Discussion

Thalassemia has become an important issue of clinical practice. With improved clinical care and increasing survival of affected individuals, complications are being increasingly recognized in older patients. Progress in our understanding of the mechanisms underlying the remarkable phenotypic variability in β-thalassemia has been made possible by a combination of analysis of the molecular basis of the different forms of thalassemia and analysis of the genotype/phenotype relationships. This phenotypic variability is mainly due to genetic factors which still have been incompletely defined. In addition, little is known about the phenotypic heterogeneity of complications of thalassemia and its pathophysiology. [8, 29]

In the present study, a significant decrease in weight and BMI were found in thalassemic patients versus the control (P 0.001). The pathogenesis of growth failure is multifactorial and is mainly due to chronic anemia and hypoxia, chronic liver disease, zinc and folic acid deficiency, iron overload, intensive use of chelating agents, emotional factors, endocrinopathies and GH-IGF-1 axis dysregulation. [224]

In agreement with our study, Salih and Mosawy [225] found decreased weight, BMI and subclinical hypothyroidism in thalassemic patients compared to normal controls. They attributed under-weight and decreased BMI in their patients to endocrinopathies secondary to iron overload. As regards height, in this study although the mean height in thalassemic patients is lower than in the control group, but it didn't reach the significant level. This is contrary to a study conducted by Pemde et al [226] where they found that 33% of thalassemic patients are short stature and they attributed that to iron overload as a significant correlation exists between high serum ferritin level and short stature. However, several studies showed high prevalence of short stature in TM children and adolescents treated intensively with desferrioxamine. [227-228]

βTM is characterized by reduced Hb level (<7 g/dl), mean MCV > 50 < 70 fl and mean MCH > 12< 20 pg. Thalassemia intermedia is characterized by Hb level between 7 and 10 g/dl, MCV between 50 and 80 fl and MCH between 16 and 24 pg. Thalassemia minor is characterized by reduced MCV and MCH, with increased Hb A2 level.[229] In the present study, blood indices showed a statistically significant lower mean Hb, PCV, MCV, MCH, RBCs, were found in patients compared to the controls (P= 0.001).

The Hb pattern in β-thalassemia varies according to β-thalassemia type. In β^0 thalassemia homozygotes, HbA is absent and HbF constitutes the 92-95% of the total Hb and Hb A2 is 2-5%. In β^+ thalassemia homozygotes or β^+/β^0 compounds heterozygotes HbA levels are between 10 and 30% and HbF between 70-90%. HbA2 is variable in β thalassemia major and it is enhanced in β thalassemia trait.[230]

In this study Hb, electrophoresis revealed that in thalassemic group HbA had a range between 5.0 and 24.0 %, while Hb F constituted 73.0 – 94.0% of total Hb. On the other hand, Hb A2 did not show significant difference than control and it ranged between 1.0 and 6.0%. These findings coincide with Paunipagar et al, who reported that Hb A2 % was found to be in normal range in β-thalassemia major, and this may be due to increased number of F cells in β-thalassemia major, that have a decrease HbA2 content. [231]

Anemia results from a combination of ineffective erythropoiesis, peripheral hemolysis and an overall reduction in hemoglobin synthesis. In addition, the present study revealed the occurrence of immune mediated hemolysis, both alloimmune or autoimmune, especially in poly-transfused patients. Moreover, intravascular hemolysis cannot be ruled out as 16% of thalassemic patients had a positive antiglobulin test, 14% of them had direct antiglobulin test reflecting autoimmunization and the remaining 2% were due to alloimmunization. This is partly attributed to lack of extended phenotyping as a standard routine in our blood banks.

