Journal Pre-proof

Oxidative status in the β-thalassemia syndromes in Sri Lanka; a cross-sectional survey

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PII: S0891-5849(21)00119-2

DOI: https://doi.org/10.1016/j.freeradbiomed.2021.02.028

Reference: FRB 15091

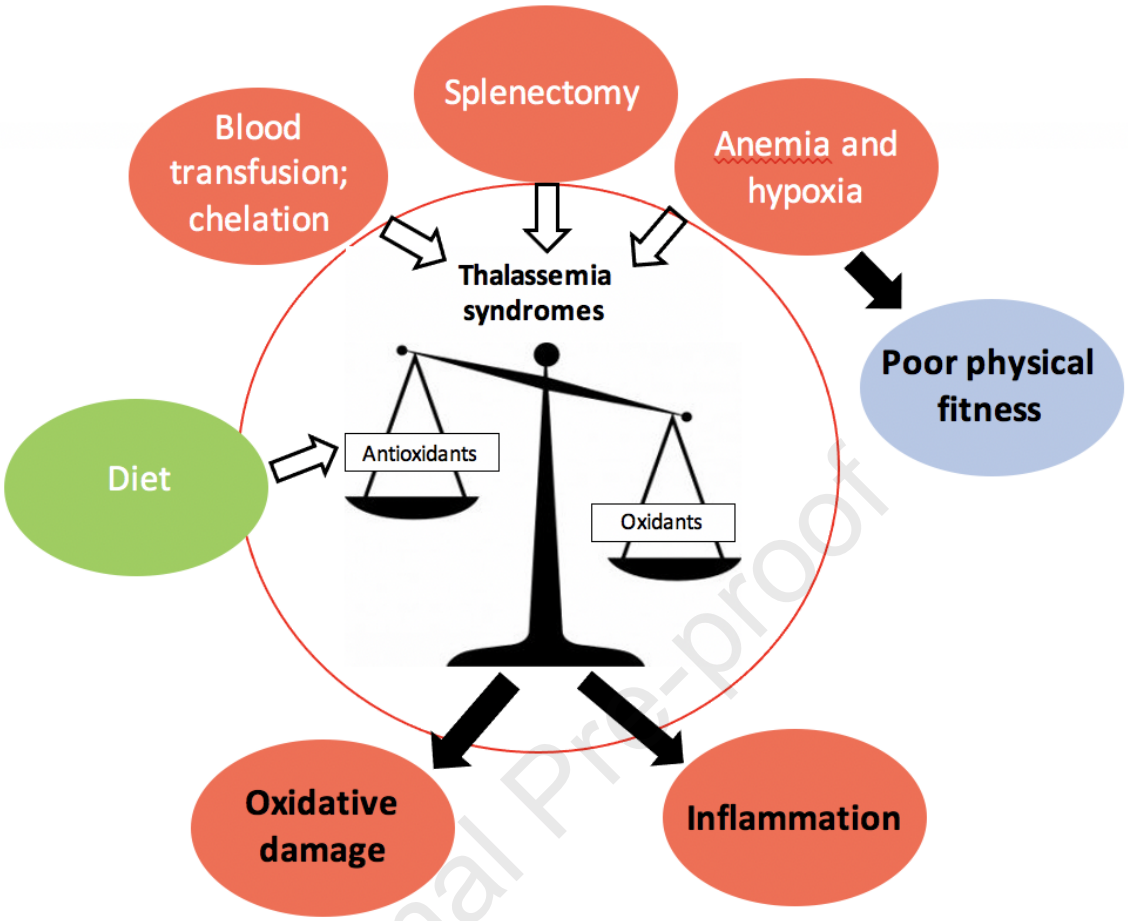
To appear in: *Free Radical Biology and Medicine*

Received Date: 11 January 2021

Revised Date: 17 February 2021

Accepted Date: 21 February 2021

Please cite this article as: A. Allen, S. Perera, S. Mettananda, R. Rodrigo, L. Perera, T. Darshana, F. Moggach, A.J. Crawford, L. Heirene, C. Fisher, N. Olivieri, D. Rees, A. Premawardhena, S. Allen, Oxidative status in the β-thalassemia syndromes in Sri Lanka; a cross-sectional survey, *Free Radical Biology and Medicine*, https://doi.org/10.1016/j.freeradbiomed.2021.02.028.



Title: **Oxidative status in the β-thalassemia syndromes in Sri Lanka; a cross-sectional survey.**

Running title: Oxidative stress and damage in thalassemia, Sri Lanka

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Key words: thalassemia; oxidative stress; oxidative damage; antioxidants; vitamins C and E.

