantly higher and haptoglobin levels were significantly lower in patients with Hp2-2 genotype. Serum ferritin was higher in the same genotype but did not reach significant value.

Conclusion: Haptoglobin polymorphism and phenotypic variability have a major influence on oxidative stress in thalassemia patients.

INTRODUCTION

Thalassemia syndromes are genetic disorders in globin chain production characterized by varying degrees of ineffective hematopoiesis and increased hemolysis. They range from asymptomatic forms to severe or even lethal entities, depending on the clinical severity [1]. The clinical phenotypes of β -thalassemia major (β TM) are remarkably distinct (homozygous or compound heterozygous states) having significant anemia from early life. The standard protocol for their treatment is life –long blood transfusion and chelation therapy. Chronic blood transfusions, despite being lifesaving, lead to organ and tissue damage due

to iron overload [2]. Due to its capacity to chelate transition metals and block their redox activity, the iron chelator deferoxamine (DFO) has been utilized for many years to prevent iron overload in thalassemia patients [3].

There is excessive synthesis of reactive oxygen species in thalassemia. All of which lead to oxidative stress. Its primary cause is iron overload, which is brought on by increased gastrointestinal iron absorption, repeated blood transfusions, and ultimately iron release from heme. Iron is incriminated to the excessive production of free radicals that might

damage erythrocytes through oxidative stress. The hastened apoptosis that could result from this oxidative stress may reduce the lifespan of erythrocytes [4]. Excessive production of malondialdehyde (MDA), a lipid peroxidation byproduct, provides evidence that thalassemic erythrocytes contain a significant amount of membrane-bound iron. Increased serum MDA and decreased antioxidant defense system are assigned to the peroxidative damage of lipids, which contributes to the pathogenesis of thalassemia [5].

The acute phase protein Haptoglobin (Hp) lowers the oxidative and peroxidative potential of free hemoglobin and removes it in intravascular or extravascular hemolysis. It acts as an inhibitor of angiogenesis and prostaglandin synthesis and is produced in the liver in response to glucocorticoids and inflammatory cytokines [6]. Haptoglobin is recognized by molecular heterogeneity brought on by genetic polymorphism. There are three common phenotypes of Hp: Hp1-1, Hp2-2 and the heterozygous Hp2-1 which are controlled by two autosomal co-dominant alleles identified as Hp1 and Hp2. They differ in their functional efficiencies, biophysical, and biochemical properties, which contribute to their varied antioxidant and immunomodulatory capacities. The peripheral blood B-cell and T-lymphocyte counts are higher with the Hp2 allele, but the anti-inflammatory effect is less prominent than with Hp1 allele [7]. Haptoglobin polymorphism is accompanied by the clinical evolution and prevalence of many autoimmune disorders, atherosclerosis, and inflammatory diseases, including infection. The antioxidant function of haptoglobin and the phenotypic dependence were validated for inhibiting the potential oxidative damage caused by free hemoglobin and iron release during its breakdown [8].

The aim of the present study was to study the effect of haptoglobin gene polymorphism on phenotypic variability in thalassemia patients and its relation to iron overload and oxidative stress.

PATIENTS AND METHODS

Fifty patients previously diagnosed with β thalassemia major were included in our study. They were on regular RBCs transfusion and iron chelation therapy using deferoxamine (DFO). Blood samples were collected before blood

transfusion. In addition, twenty-five healthy individuals were included as control. After obtaining approval from Ethics Committee, patients or their parents provided informed consent in order to participate in the study,

All patients as well as controls were subjected to: history taking, physical examination and Laboratory investigations including Complete Blood Count (CBC), Reticulocytes count [9], Hb electrophoresis [10], Iron profile (serum iron, total iron binding capacity, transferrin saturation), Serum ferritin [11], C- reactive protein (CRP) [12] and Coombs' test [13]. In addition to: Serum haptoglobin (Hp) using immune-nephelometry automated chemistry analyzer [14], Serum malondialdehyde (MDA) by spectrophotometric thiobarbituric acid (TBA) test method [15] and Haptoglobin gene polymorphism by polymerase chain reaction (PCR) [14].