Our findings coincide with Salama et al [189] who reported autoimmune RBCs destruction in poly-transfused patients which they proved to be autoantibodies mounted against CD59 with decreased expression of this complement stabilizing protein. On the other hand, complement activation depends on the level of serum haptoglobin, as higher haptoglobin levels prevent complement activation. Hence, with intravascular hemolysis and release of free Hb, haptoglobin level further decreases which aggravates the immune mediated hemolytic process. [232]

The DAT detects antibodies, most commonly IgG, or complement on the surface of red cells. It has been theorized that loss of sialic acid in aged RBCs creates a "senescent cell antigen" that binds to an autologous IgG antibody, specific for that antigen with subsequent phagocytosis by macrophages and removal from the circulation. [233]

It seems that in thalassemic patients, it is rather a defective membrane biogenesis or over balanced rate of removal of sialic acid from thalassemic RBCs membrane and not the mere red cell senescence which induce IgG binding. The question still remains why a small percentage of thalassemic RBCs have reduced sialic acid and consequently a positive DAT? The answer to this question may be found in the complexity and diversity of the immune system, as well as in the clinical and molecular heterogeneity of thalassemia. Moreover, epigenetic and genetic factors most likely play a role in RBCs autoimmunization as a positive DAT differed by blood group. [233]

Our findings agree with Arinsburg et al [233] as only 2/8 patients with positive DAT had blood group B compared to the other groups. They reported that blood group B patients were 2.5 times less likely to have a positive DAT. They added that a positive DAT was significantly associated with splenectomy; this could be explained by the fact that splenectomized patients have higher IgG levels.

In a study conducted by Singer et al, [188] splenectomy was found to be a risk factor for alloimmunization in 36% vs.12.8% non splenectomized, and 25% of their patients had positive Coombs' test, while Ameen et al [190] reported alloimmunization in 30% of patients aged 2-10 years. On the other hand, Shamsian et al [187] and Hok-Kung et al [234] found that splenectomy did not affect the incidence of alloimunization, which coincides with our results.

On examining the impact of splenectomy on the blood indices, the present study revealed a significantly lower PCV in non splenectomized patients (P=0.033).This could be ascribed to the hemodilutional effect of splenomegaly.
As regards, the significantly high MCV in splenectomized patients (P=0.008), it could be attributed to the increased number of circulating reticulocytes that are usually removed by the spleen. While the significant higher platelets count in splenectomized patients (P=0.008) might be due to altered splenic function in pooling of platelets. The duration of this post splenectomy thrombocytosis is not well defined.[235]

Worldwide, the estimated prevalence of HCV infection is 2.2% corresponding to about 130 million HCV positive persons. [236] The lowest prevalence (0.01-0.1%) has been reported from countries in United Kingdom and Scandinavia , the highest prevalence (15-20%) has been reported in Egypt. [237] This study showed that 32% of thalassemic patients are HCV positive, which may be attributed to the fact that thalassemic patients undergo many transfusions over years and thus have repeated exposure to very high prevalence of HCV infection.

In harmony with our results, Louagie et al [238] reported that no significant differences were found between Hp phenotype distribution and HCV infection. On the other hand, multivariate analysis revealed that HCV was independently associated with a positive DAT. They added that a positive DAT did not correlate with decreased response to transfusion, RBCs survival, hemolysis or increased transfusion requirements.[233] Angelucci and Pilo [239] reported that, in thalassemia the severity of HCV related liver disease is aggravated by iron overload. Both lead, albeit through different mechanisms to hepatocellular necrosis, fibrosis and cirrhosis. They added that reduction of liver inflammation is paralleled by improved iron chelation.

In the present study, a statistically significant relation was found between a positive DAT and a lower serum haptoglobin level (P=0.017) being consumed in the trapping of free Hb from the circulation. On the other hand, the lower levels of serum haptoglobin in thalassemic patients could be attributed to the presence of different polymorphisms of the haptoglobin gene. In this study, the genotype Hp1-1results in the highest level of serum haptoglobin, while Hp2-2 was associated with lower serum haptoglobin level which was statistically significant ($P< 0.05$).