Highlights:

- Oxidative stress and damage were common in the β-thalassemia syndromes in Sri Lanka
- Oxidative stress and damage varied both between and within thalassemia syndromes
- Oxidative stress and damage tended to be worse in patients with HbE β-thalassemia
- Oxidative stress and damage were associated with splenectomy and chelation therapy
- Poor diets likely further exacerbate antioxidant deficiency

Abstract

In the β-thalassemias, oxidative stress, resulting from chronic hemolysis, globin chain imbalance, iron overload and depleted antioxidant defences, likely contributes to cell death, organ damage, anemia, hypoxia and inflammation. We assessed variations in these parameters in β-thalassemia syndromes in Sri Lanka.

Between November 2017 and June 2018, we assessed children and adults attending two thalassemia centres in Sri Lanka: 59 patients with HbE β-thalassemia, 50 β-thalassemia major, 40 β-thalassemia intermedia and 13 HbS β-thalassemia. Median age was 26.0 years (IQR 15.3-38.8), 101 (62.3%) were female and 152 (93.8%) of Sinhalese ethnicity. Methemoglobin, plasma hemoglobin, heme and ferritin were measured as sources of oxidants; plasma total antioxidant capacity, haptoglobin, hemopexin and vitamins C and E assessed antioxidant status; plasma thiobarbituric acid reactive substances and 8-hydroxy–2'–deoxyguanosine assessed oxidative damage; hemoglobin, plasma erythropoietin and transferrin receptor assessed anemia and hypoxia and plasma interleukin-6 and C-reactive protein assessed inflammation. Fruit and vegetable intake was determined by dietary recall. Physical fitness was investigated using the six-minute walk test and measurement of handgrip strength.

Oxidant sources were frequently increased and antioxidants depleted, with consequent oxidative damage, anemia, hypoxia and inflammation. Biomarkers were generally most abnormal in HbE β-thalassemia and least abnormal in β-thalassemia intermedia but also varied markedly between individuals with the same thalassemia syndrome. Oxidative stress and damage were also more severe in splenectomized patients and/or those receiving iron chelation therapy. Less than 15% of patients ate fresh fruits or raw vegetables frequently, and plasma vitamins C and E were deficient in 132/160 (82.5%) and 140/160 (87.5%) patients respectively. Overall, physical fitness was poor in all syndromes and was likely due to anemic hypoxia.

Studies of antioxidant supplements to improve outcomes in patients with thalassemia should consider individual patient variation in oxidative status both between and within the thalassemia syndromes.

Introduction

Despite being the most common monogenic diseases, the true disease burden of the inherited hemoglobin disorders is unknown. It is estimated that >7% of the world's population carry a hemoglobin variant resulting in 300,000-500,000 annual births with a serious hemoglobin disorder accounting for at least 3.4% of under-five deaths [1].

A recent survey of 23 thalassemia treatment centres in Sri Lanka identified 1219 patients with β-thalassemia major, 360 with HbE β-thalassemia and 50 with HbS β-thalassemia [2]. Patients with β-thalassemia major require life-long intensive clinical management including at least monthly blood transfusion. The clinical course of HbE β-thalassemia is more variable, ranging from mild to severe anemia. In both conditions, transfusional iron overload in the absence of early and effective iron-chelation therapy results in liver, cardiac and endocrine dysfunction including impaired growth and glucose intolerance. Sickle cell disorders are less common in Sri Lanka and the majority of patients have the Asian haplotype associated with a relatively mild phenotype [3-5].

In the β-thalassemias, β globin chains are either absent or produced at a reduced rate, resulting in globin chain imbalance. Within red cells, free α-globin chains and unstable hemoglobins such as HbE and HbS auto-oxidise, producing methemoglobin (MetHb), hemichromes and free radicals. Hemichromes bind to the red cell membrane sequestering the band 3 protein and resulting in cell lysis [6]. Following hemolysis, free hemoglobin (Hb) in the plasma undergoes further oxidative reactions, generating free radicals and releasing free heme and iron, which are toxic. In the absence of an adequate supply of antioxidants, oxidant damage occurs to cell membranes, vascular endothelium and various organs, and a pro-inflammatory state can ensue [7]. In addition to host antioxidant enzyme systems, the dietary antioxidants vitamins C (ascorbate) and E (alpha-tocopherol) scavenge free radicals [8]. Vitamin C reduces MetHb, ferryl hemoglobin and globin radicals, preserves alpha-tocopherol in lipoproteins, and generates and preserves nitric oxide needed for vascular regulation. Vitamin E, a scavenger of peroxyl radicals, protects cell membranes from lipid peroxidation and lysis. As well as increased utilisation in thalassemia, dietary deficiency may also contribute to low concentrations of these vitamins [9,10].

We undertook a comprehensive assessment of oxidant, antioxidant and inflammatory status, and their underlying contributory factors, in patients attending thalassemia services in Sri Lanka.

Materials and Methods

Between November 2017 and May 2018, we undertook a cross-sectional study of children with thalassemia attending Colombo North Teaching Hospital, Ragama and adolescents and adults attending Hemals Thalassemia Care Unit, Mahara, Sri Lanka. Participation was voluntary and informed, signed consent from patients or

parents/guardians was obtained. Patients who were unwell on the day of recruitment were excluded.