Statistical analysis

IBM SPSS software package version 23.0 was used to analyze data (Armonk, NY: IBM Corp). The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests. We evaluated normally distributed quantitative data by independent t-test and ANOVA while not normally distributed data were evaluated by Mann Whitney and Kruskal-Wallis tests. Qualitative data were tested by the Chi-square and Fisher's exact tests. The level of significance was set as $p \leq 0.05$.

RESULTS

The study comprised fifty patients with established β TM. They were 22 (44%) males and 28 (56%) females, their age ranged from (12.0-20.0 years) with mean age of 16.38 ± 2.65 years. Twenty-five healthy individuals were included as a control group, 15 (60%) females and 10 (40%) males, ranging in age from (13 to 20 years) with mean of 16.40 ± 2.60 years.

On clinical examination, forty-two (84%) patients had hepatomegaly, twenty-two (44%) patients had undergone splenectomy, while eighteen patients (36%) had splenomegaly. On the other hand, sixteen (32%) patients had hepatitis C infection.

As regards CBC results, table 1 showed the hematological data of β TM patients & the control subjects. There was significant decrease

in the mean Hb, PCV, MCV, MCH and RBCs but the mean platelets and reticulocyte counts were significantly higher in thalassemia patients compared to controls ($P < 0.001$).

Iron profile

The mean serum iron and transferrin saturation in addition to serum ferritin were significantly higher in thalassemia patients compared to control group, ($P < 0.001$) whereas, TIBC was significantly higher in the control group in comparison to thalassemic patients ($P < 0.001$). (Table 2)

Haptoglobin & Malondialdehyde (MDA):

The study also revealed significant decrease in mean serum haptoglobin level in β TM patients than in controls (Mean \pm SD 21.74 \pm 23.53 and 96.84 \pm 30.19 mg/dl respectively), ($P < 0.001$), while the mean serum MDA level was significantly higher in thalassemia patients than control group (Mean \pm SD 3.47 \pm 1.90 and 0.90 \pm 0.28 nmol/l respectively), ($P < 0.001$). (Table 3).

Haptoglobin polymorphism

There was higher incidence of Hp 2-2 (56%) than other types Hp2-1 (30%) and Hp1-1 (14%)

in thalassemic group with allele frequency of 71% for Hp-2 and 29% for Hp-1, while the control group showed more prevalence of Hp2-1(44%) followed by Hp2-2 (36%) then Hp1-1(20%) with allele frequency of 58% for Hp2 and 42% for Hp1. (Table 4)

Relation between haptoglobin, MDA and ferritin with haptoglobin genotype in thalassemic group.

The mean serum level of MDA was significantly higher in Hp2-2 genotype patients (Mean \pm SD 3.92 \pm 1.97) in comparison with Hp1-1 genotype (Mean \pm SD 2.31 \pm 1.27). ($p \leq 0.05$), while mean serum haptoglobin levels were significantly lower in Hp2-2 genotype (Mean \pm SD 18.65 \pm 23.59) compared with that of Hp1-1 genotype (Mean \pm SD 41.76 \pm 30.17) ($P \leq 0.05$). However mean serum ferritin was higher in thalassemic patients with Hp2-2 genotype than in those with Hp1-1 and Hp2-1, but did not reach the significant value, (Mean \pm SD 3502.8 \pm 2314.6, 3028.7 \pm 2093.5 and 3005.4 \pm 2055.7 respectively). (Table 5)