This is in agreement with Kasvosve et al [240] who reported that the reference values of haptoglobin depend on the Hp phenotype. In the same context, Imrie et al [241] reported that HP levels were related to age, Hp genotype, Hb levels, parasitemia, splenomegaly, and α+- thalassemia genotype. It also appears that the rate of clearance of free hemoglobin from circulation is phenotype-dependent which has been demonstrated by Van et al [242], hence the clearance of Hp1-1-Hb complex is faster than that of Hp2-Hb complex.

In the present study, 56% of thalassemic patients had the Hp2-2 genotype, followed by Hp 2—1 genotype,(30%) and Hp1-1(14%) while in the control, Hp2-1 predominated (44%) followed by Hp2-2(36%) and Hp1-1(20%) while Hp0 did not exist in both groups. This coincides with the results mentioned by Koch et al [221] ,who found in a group of 249 individuals, that haptoglobin genotype distribution was as follows : 48.2% Hp 2-1, 37.3% Hp 2-2 and 14.5% for Hp1-1. Study of the genotypic distribution among the northwest European population showed that, 16% of their individuals are Hp1-1, 48% Hp2-1 and 36% Hp2-2 which corresponds to allele frequencies of 0.4 (Hp1)and 0.6 for (Hp-2).[243, 244]

In the present study, we observed a discrepancy in the prevalence of Hp genotypes in thalassemic patients versus the control. While Hp2-2 was more common in thalassemic patients, Hp 2-1 predominated in the control group. We might speculate that being encoded on chromosome 16 q as the α hemoglobin chain, this specific region is extremely sensitive to genomic DNA rearrangement, hence its proximity to this gene could be responsible for favoring the Hp2-2 genotype.

Moreover, genetic determinants mapping outside the globin gene cluster encode transcription factors that are involved with the activation or repression of haptoglobin synthesis. It is synthesized as a single precursor protein that is proteolytically processed after translation to form α and β subunits. The Hp2 allele arises from an internal duplication of a 7 kb DNA fragments that includes exons 3 and 4 of the Hp1 gene. It seems that the Hp 2 allele is the result of a breakage and reunion event at non homologous positions within the second and fourth introns of two Hp1 genes. On the other hand, this proximity of Hp 2 to the globin chain could produce triplication or quadruplication of the α globin chains with more alleles hence tipping the balance towards enhanced hemolysis and consequently more oxidative stress. This imbalance could be maximized in the absence of α- Hb stabilizing protein, a chaperone of α globulin chain. This area of research is warranted in patients with thalassemia. [245]

Free hemoglobin is highly toxic to renal tissues especially in cases of low Hp levels. It induces oxidative damage to erythrocytes [243] and protection is greater for Hp1-1 and least for Hp2-2. [244] These data are consistent with earlier findings showing that the consumption of vitamin C in the plasma in persons with Hp2-2 was more rapid than in the plasma of those with Hp 1-1, and that vitamin C levels are significantly lower in those with Hp 2-2. [246]

The difference between both types of haptoglobin proteins may be amplified in the vessel wall because of differences in the sieving capacity of haptoglobin. A key site of action of haptoglobin in neutralizing the oxidative capacity of free hemoglobin is the extravascular space, particularly after endothelial injury.

Haptoglobin 1-1 and 2-2 differ in their ability to sieve into the extravascular compartment across the endothelial barrier. [125,247] Moreover, a specific binding of Hp towards neutrophils has been reported in a demonstration that neutrophils respiratory burst activity can be inhibited by haptoglobin. Haptoglobin is concentrated within granules of neutrophils and macrophages, and then exocytosed after neutrophil activation at sites of inflammation in an attempt to modulate granulocyte activity. [248] Apparently, Hp1-1 is a ligand for the Mac-1 (complement receptor type 3, CD11b/CD18) Integrin dimers on granulocytes and monocytes. These integrins are involved in cell-cell and cell-matrix interactions, including binding of fibrinogen and ICAM-1. [249]