Sampling/ recruitment procedure

The study clinician examined the patients and reviewed case records. The primary diagnosis, spleen status, chelation regime, transfusion history and known genetic modifiers of disease severity such as α-thalassemia were recorded. Height and weight were measured and body mass index (BMI) in adults and BMI percentile in children and adolescents (<20 years) determined according to standard reference data [11].

Fruit and vegetable intake was assessed using a dietary recall questionnaire. Physical function was assessed by the six-minute walk test [12] and handgrip strength [13]. Briefly, participants walked at their own pace for 6 minutes along a 100m level corridor whilst observed and encouraged by a study staff member and the total distance walked was recorded. Using their dominant hand, each patient was asked to squeeze a bulb dynamometer (Baseline^{R}, New York, USA) as tightly as they could. The average total amount of static force applied of three readings was recorded (pounds per square inch; psi).

Finally, five millilitres of venous blood was collected and MetHb (%) and carboxyhemoglobin (%; CoHb) and p50 (mmHg) measured immediately (ABL-FLEX 80 blood gas analyser with integral co-oximeter; Radiometer, Medical ApS, Akandavec, Denmark). Equal volumes of the remaining blood sample were transferred into EDTA and lithium heparin and transported immediately to the laboratory for analysis. Lithium heparin venous blood (2 ml) was also collected from 26 healthy adult volunteers for the measurement of plasma vitamin C and E.

Laboratory analyses

Routine hematological indices were measured in EDTA whole blood (Ac.T differential analyser; Beckman Coulter, Luton, UK). Anemia was defined as Hb concentration <11.5 g/dl in children <12 years, Hb <12.0 g/dl in females ≥12 years and males aged 12-14 years and Hb <13.0 g/dl in males ≥15 years, and severe anemia was defined as Hb concentration <8.0 g/dl in adults and children > 5years or <7.0 g/dl in pregnant women and children < 5years [14]. Hemoglobin variants were quantified by capillary electrophoresis (Capillarys 2 Flex Piercing Instrument; Sebia, Lisses, France). EDTA samples were then centrifuged and the plasma and buffy coats removed.

DNA was extracted from buffy coat samples (QIAamp DNA mini –kit; # 51304, QIAGEN, Manchester, UK) and genotyped for the presence of triplicated α –globin genes and deletional forms of α-thalassemia (-$\alpha^{3.7}$/αα and $\alpha^{4.2}$/αα) using previously described methods [15,16].

Lithium heparin blood samples were centrifuged and 250ul aliquots of plasma transferred into light protective cryo-storage tubes. One aliquot from each patient was stabilised in an equal volume of 6% metaphosphoric acid within 4 hours of

collection for the vitamin C assay. All plasma samples were stored at -20°c until further analyses.

Plasma hemoglobin, heme and ferritin were measured as sources of oxidants. Antioxidant status was evaluated by measurement of plasma total antioxidant capacity, haptoglobin, hemopexin and vitamins C and E. Oxidant damage was assessed by measurement of plasma thiobarbituric acid reactive substances (TBARS) and 8-hydroxy–2'–deoxyguanosine (8-oxo-dG). Plasma erythropoietin, transferrin receptor and nitrite assessed response to anemia and hypoxia, and inflammation was assessed by measurement of plasma Interleukin- 6 (IL-6) and C-reactive protein (CRP). Details of all assays and kits used are summarised in supplementary table S1. All assays were performed in accordance with the manufacturers' guidelines and all samples were tested in duplicate for each biomarker. Quality controls were included with each batch of tests.

Stabilised plasma samples and heparin plasma were shipped back to Oxford, UK on dry-ice

Stabilised plasma samples were transferred to the Scottish Trace Element and Micronutrient Diagnostic and Research laboratory, Glasgow, UK for measurement of vitamin C, by high performance liquid chromatography (HPLC), [17] using a Waters auto-sampler, pump and electrochemical detection system; (Waters, Massachusetts, USA).

Heparin plasma samples were transferred to the Department of Clinical Biochemistry, Royal Gwent Hospital, Newport, UK, for the measurement of vitamin E, by HPLC using an Agilent 1200 analyser and multi-wavelength detection system; (Agilent, California, USA).

Statistical methods

Demographic, clinical and laboratory categorical variables were summarized using counts and percentages. Continuous variables tended to have skewed distributions; they were described using median and interquartile range and compared using the Mann-Whitney U and Kruskall Wallis tests. Data analysis was performed using Statistical Package for Social Sciences Software (SPSS), version 26 (New York, USA).

Ethical approval

The study was approved by the Ethics Committee, University of Kelaniya, Sri Lanka (P/225/09/2017) and Oxford University Tropical Research Ethics Committee, Oxford, UK (OXTREC 515-13). The study was conducted in accordance with the declaration of Helsinki (2013) [18].