Table 1: Hematological data of β TM patients and the control group

Item	Cases (n=50)	Control (n=25)	P-value
Hb (gm/dl)			
Min. – Max	3.50-10.0	11.70-14.70	
Mean \pm SD.	6.69 \pm 1.22	13.18 \pm 0.89	<0.001*
Median	6.65	13.0	
PCV (%)			
Min. – Max	9.90-30.50	35.0-45.60	
Mean \pm SD.	21.19 \pm 3.94	40.97 \pm 3.02	<0.001*
Median	21.15	41.50	
MCV (fl)			
Min. – Max	53.40-88.20	75.0-100.0	
Mean \pm SD.	72.73 \pm 8.67	82.28 \pm 4.98	<0.001*
Median	74.25	81.0	
MCH (pg)			
Min. – Max	16.30-27.70	20.40-32.30	
Mean \pm SD.	23.12 \pm 3.18	28.65 \pm 2.63	<0.001*
Median	23.95	28.60	
MCHC (gm/dl)			
Min. – Max	23.70-38.10	30.10 – 36.0	
Mean \pm SD.	31.93 \pm 2.75	32.34 \pm 1.57	0.406
Median	32.70	32.0	
RBCs (x10¹²/l)			
Min. – Max	1.35-4.50	4.28-5.70	
Mean \pm SD.	2.94 \pm 0.71	5.03 \pm 0.38	<0.001*
Median	2.86	5.10	
Retic count(x10⁹/l)			
Min. – Max.	54.0 – 364.0	22.0 – 77.0	
Mean \pm SD.	124.66 \pm 69.43	39.44 \pm 15.15	<0.001*
Median	98.0	43.0	
WBCs (x10⁹/l)			
Min. – Max	3.84 – 9.73	4.0-9.80	
Mean \pm SD.	6.64 \pm 1.65	6.83 \pm 1.64	0.744
Median	6.55	7.10	
Plts (x10⁹/l)			
Min. – Max	133.0-1056.0	131.0-455.0	
Mean \pm SD.	457.28 \pm 263.80	243.80 \pm 89.33	<0.001*
Median	354.50	225.0	

Hb: hemoglobin, PCV: packed cell volume, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RBCs: red blood cells count; Retic count: reticulocyte count; WBCs: white blood cells count; Plts: Platelets count

*: Statistically significant at $p \leq 0.05$

Table 2: Iron profile in thalassemic & control groups

Item	Cases (n = 50)	Control (n = 25)	P value
Serum iron ($\mu\text{mol/l}$)			
Min. – Max	22.40-64.40	10.70-23.30	<0.001*
Mean \pm SD.	42.30 \pm 9.81	15.53 \pm 2.83	
Median	41.20	15.0	
TIBC ($\mu\text{mol/l}$)			
Min. – Max	33.50-64.40	42.90-62.80	<0.001*
Mean \pm SD.	41.72 \pm 7.87	51.34 \pm 5.11	
Median	38.75	50.50	
TS (%)			
Min. – Max	38.0-160.0	17.0-47.0	<0.001*
Mean \pm SD.	106.66 \pm 33.28	30.88 \pm 7.42	
Median	112.0	30.0	
Serum ferritin ($\mu\text{g/l}$)			
Min. – Max	890.0-9830.0	30.0-210.0	<0.001*
Mean \pm SD.	3287.22 \pm 2180.96	95.04 \pm 46.40	
Median	2705.0	82.0	

TIBC: total iron binding capacity; TS: transferrin saturation

*: Statistically significant at $p \leq 0.05$

Table 3: Comparison between the two studied groups according to serum haptoglobin & MDA

Item	Cases (n = 50)	Control (n = 25)	P value
Serum haptoglobin (mg/dl)			
Min. – Max	1.90-99.0	48.50-158.0	<0.001*
Mean \pm SD.	21.74 \pm 23.53	96.84 \pm 30.19	
Median	10.20	90.0	
Serum MDA (nmol/l)			
Min. – Max	1.10-9.10	0.20-1.30	<0.001*
Mean \pm SD.	3.47 \pm 1.90	0.90 \pm 0.28	
Median	3.25	0.80	

MDA: malondialdehyde

*: Statistically significant at $p \leq 0.05$

Table 4: Haptoglobin Polymorphism in the studied groups

Item	Cases (n = 50)		Control (n = 25)		χ^2	P value	OR	95% CI	
	No.	%	No.	%				LL	UL
Haptoglobin									
Hp1-1	7	14.0	5	20.0	0.446	0.519	1.000	-	-
Hp2-1	15	30.0	11	44.0	1.442	0.230	0.974	0.243	3.897
Hp2-2	28	56.0	9	36.0	2.667	0.102	2.222	0.564	8.759
Hp 0-0	0	0.0	0	0.0	-	-	-	-	-
Allele frequency									
Hp1	29	29.0	21	42.0	2.535	0.111	1.000	-	-
Hp2	71	71.0	29	58.0					

χ^2 : Chi square test

Table 5: Relation between MDA, haptoglobin and ferritin with haptoglobin genotype in thalassemic group.