The toxicity of free Hb arises from the heme iron which can react with endogenous hydrogen peroxide to produce free radicals, which in turn may produce severe oxidative damage. In addition, Hb is a potent scavenger of NO, a signaling molecule that regulates smooth muscle relaxation, endothelial adhesion molecule expression, platelet activation and aggregation.[115,250,251,)]

The protective effect of haptoglobin against free Hb extends to protect the permeability of RBCs. Gueye et al [252] measured the release of potassium (K+), lactate dehydrogenase (LDH) and pH variation in the extracellular medium. In the presence of Hb, K+ and LDH concentrations increased slowly and regularly for at least 24 hours. Addition of external Hp, regardless of its phenotypes, significantly reduced the release of K+ and LDH induced by free Hb. This protective effect was more marked for Hp 1-1 than for Hp 2-1 and Hp 2-2.

The variable capacity of the three Hp phenotypes to prevent oxidative stress induced by free Hb could be related to differences in affinity of the three Hp phenotypes for Hb. However, Asleh et al [253] showed no significant difference in affinity of Hb , in contrast to others [124,125].It seems that the mechanism of protection of Hp and its phenotype dependency is not yet elucidated.

Iron overload depends on the volume of transfused blood, iron absorbed from gastrointestinal tract which is mediated by down regulation of hepcidin and up regulation of ferroportein, in addition to intravascular hemolysis. Moreover, the ability of chelator to remove excess iron depends on two factors: the rate at which the chelator depletes storage iron and the rate of continued iron accumulation. [88] There are two broad objectives of iron chelation therapy. The first is to maintain body iron at safe levels and the second is to detoxify excess labile iron. If high iron loading has already developed prior to chelation, there will be a period of negative iron balance which will initially be necessary for months or even years before body iron is reduced to safe level.[254]

In the present study, the majority of the patients had very high ferritin levels, with a mean of 3287 µg/l, 40% of them had serum ferritin between 1000 to 2500 µ/l, while 56% of patients had values above 2500 µg/l. We also found that, the Hp 2-2 was associated with the highest serum ferritin which is congruent with Van et al. [255]

On the other hand, patients with lower serum ferritin may be receiving additive drugs that act as iron chelators namely silymarin which is prescribed to patients with hepatitis and elevated liver enzymes. Haggag et al [256] studied the therapeutic value of silymarin as iron chelator in iron overloaded patients. They concluded that combined use desferroxamine or deferasirox and silymarin achieves a better iron chelation than single drug use as reflected by lower serum ferritin.

The sequestration of Fe^{2+} by ferritin inhibits Fe^{2+} catalyzed generation of ROS via the Fenton reaction and thereby protects against oxidative stress. ROS are incriminated in injury to the liver, heart and endocrine glands. Oxidation of ferrous ions to ferric form enhances its binding with transferrin and its subsequent transport to storage or utilization sites. Free radicals or their derivatives are also involved in important physiological functions including regulation of vascular tone, sensing of oxygen tension and regulation of functions that are controlled by oxygen concentrations, enhancement of signal transduction from various membrane receptors including the antigen receptors of lymphocytes and oxidative stress responses that ensure the maintenance of redox homeostasis. [257]

In the present study, a significantly higher level of MDA, a marker of lipid peroxidation and oxidative stress was found in thalassemic patients versus the control, ($P < 0.001$) reflecting a state of significant oxidative stress in patients group. More important, is the highest level of MDA significantly found in patients with the Hp2-2 phenotype as compared with the other phenotypes ($P=0.005$).

Our results support the findings of Blum et al [258] who reported that the Hp-2 protein is associated with increased generation of oxidative active iron, while the Hp-1 protein is associated with increased production of the antioxidant cytokine IL 10 in diabetic mice with myocardial infarction, which is reflected on the infarct size. They stated that the Hp1 phenotype has an antioxidant and anti-inflammatory properties.