Results

The study group comprised 162 patients: 59 with HbE β-thalassemia, 50 β-thalassemia major, 40 β-thalassemia intermedia, and 13 HbS β-thalassemia. 1 patient with δβ-thalassemia and 1 with sickle cell disease were excluded.

In 128 (79.0%) patients in whom α-thalassemia genotype was determined, triplicated α-globin genes were present in 3/45 (6.7%) patients with β-thalassemia major, 3/42 (7.1%) HbE β-thalassemia, 19/28 (67.9%) with β-thalassemia intermedia and 0/13 (0.0%) with HbS β-thalassemia. Heterozygous deletional α-thalassemia was present in 3/45 (6.7%) patients with β-thalassemia major, 4/42 (9.5%) HbE β-thalassemia, 3/28 (10.7%) β-thalassemia intermedia and 1/13 (7.7%) HbS β-thalassemia. Homozygous deletional α-thalassemia was present in 1 (2.2%) patient with HbE β-thalassemia and 1 (3.6%) with β-thalassemia intermedia.

Demography, anthropometry and clinical assessment according to diagnostic group are shown in table 1. In all patients, median age was 26.0 years (IQR 15.3-38.8) years and was lower in HbS β-thalassemia and higher in β-thalassemia intermedia than in the other groups. There were more females (101; 62.3%) than males overall but males predominated in HbS β-thalassemia. One hundred and fifty-two (93.8%) patients were of Sinhalese ethnicity. Low body weight occurred in 49 adults (BMI< 18.5 kg/m^2) and 15 children/adolescents (BMI percentile <5%) and was more common in patients with HbS β-thalassemia or HbE β-thalassemia. Only 13 adults (BMI >23.0 kg/m^2) and 2 children/adolescents (BMI percentile >85.0%) were overweight.

As expected, patients with β-thalassemia major had received more frequent blood transfusions in the previous 12 months than those in the other groups, with many receiving more than one blood transfusion per month. Chelation data was available for 160 patients, of whom 111 (69.4%) were receiving iron chelation therapy. Overall, 45/162 (27.8%) patients had undergone splenectomy; splenectomy was more common in HbE β-thalassemia and β-thalassemia major, where approximately 1 in 3 participants were splenectomized.

Oxidant and antioxidant status, oxidative damage, anemia, hypoxia and inflammation are summarised according to the proportion of patients with abnormal values in each diagnostic group (figure 1) and data summarised in figures 2-6 and supplementary table S2. Overall, values for these biomarkers varied both between diagnostic groups and between individuals with the same thalassaemia syndrome.

Sources of oxidants (Fig 1, Fig 2a-d; Suppl Table S2)

Nearly all patients had markedly increased concentrations of markers of hemolysis and iron overload. The exception was ferritin that was raised in just over 50% HbS β-thalassemia patients (Fig 1). Median values varied significantly according to diagnostic group. Patients with HbE β-thalassemia had the highest concentration of plasma hemoglobin and heme and percentage of MetHb in whole blood. However, plasma ferritin concentration was greatest in β-thalassemia major, where more than

half of patients had concentrations more than 10 fold (>2830 ng/ml) above the upper limit of the normal range.

Antioxidants (Fig 1, Fig 3a-e; Suppl Table S2)

Total antioxidant capacity varied significantly according to diagnosis with marked deficiency in all groups except for HbS β–thalassemia where capacity was low in 46% patients. Concentrations of individual antioxidants also varied significantly according to diagnostic group. Median plasma haptoglobin was very low in all groups except for β-thalassemia major where 42% patients had a normal value. Hemopexin concentrations were low in nearly all patients with marked deficiency in HbE β-thalassemia and β-thalassemia major.

Vitamins C and E were markedly deficient in all diagnostic groups except for β -thalassemia intermedia, where around half had normal vitamin C and one-third had normal vitamin E levels. Plasma vitamin C and E were below the lower level of quantitation in 86/160 (53.8%) and 130/160 (81.3%) patients respectively. In healthy adult volunteers, plasma vitamin C was low (<14 μmol/L) in 23/26 (88.5%) and vitamin E was low (<12 μmol/L) in 6/25 (24.0%) with low concentrations of both vitamins in 5/25 (20%; data not shown).

Oxidant damage (Fig 1, Fig 4a -b; Suppl Table S2)

Oxidant damage also varied markedly according to diagnosis. Plasma TBARS was raised in approximately three-quarters of patients with HbE β-thalassemia, two-thirds of patients with β-thalassemia, one in five β thalassemia intermedia but was normal in HbS β-thalassemia. In contrast, median plasma 8-oxo-dG was raised in all diagnostic groups and especially in HbS β-thalassemia.