Item	Haptoglobin genotype			P value
	Hp1-1 (n=7)	Hp2-1 (n=15)	Hp2-2 (n=28)	
Serum MDA (nmol/l)				
Min. – Max	1.10 – 4.60	1.10 -7.60	1.10 – 9.10	≤ 0.05
Mean ± SD.	2.31 ± 1.27	3.17 ± 1.80	3.92 ± 1.97	
Median	1.90	2.70	3.45	
Sig. bet. Grps. #	1-1 with 2-2*			
Serum haptoglobin (mg/dl)				
Min. – Max	5.0 – 84.0	2.80 – 49.50	1.90 – 99.0	≤ 0.05
Mean ± SD.	41.76 ± 30.17	18.17 ± 15.51	18.65 ± 23.59	
Median	29.60	12.0	7.0	
Sig. bet. Grps. #	1-1 with 2-2*			
Serum ferritin (µg/l)				
Min. – Max	1200 – 7000	900 – 8400	890 – 9830	0.608
Mean ± SD.	3028.7±2093.5	3005.4±2055.7	3502.8±2314.6	
Median	2400.0	2600.0	3091.5	

MDA: malondialdehyde

Pair-wise comparison was done using Mann Whitney test

*: Statistically significant at $p \leq 0.05$.

DISCUSSION

Thalassemia has emerged as a significant problem in clinical practice. Analysis of the molecular principles of the different types of thalassemia and the phenotype / genotype relationships have made great advances in understanding the processes behind the phenotypic variability in β -thalassemia [16]. This phenotypic variability is mainly due to genetic factors which still have been incompletely defined. In addition, little is known about the phenotypic heterogeneity complications of thalassemia and its pathophysiology. Repeated blood transfusions are necessary for the survival of thalassemia patients, but lead to excessive iron overload, altered trace element levels and antioxidant enzymes, all of which may cause oxidative stress [17].

Nitric oxide (NO) can oxidize heme, the free hemoglobin molecule carrying the iron moiety, causing oxidative stress-mediated damage via hydrogen peroxides and lipid. Plasma Haptoglobin (HP) binds to free hemoglobin to scavenge it from oxidation, possibly halting additional heme-mediated nitric oxide damage, iron renal excretion, and additional oxidative damage [18].

In the current study, a significant decline in mean serum haptoglobin level in β TM patients compared to the control group was observed.

Ragab et al [19] confirmed our results. They found that thalassemic children had intense Hp depletion when compared to the control group. They concluded that the presence of ineffective erythropoiesis as well as hemolysis results in a decrease in the level of plasma haptoglobin. Regardless of hemolysis site (extravascular or intravascular) or the existence of concordant inflammation, its depletion is an indication for the rapid diagnosis of enhanced destruction of red cells. Thus, Hp depletion in patients with thalassemia is assigned to hemolysis and ineffective erythropoiesis.

MDA is a lipid peroxidation and oxidative stress marker that is produced as the final product of oxygenation of polyunsaturated fatty acid [5]. Our results revealed a significant increase in MDA levels in thalassemia patients compared to controls which reflects the patient's state of oxidative stress. This was in accordance with Atmakusuma et al [20] who demonstrated that thalassemia patients show increased MDA levels compared to healthy controls reflecting the state of oxidative stress of these patients. In addition, Basu et al [21] reported that Malonaldehyde (MDA) is increased significantly in both transfusion and non-transfusion-dependent β -thalassemia. Regardless of severity and blood transfusion dependency they signified MDA as a reliable surrogate indication of iron overload in β -thalassemia. As a result, it can be utilized

instead of ferritin as a predictive biomarker of management, particularly in the consequence of chelation therapy.