Moreover, Awadallaha et al [259] and others [99,244,245,260] demonstrated decreased antioxidant capacity and increased products of peroxidative damage in plasma of β-thalassemic patients. Once Hp-Hb complex is formed, it is quickly quenched from the circulation and scavenged by the CD163 receptors of hepatocytes and macrophages. [152]

Studies have demonstrated that internalization of the Hb-Hp complex by the CD163 receptors is more potent for Hb-Hp 2-2 complex than for that bound to Hp1-1or Hp 2-1. Hence, it was proposed that individuals with Hp 2-2 phenotype are under greater oxidative stress as a result of higher iron accumulation within the macrophages. [242,243]

It has been suggested that the increased levels of plasma MDA in thalassemia may result from several mechanisms. First, plasma MDA could be enhanced in thalassemia patients because it may be dependent on the amount of circulating erythroid precursors and peripheral blood erythrocytes that have a high density of unpaired α-globin chains, these are prone to denaturation and oxidation. The chains in thalassemic RBCs can auto-oxidize, releasing heam and generating superoxide .In addition, perioxidation of tissues such as liver lipids ensures MDA leak into the plasma. [99]

As regards CRP, although thalassemic patients had a higher significant level than the control, yet their mean value was within normal range. This highlights that the excessively elevated serum ferritin was attributed to frank iron overload and not to acute phase. In addition, we observed a higher mean NLR in thalassemic patients than the control as well as a higher NLR in splenectomized compared to non splenectomized, although both differences were not statistically significant. The higher NLR is attributed to relative increase in neutrophils compared to lymphocytes with subsequent neutrophil activation which is responsible for the vasculopathy and oxidative stress in thalassemia. This is reflected by the presence of significant positive correlation between CRP and NLR in our study (P=0.022).

On the other hand, Ludwiczek et al [261] demonstrated that inflammation is a known regulator of iron transport and storage which added another explanation for the raised plasma MDA. Inflammation stimulates the uptake and retention of iron into monocytes and reticuloendothelial cells. This is supported by our findings as a significant positive correlation was found between serum ferritin and CRP reflecting the role of inflammation in enhancing the oxidative stress in thalassemic patients and vice versa. Papanikolaou et al[262] forwarded another fact that hepcidin which is increased in inflammation has been identified as a critical participant in cellular regulation of iron storage.

The state of oxidative stress is as well attributed to lifelong blood transfusion, increased intestinal absorption of iron and intravascular hemolysis .[263] Therefore, the combination of effective iron chelator agents with natural or synthetic antioxidants can be very helpful in clinical practice and the regulation of the antioxidant status of patients with β-thalassemia major.

On the other hand, epidemiological studies have found numerous cases of anhaptoglobinemia all over the world. True anhaptoglobinemia (Hp 0-0) in which the expression of the Hp gene is absent, approximately present in 1 of every 1000 whites. Next to the occurrence of Hp 0-0, secondary hypohaptoglobinemia can occur as a consequence of congenital diseases such as hemolytic disorders (eg, hereditary red cell membrane and enzyme defects, thalassemia, sickle cell anemia).[137] After destruction of erythrocytes, Hp is saturated when approximately 500 to 1,500 mg/L of free hemoglobin is released into the plasma, which corresponds to only a moderate degree of hemolysis. [124]

The clinical importance of determining haptoglobin genotyping and its level in serum relies in detecting patients with Hp 0 phenotype whom are at great risk of developing fatal transfusion anaphylaxis. In Japanese and Chinese, Hp 0 allele occurs more often than that of IgA deficiency which is a known risk factor for post transfusion sensitization and anaphylaxis. [264] In the present study, the 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 reported by Koda et al. [265] Similarly, Shimada et al [266] reported on patients with haptoglobin deficiency associated with Hp IgG antibodies that experienced fatal anaphylactic non hemolytic transfusion reactions. It is worthmentioning that the Hp- 2 allele has a superior ability to form antibodies than the Hp-1 allele.