Anemia, hypoxia and erythropoiesis (Fig 1, Fig 5a -f; Suppl Table S2)

Biomarkers of anemia, hypoxia and erythropoiesis also varied significantly according to diagnostic group. Apart from 3 patients with β-thalassemia major who had been recently transfused, all patients were anemic. Hemoglobin concentration was lowest in HbE β-thalassemia.

CarboxyHb (%) was raised in about three quarters of patients with HbE β-thalassemia and HbS β- thalassemia and about half of patients with β-thalassemia intermedia. Plasma nitrite concentration was normal in most patients, but deficiency occurred in about one in three patients with HbE β-thalassemia and one in five with β-thalassemia major.

Plasma erythropoietin and soluble transferrin receptor were markedly raised in nearly all patients and greatest in those with HbE β-thalassemia, where concentrations of each biomarker were up to 5 times greater than the upper limit of normal in many individuals. A right shift in the oxygen dissociation curve was observed in more than half of patients, and p50 was greatest in patients with HbS β-thalassemia.

Inflammation (Fig 1, Fig 6a -b; Suppl Table S2)

Plasma concentrations of IL-6 varied significantly according to diagnosis and were within the normal range in about two-thirds to half of patients, except for those with HbS β-thalassemia, where levels were markedly elevated. In contrast, CRP was raised in only about 1 in 4 patients and was similar according to diagnosis (P=0.24).

Dietary antioxidants

Amongst 158/162 (97.5%) participants who completed dietary questionnaires, only 40 (25.3%) participants reported that they ate one or more portions of fruit and/or raw vegetables each day. Nearly all participants (149/158; 94.3%) reported that they ate at least one portion of cooked vegetables each day. Dietary intake was similar in each diagnostic group (data not shown).

Physical fitness (Table 2)

Physical fitness was assessed in 54 males and 93 females. Only 2/147 (1.3%) patients were able to achieve ≥80% of their expected walking distance and only 2/147 (1.3%) patients achieved normal handgrip strength (20 psi). Walking distance and handgrip strength were greater in males than females (χ^2 =7.59, p=0.006 and χ^2 =4.23, p=0.04 respectively) but were similar according to diagnosis.

Effects of splenectomy (Table S3)

Blood MetHb and plasma ferritin were significantly greater and plasma antioxidants hemopexin, vitamin C and vitamin E were significantly lower in splenectomized patients than those with intact spleens. Plasma TBARS and CRP were also significantly greater in splenectomized patients.

Effects of iron chelation (Table S3)

Plasma ferritin and haptoglobin were significantly greater and plasma antioxidants hemopexin, vitamin C and vitamin E were significantly lower in patients receiving iron chelation therapy compared to patients who were not. Plasma TBARS was also significantly greater in patients who were chelated. The fraction of Carboxyhb in whole blood and plasma soluble transferrin receptor were both significantly lower in chelated patients.

Effects of α–thalassemia and triplicated α–globin genes

There were too few patients with concomitant α-thalassemia in each diagnostic group to assess the effects of α-globin genotype on oxidative stress and damage. Triplicated α-globin genes were detected in 25/128 (19.5%) DNA samples but most (19/25; 76%) were from patients with β-thalassemia intermedia, which meant it was also not possible to assess the effects of excess α-globin genes in the study group as a whole.

Discussion

As far as we are aware, this is the most comprehensive study of oxidative status in the β-thalassemia syndromes in South Asia. Overall, the combination of markedly increased sources of oxidants with antioxidant deficiency contributed to the DNA damage, assessed by 8-oxo-dG, in all patient groups and lipid peroxidation, assessed by TBARS, in HbE β-thalassemia and β-thalassemia major. Dietary deficiency of vitamins C and E likely contributed to poor antioxidant status. In addition, values for many biomarkers varied significantly both between diagnostic groups and between individuals with the same thalassemia syndrome.

The main instigator of oxidative stress in β-thalassemia is likely to be iron, which participates in the Fenton reaction with hydrogen peroxide to produce reactive oxygen species (ROS). Iron homeostasis is controlled by hepcidin, a regulatory hormone produced primarily by the liver. Hepcidin synthesis is down-regulated by erythropoiesis and up-regulated by inflammation and increased iron. In the β-thalassemias, hepcidin synthesis is suppressed due to increased erythropoiesis, which results in increased gastrointestinal iron absorption [19,20]. Iron from blood transfusion further contributes to increased iron stores in β-thalassemia.