In the present study, 56% of thalassemic patients had the Hp2-2 genotype, while in the control group Hp2-1 genotype predominated. Our results agree with Melamed- Frank et al, [22] who reported that Hp2-2 persons are more susceptible to oxidative stress than Hp1-1 and Hp2-1 persons. Moreover, our results showed that Hp2-1 and Hp2-2 genotype are associated with lower serum haptoglobin levels while the genotype Hp1-1 is linked with the highest serum haptoglobin level. This result was confirmed by Van et al [23] who stated that persons with the Hp 2-2 phenotype have lower reference range for haptoglobin concentration than those with the Hp 1-1 and Hp 2-1 phenotypes.

Additionally, Kasvosve et al [24] described that the reference values of haptoglobin depend on the Hp phenotype. In the same context, Imrie et al [25] reported that Hp levels were related to Hb levels, Hp genotype, splenomegaly and age. Additionally, it appears that the rate of free hemoglobin clearance from circulation is phenotype-dependent as shown by Van et al [23] hence the clearance of Hp1-1-Hb complex is faster than that of Hp2-2Hb complex. Thus, the lower levels of serum haptoglobin in thalassemic patients could be attributed to presence of different polymorphisms of the haptoglobin gene. Kaiser et al [7] found that people with Hp2-2 have the lowest concentrations of Hp, while those with Hp1-1 have the highest, and the ones with Hp2-1 have concentrations fall somewhere in between.

Our study also revealed that thalassemic patients with Hp2-2 have the highest serum ferritin than other phenotypes which agrees with Ragab et al [19] who demonstrated that Hp2-2 genotype patients had higher serum ferritin compared to their analogues. When coupled to Hp 2-2 rather than Hp 1-1 or Hp 2-1, the Hb-Hp complex is more effectively internalized by CD163 receptors. Therefore, it was hypothesized that people with the Hp 2-2 phenotype experience more oxidative stress as a result of higher iron buildup in macrophages [26].

On the other hand, we demonstrated that patients with the Hp2-2 phenotype had the highest level of MDA when compared to the other phenotypes. Our results corroborate the findings of Blum et al [27] who stated that the Hp-1

protein is allied with enhanced production of the antioxidant cytokine IL 10, whereas Hp-2 protein is accompanied with excess of oxidative active iron production. Subsequently, the Hp1 phenotype has anti-inflammatory and antioxidant properties. Moreover, Awadallah et al [28] reported that upon formation of the Hp-Hb complex, it is rapidly quenched from the circulation and hunted by the macrophages and hepatocytes CD163 receptors.

In the current study, lower level of serum haptoglobin could be interpreted by the presence of anti Hp antibody which needs to be further studied. The risk to produce anti Hp antibody by blood transfusion has been investigated by Shimada et al [29] who reported that haptoglobin deficiency was associated with Hp IgG antibodies and those subjects could experience fatal anaphylactic non hemolytic transfusion reactions. They also reported that Hp-2 allele has a superior ability to form antibodies than the Hp-1 allele. It is worth mentioning that our results revealed high Hp-2 allele frequency than Hp-1. Previous studies suggest that Hp- 1 allele may be beneficial from a biological standpoint due to its smaller size, faster Hb exportation, and increased antioxidative capabilities, so Hp 1-1 can be taken up by cells faster and pass the blood-brain barrier. On the other hand, Hp-2 genotype is associated with a lower Hp concentration and a diminished competency to bind to Hb [30].

CONCLUSION

We found functional variations in the antioxidant ability of different haptoglobin proteins towards hemoglobin, proposing that those with haptoglobin 1-1 protein may have better antioxidant protection than those with Hp 2-2 protein. Haptoglobin polymorphism and phenotypic variability have a major influence on oxidative stress in patients with thalassemia and those with the Hp 2-2 phenotype are more vulnerable to iron-steered oxidative stress than those with different phenotypes.

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Conflict of interest:None.

Ethical consideration

This study was approved by Research Ethics Committee of Medical Research Institute, Alexandria University following principles of the declaration of Helsinki (IORG0008812). All

studied patients signed for informed and written consent.