Another difficulty in interpreting low serum haptoglobin levels is the fact that its reference values depend on the Hp phenotype. The reference values for Hp 2-2 in serum are considerably lower (0.38 to 1.50 g/L) than for Hp 2-1 (0.44 to 1.83 g/L) and Hp 1-1 (0.57 to 2.27 g/L). Therefore, Hp 2-2 subjects (30% to 50% of whites) may be in a state of relative hypohaptoglobinemia compared with the other phenotypes. [240]

Several analytical techniques have been used to assay serum haptoglobin. Initially, methods for determining Hp were based on enhancement of the peroxidase activity of Hb by Hp-Hb binding.[267,268]Other methods are based on the altered spectrophotometric properties of Hp-bound Hb or on the separation of the Hp-Hb complex from unbound Hb.[269] In addition, immunological techniques such as radial immunodiffusion have also been developed. [270] However, many of these methods suffer from lack of sensitivity, slow turnaround time, interference from in vitro hemolysis, large sample requirement, and/or dependence on knowledge of haptoglobin phenotypes.

Nephelometric technique which has been used in this study for assaying serum haptoglobin offers the advantages of requiring much shorter time for analysis, higher sensitivity, precision, small sample requirement, independence of knowledge of phenotype, and lack of significant interference from in vitro hemolysis. [271]

Hp phenotype can be determined by gel electrophoresis, isoelectric focusing, chromatography or ELISA. [272-275] Although these phenotyping methods have been used for a relatively long time and many studies have been conducted based on these methods, these techniques are not designed to detect patients harboring the $Hp\ del$ allele that is, they cannot discriminate true anhaptoglobinemia from conditions of acquired undetectable haptoglobin levels. [137]

Conventional PCR can determine haptoglobin genotyping fairly well when appropriately designed. However, multiple primers and reactions are required to distinguish between the $Hp1$, $Hp\ 2$, and $Hp\ del$ alleles. [276]

Koch et al. [221] designed 4 primers to distinguish the Hp 1 from the Hp 2 alleles. They suggested 3 protocols for genotyping, each yielding different patterns of PCR products. In the simplest protocols (protocol 1), which used just 1 set of primers (A and B), the Hp 1 allele and the Hp 2 allele were amplified to generate bands of 1757 bp and 3481 bp, respectively.

In some instances where a band of the *Hp* 2 allele might not be easily detected due to its large size, another set of primers was applied to create a *Hp* 2-specific small amplicon of 349 bp. Protocol 2, consists of two separate reactions, one reaction, using primers A and B, was aimed at detecting the 1757-bp Hp 1-specific product, and the other reaction, using primers C and D, was aimed at detecting the 349-bp Hp 2-specific product.

They also tried to use all the 4 primers simultaneously (protocol 3) to yield an *Hp*1-specific band (1757 bp), 3 *Hp* 2-specific bands (349 bp, 1910 bp, and 1923 bp), and 2 nonspecific products (195 bp and 196 bp). In this case, the product pattern was more complex than that obtained with the combined samples of the simplex reactions. Comparative testing with DNA samples showed that the three protocols for haptoglobin genotyping yielded identical results. [221]

With protocol 1, 10 ng of genomic DNA was required for genotyping, whereas with protocols 2 and 3, only 1 ng of DNA was sufficient. Protocol 2 or 3 were especially useful in situations in which the presence or absence of the 3481-bp product could not be determined conclusively. We observed that such a problem occurred in cases in which the PCR was run under suboptimal conditions, with extensively degraded DNA, or with limited quantities of DNA.

In this study we used protocol 2, which was more laborious than protocol 3. However, protocol 2 gave rise to relatively simple patterns of DNA bands. In contrast, although protocol 3 involved only one amplification reaction, it produced a relatively complex pattern of DNA bands.

To the best of our knowledge ,this is first study relating Hp polymorphism to the oxidative stress in patients with β thalassemia .It might be exploited in the future in providing insights in the therapeutic potential of haptoglobin preparations in the relief of oxidative stress.