Oxidative status according to clinical group

Raised plasma ferritin concentration, indicating increased iron stores, was present in the majority of patients and greatest in those with β-thalassemia major who are regularly transfused. In addition to excess iron, free radicals are also generated as a consequence of the complex interplay between unstable hemoglobins, globin chain imbalance and chronic haemolysis that occurs in the β-thalassemias [21].
We found that MetHb, plasma hemoglobin and heme were greatest and plasma haptoglobin and hemopexin (chaperones for free Hb and heme) were lowest in HbE β-thalassemia, reflecting the greater degree of haemolysis in these patients. We have previously reported raised MetHb in HbE-β thalassemia in Sri Lanka [22]. These findings are consistent with the greater degree of lipid peroxidation (assessed by TBARS) found in the HbE β-thalassemia group. The structurally and oxidatively unstable HbE molecule likely underlies the greater oxidant stress and damage [23-25]. Hemoglobin E binds to the red cell membrane band 3 with greater affinity than HbA or HbS, promoting cell lysis [26], and Ferryl ($Fe4^{+}$) forms of HbE remain longer in solution and promote hemolysis and further free radical generation [27]. Anemia was also more severe in patients with HbE-β thalassemia than in the other diagnostic groups. The increased fractions of both CoHb and MetHb (unable to bind oxygen) would have contributed to tissue hypoxia. This is reflected in the greatest concentrations of erythropoietin and transferrin receptor in HbE β-thalassemia as responses to anemia and hypoxia.

The structural and oxidative instability of HbS [28-30] also likely contributed to oxidant stress in HbS β-thalassemia and the greatest degree of DNA damage as assessed by plasma 8-oxo-dG. The absence of lipid peroxidation in these patients may be related to their greater total antioxidant capacity, younger age and much lower iron stores due to fewer transfusions [31,32]. Our findings relating to oxidant damage are in keeping with Walter et al., who described lower malondialdehyde (a

naturally occurring product of lipid peroxidation) and increased 8-oxo-dG in sickle cell disease compared with β-thalassemia major [33]. A right shift in the oxygen dissociation curve compensated for hypoxia, and was most marked in patients with HbS β-thalassemia (Suppl Table S2).

Abnormalities were least in patients with β-thalassemia intermedia. These patients were less regularly transfused and iron stores were lower. The greater plasma concentrations of vitamins C and E may explain, in part, the low or normal concentration s of plasma TBARS in the majority of these patients. Interestingly, 19/28 (68%) patients with normal plasma vitamin C and 13/15 (87%) with normal plasma vitamin E concentration were from the β -thalassemia intermedia group.

Effects of splenectomy and chelation therapy

Splenectomized patients had significantly greater sources of oxidant free radicals and decreased antioxidants, resulting in greater oxidant damage. We have previously reported that % MetHb was increased in splenectomized thalassemia patients compared to those with intact spleens [22]. Increased ferritin concentration has been reported in splenectomized patients with HbE β-thalassemia, β-thalassemia intermedia and Hb H syndromes [34,35]. It is likely that abnormal red cells that would ordinarily be sequestered by the spleen remain in the circulation, increasing the potential for free radical generation.

Decreased concentrations of antioxidants and increased oxidant damage were evident in patients who were chelated. This likely reflects the increased iron stores in this group, despite chelation therapy.

Inflammation

Both free heme and MetHb can activate endothelia to increase pro-inflammatory cytokines, E-selectin, adhesion molecules and platelets [36]. Increased IL-6 was present in about half of the patients in this study, and concentrations were greatest in patients with HbS β-thalassemia. IL-6 stimulates the acute phase response by inducing the production of CRP, which was increased in about a quarter of patients in each group. Our findings of greater concentrations of inflammatory cytokines in patients with HbS are in keeping with the findings in patients with sickle cell disease [33]. We have reported previously that the pro-inflammatory cytokine interleukin-8 was elevated in many patients with HbE-β thalassemia [22].

Physical fitness

It was surprising that the patients in this study had such poor physical fitness, with only 2 patients able to achieve a distance ≥ 80% of the expected walking distance and a normal handgrip strength. This likely relates to anemia, hypoxia, inflammation, and may also reflect underlying organ damage and vasculopathy, such as pulmonary hypertension.

Deficiency of dietary antioxidants vitamins C and E

Antioxidant deficiency was common across all groups and especially in HbE β-thalassemia and β-thalassemia major. We have reported previously low plasma vitamin C concentrations in patients with HbE β-thalassemia in Sri Lanka [22] and concentrations were remarkably low in this study. Low vitamin C has also been reported in β-thalassemia major and sickle cell disorders [9,10,37] and is likely due to the increased demand in protecting against oxidant stress. Additionally, the haptoglobin 2-2 genotype is very common in Sri Lanka and may also have contributed to the very low plasma vitamin C concentrations [22,38,39].

Despite their abundance in Sri Lanka [40], a low intake of fresh fruit and raw vegetables was reported by the majority of patients suggesting that dietary insufficiency may also have contributed to vitamin C deficiency. Low intake of fresh fruit and vegetables has been reported previously in Sri Lanka; <1% of adults and <14% of children met National daily intake recommendations [41-44]. Severe vitamin C deficiency results in scurvy, and we have previously reported that following treatment with a cautious dose of ascorbate, MetHb concentration normalised and clinical symptoms of scurvy improved in a patient with HbE β-thalassemia [22]. Similarly, in 3 patients with β-thalassemia major in India, treatment with ascorbate reversed purpura and joint effusions [45]. However, a concern regarding supplementation of all patients is that this may exacerbate iron overload through increased intestinal absorption of iron [46].