HIGHLIGHTS

- This study assessed the effect of haptoglobin gene polymorphism on phenotypic variability in thalassemia patients and its relation to iron overload and oxidative stress.
- The study revealed higher incidence of haptoglobin 2-2 genotype in thalassemic patients. Serum level of MDA & ferritin were higher and haptoglobin levels were lower in patients with Hp2-2 genotype.
- Haptoglobin polymorphism and phenotypic variability have a major influence on oxidative stress in patients with thalassemia and those with the Hp 2-2 phenotype are more vulnerable to iron-steered oxidative stress than those with different phenotypes.

REFERENCES

1. Angastiniotis M, Lobitz S. Thalassemias: An Overview. *Int J Neonatal Screen* 2019;5(1):16. doi: 10.3390/ijns5010016 .
2. Karimi M, Cohan N, De Sanctis V, Mallat NS, Taher A. Guidelines for diagnosis and management of Beta-thalassemia intermedia. *Pediatr Hematol Oncol* 2014; 31(7):583-96 .
3. Porter JB, Garbowski MW. Interaction of Transfusion and Iron Chelation in Thalassemias. *Hematol Oncol Clin North Am.* 2018;32(2):247-259. doi: 10.1016/j.hoc.2017.11.010 .
4. Naji NA, Saleh SS, Taher GN. Studying of Oxidative Stress and Some Biochemical Parameters in Patients with β -Thalassemia Major in Kirkuk City. *IJSBAR.* 2018;42(2):239-51 .
5. Alaydrus SRM, Ridha NR, Alasiry E, Amiruddin L, Angriani H, Satrio KJ. Analysis of malondialdehyde levels in children with β thalassemia: a cross –sectional study. *IJHSMR* 2023; 2(2):76-84
6. Fotsing CBK, Pieme CA, Nya PCB, Chedjou JP, Dabou S, Nguemeni C et al. Relation between haptoglobin polymorphism and oxidative stress status, lipid profile, and cardiovascular risk in sickle cell anemia patients. *Health Sci Rep* 2022; 5(1):465. doi: 10.1002/hsr2.465 .
7. Kaiser M, Thurner EM, Mangge H, Herrmann M, Semeraro MD, Renner W et al. Haptoglobin polymorphism and prostate cancer mortality. *Sci Rep.* 2020;10(1):13117. doi: 10.1038/s41598-020-69333-z .
8. Naryzny SN, Legina OK. Haptoglobin as a Biomarker. *Biochem Mosc Suppl B Biomed Chem.* 2021;15(3):184-198. doi: 10.1134/S1990750821030069. Epub 2021 Aug 16.
9. Bain BJ, Bates I, Laffan MA. Basic hematological techniques In B. J. Bain, S. M. Lewis & I. Bates. *Dacie and Lewis practical hematology* (12th ed p.p. 19-47). Philadelphia: Churchill Livingstone Elsevier. 2017
10. Wild BJ, mBain BJ. Investigation of Variant Haemoglobins and Thalassaemias In B. J. Bain, I. Bates & M. A. Laffan. *Dacie and Lewis Practical Haematology* (12th ed p.p. 283-231). Churchill Livingstone Elsevier. 2017
11. Pfeiffer CM, Looker AC. Laboratory methodologies for indicators of iron status: strengths, limitations, and analytical challenges. *Am J Clin Nutr.* 2017;106:1606S-1614S. doi: 10.3945/ajcn.117.155887 .
12. Nehring SM, Goyal A, Patel BC. C Reactive Protein. *StatPearls.* Treasure Island (FL): StatPearls Publishing. 2023
13. Jaime-Pérez JC, Almaguer-Gaona, C. Rediscovering the Coombs test. *Medicina Universitaria.* 2016; 18(72):185-186.
14. Shih AW, McFarlane A, Verhovsek M. Haptoglobin testing in hemolysis: measurement and interpretation. *Am J Hematol.* 2014;89(4):443-7. doi: 10.1002/ajh.23623.
15. Papastergiadis A, Mubiru E, Van Langenhove H, De Meulenaer B. Malondialdehyde measurement in oxidized foods: evaluation of the spectrophotometric thiobarbituric acid reactive substances (TBARS) test in various foods. *J Agric Food Chem.* 2012;60(38):9589-94. doi: 10.1021/jf302451c .
16. Taher AT, Weatherall DJ, Cappellini MD. Thalassemia. *Lancet.* 2018;391(10116):155-167. doi: 10.1016/S0140-6736(17)31822-6 .
17. Baruah A, Baruah MK. Phenotypic Diversity and Clinico-Hematological Profile of Hb E-Beta Thalassemic Children. *Indian J Hematol Blood Transfus.* 2020; 36(1):117-122. doi: 10.1007/s12288-019-01150-5 .
18. Abah, SE, Burté F, Marquet S, Brown BJ, Akinkunmi F, Oyinloye G et al. Low plasma haptoglobin is a risk factor for life-threatening childhood severe malarial anemia and not an exclusive consequence of hemolysis. *Sci Rep.* 2018;8(1): 17527. doi:10.1038/s41598-018-35944-w
19. Ragab SM, Safan MA, Badr EA. Study of serum haptoglobin level and its relation to erythropoietic activity in Beta thalassemia