Remarkably low plasma vitamin E was also found in the majority of patients. Again, this is likely due to the increased demands of oxidant stress. Also, as vitamin C is involved in vitamin E recycling [47], it follows that if vitamin C concentration is low, less vitamin E will be recycled and concentrations will fall.

Although plasma concentrations of vitamins C and E are reduced during the acute phase response [48], the normal CRP levels in most of our patients indicate true deficiency of these micronutrients. Low concentrations of both vitamins have been reported previously in β-thalassemia, sickle-cell anemia, HbE β-thalassemia and α-thalassemia [9,10,49-51]; and low plasma vitamin E has been described in Sri Lankan females diagnosed with phrynoderma [52].

Antioxidant intervention studies

Antioxidant interventions in patients with hemoglobinopathies have been evaluated in several studies with mixed results. Fermented papaya ameliorated oxidant stress in HbE β-thalassemia, β-thalassemia major and β-thalassemia intermedia [53]. Vitamin E supplements are considered safe, unless given in excess [54], and oral vitamin E improved oxidant stress in β-thalassemia intermedia [55,56] and total antioxidant capacity in HbE-trait [57]. Vitamin C and E supplements worsened hemolysis in sickle cell anemia [58] and did not reduce oxidant damage in β-thalassemia major although plasma concentrations of both vitamins increased and bilirubin concentration decreased [59]. In a recent study of Thai patients with HbE β-thalassemia, antioxidant cocktails increased hemoglobin concentration and decreased oxidant stress [60]. Our findings of marked variability in biomarkers of

oxidative status and damage both between and within the thalassemia syndromes indicate that participants should be selected on an individual basis for inclusion in future intervention studies.

Strengths and limitations

A strength of our study is that we evaluated a broad range of variables related to oxidative status in four important hemoglobinopathy syndromes and, to our knowledge, this is the first study to describe oxidative status in patients with HbS β-thalassemia. Employing single methods and same kit lot numbers and quality controls for each biomarker assay and minimizing variation in sample collection, storage, preparation and analysis allowed for us to compare results between the diagnostic groups. However, our study had several limitations. Limited sample volume and laboratory resources restricted the choice of laboratory assays, including state-of-the-art methodologies for determining parameters of lipid peroxidation. Also, measurement of other antioxidants such as albumin, free thiols and uric acid would have added to our assessment of oxidative status and helped in interpreting total plasma antioxidants. We were also unable to assess the effects of coinheritance of α–thalassemia or triplicated α-globin genes because the number of patients with abnormal α–globin genotypes in each diagnostic group was too few.

Conclusion

Markedly increased oxidant stress results in significant oxidant damage in the thalassemia syndromes in Sri Lanka. Antioxidant deficiency was evident in all syndromes and is likely compounded by dietary vitamin C and E deficiency. There is an urgent need for clinical trials of antioxidants, such as vitamins C and E, in thalassemia, as they offer the potential for significant and affordable clinical benefits. However, sources of oxidants, antioxidant status and oxidant damage varied both between and within the thalassemia syndromes, emphasising the need for individual patient selection in future intervention studies.

Acknowledgements

This article is dedicated to the memory of the late Professor Sir David Weatherall, who not only played a pivotal role in this study, secured its funding and provided great insight and contributions to the study design, but also cared deeply for the many patients with thalassemia in Sri Lanka. We would like to thank Miss Theja Gayathri Perera and Mrs Iresha Priyadarshani Wijethunga for technical assistance and Drs Ishari Silva and Hamid Nizri and all staff at the Thalassemia Care Unit, North Colombo Teaching Hospital, Ragama, Sri Lanka, for their valuable support. Special thanks go to the patients and their parents and the volunteers who participated in this study.

Author contributions

AA, DW, SA, AP, SP, DR and NO designed the study, AA, SP, LP, RR, FM, LH, CF and AJC performed the laboratory work, SP, SM, DT and AP performed the clinical work, SP, RR and AA performed the data entry, AA and SA performed the statistical analysis, AA and SA wrote the manuscript and all authors reviewed and approved the final draft.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding statement: The study was supported by The Anthony Cerami and Ann Dunne Foundation for World Health. The funders had no role in study design, data collection, analysis, decision to publish, or preparation of the manuscript.