- children. *Mediterr J Hematol Infect Dis*. 2015; 7(1):e2015019. doi: 10.4084/MJHID.2015.019 .
20. Atmakusuma TD, Nasution IR, Sutandyo N. Oxidative Stress (Malondialdehyde) in Adults Beta-Thalassemia Major and Intermedia: Comparison Between Before and After Blood Transfusion and Its Correlation with Iron Overload. *Int J Gen Med*. 2021;14:6455-6462. doi: 10.2147/IJGM.S336805 .
 21. Basu D, Adhya DG, Sinha R, Chakravorty N: Role of malonaldehyde as a surrogate biomarker for iron overload in the β -thalassemia patient: A systematic meta-analysis. *Advances in Redox Research*. 2021; 3:100017.
 22. Melamed-Frank M, Lache O, Enav BI, Szafranek T, Levy NS, Ricklis RM, et al. Structure-function analysis of the antioxidant properties of haptoglobin. *Blood*. 2001; 98(13):3693-8. doi: 10.1182/blood.v98.13.3693.
 23. Van VH, Langlois M, Delanghe J. Haptoglobin polymorphisms and iron homeostasis in health and in disease. *Clin Chim Acta* 2004; 345:35–42.
 24. Kasvosve I, Gomo ZA, Gangaidzo IT, Mvundura E, Saungweme T, Moyo AV, et al. Reference range of serum haptoglobin is haptoglobin phenotype-dependent in blacks. *Clinica Chimica Acta* 2000;296:163-70 .
 25. Imrie H, Fowkes FJ, Michon P, Tavul L, Hume JC, Piper KP et al. Haptoglobin levels are associated with haptoglobin genotype and alpha+ -Thalassemia in a malaria-endemic area. *Am J Trop Med Hyg*. 2006;74(6):965-71.
 26. Naryzny SN, Legina OK. Haptoglobin as a Biomarker. *Biochem Mosc Suppl B Biomed Chem*. 2021;15(3):184-198. doi: 10.1134/S1990750821030069.
 27. Blum S, Asaf R, Guetta J, Miller L, Asleh R, Kremer R, et al. Haptoglobin genotype determines myocardial infarct size in diabetic mice. *J Am Coll Cardiol* 2007; 49 (1):82-7.
 28. Awadallah MS, Nimer AN, Atoum FM, Saleh AS. Association of haptoglobin phenotypes with ceruloplasmin ferroxidase activity in β -thalassemia major. *Clin Chim Acta* 2011; 412:975-79.
 29. Shimada E, Tadokoro K, Watanabe Y, Ikeda K, Niihara H, Maeda I, et al. Anaphylactic transfusion reactions in haptoglobin-deficient patients with IgE and IgG haptoglobin antibodies. *Transfusion* 2002; 42:706–73. doi: 10.1046/j.1537-2995.2002.00117.x.
 30. Edwards O, Burris A, Lua J, Wilkie DJ, Ezenwa MO, Doré S. Influence of Haptoglobin Polymorphism on Stroke in Sickle Cell Disease Patients. *Genes (Basel)*. 2022 Jan 14;13(1):144. doi: 10.3390/genes13010144..