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Table 1. Demography, anthropometry, transfusion history, chelation and spleen status according to diagnosis

	β-thal major n=50	**HbE-β thal n=59**	**HbS –β thal n=13**	**β-thal intermedia n=40**
Age Years				
Median	22	30	13	40
(IQR)	(15.8-27)	(18-40)	(11.5-23.5)	(23.5-54.5)
[Range]	[4-35]	[1-61]	[6-37]	[6-75]
Male				
n	20/50	17/59	10/13	14/40
(%)	(40.0%)	(28.8 %)	(76.9%)	(35.0%)
Ethnic group				
Sinhala				
n	47/50	52/59	13/13	40/40
(%)	(94.0%)	(88.1%)	(100.0%)	(100.0%)
Muslim				
n (%)	1/50 (2.0%)	6/59 (10.2%)		
Tamil				
n (%)	2/5(4.0%)	1/59 (1.7%)		
Body mass index (BMI) [$]				
Adults	31	43	4	33
(18.5-23.0)	19.7	17.8	17.6	20.0
	(16.9-21.40)	(16.1-19.9)	(16.6-22.1)	(17.5-22.9)
	[14.3-28.1]	[14.1-22.4]	[16.5-23.3]	[13.6-29.2]
No (%) underweight (<18.5)	10/31 (32.3)	26/43 (60.5)	3/4 (75.0)	10/33 (30.3)
No (%) overweight/obese (>23.0)	4/31 (12.9)	0/43 (0.0)	1/4 (25.0)	8/33 (24.2)
Children and adolescents <20 years of age	18	16	9	7
	11.2	12.4	3.8	13.7
	(4.2-37.0)	(0.43-27.8)	(0.96-41.8)	(11.4-59.8)
(5^{th} -85^{th} centile)	[.09-99.5]	[0.09-77.4)	(0.14-97.5)	(0.09-99.5)
No (%) underweight (<5^{th} centile)	4/18 (22.2)	5/16 (31.3)	5/9 (55.6)	1/7 (14.3)
No (%) overweight/obese (>85^{th} centile)	1/18 (5.6)	0/16 (0.0)	1/9 (11.1)	0/7 (0.0)
Transfusion history*				
No. transfusions in last 12 months				
n	37	50	9	22
Median	12	6	3	3
(IQR)	(12-18)	(2.8-10.5)	(0.5-5.0)	(0.75-10)
[Range]	[12-24]	[0-24]	[0-12]	[0-24]

Iron chelation**	50/50 (100%)	42/57 (73.7%)	4/13 (30.8%)	15/40 (37.5%)
Splenectomy	16/50 (32.0%)	23/59 (39.0%)	2/13 (15.4%)	4/40 (10.0%)

- [$] WHO BMI cut-offs for Asia Pacific countries.
- *It was not possible to obtain a complete transfusion history for some patients because medical records were either incomplete or unavailable.

** Iron chelation data not recorded for 2 patients.

Table 2. Assessment of physical fitness

Physical function	β-thalassemia major	HbE-β thalassemia	HbS –β thalassemia	β-thalassemia Intermedia
Total Distance walked in 6 minutes (metres)				
Males	N=14	N=16	N=10	N=14
• Median (IQR)	410.4 (331.0–450.2)	403.8 (347.55-460.09)	436.92 (390.58 – 466.71)	397.2 (350.9 – 426.99)
[Range]	[145.6-595.8]	[264.8-516.4]	[370.7-489.9]	[278.0-476.6]
• % of expected[1]	56.5 (43.8-67.3) [22.0-97.0]	54.5 (48.5-64.0) [35.0-79.0]	63.5 (57.5-68.3) [51.0-70.0]	65.0 (57.5-72.8) [50.0-91.0]
Females	N=27	N=39	N=3	N=24
• Median (IQR)	357.48 (331.0 – 410.44)	370.27 (321.07-408.79)	397.2 (344.2, 397.2, 503.1)	397.2 (334.31 – 423.68)
[Range]	[185.4-489.9]	[198.6-463.4]	[344.2-503.1]	[158.9-503.1]
• % of expected[1]	50.0 (46.0-58.0) [28.0-74.0]	53.0 (44.0-61.0) [26.0-76.0]	52.0 52.0, 52.0, 61.0 [52.0-61.0]	60.0 (53.5-67.5) [32.0-74.0]
Handgrip strength [psi]				
Males	N=13	N=16	N=10	N=15
	11.0 (5.5-15.0) [0.0-20.0]	11.28 (6.25-15.75) [4.0-20.0]	6.25 (5.75 - 12.0) [3.0-17.0]	10.0 (6.0 -12.0) [2.0-15.0]
Females	N=26	N=41	N=2	N=24
	26 7.0 (6.0 -9.25) [2.0-14.0]	41 7.0 (6.0 – 9.0) [0.0-11.0]	2 - 8.0, 10.0	24 5.0 (4.0 – 7.25) [1.0-10.5]

Notes

1. Distance achieved expressed as % of the expected distance for an age and sex matched individual

Figure legends.

Figure 1. Heat map to show percentage of patients with abnormal values in each diagnostic group.

Figure 2. Sources of oxidants according to diagnostic group. Horizontal lines show the median value, box length is the interquartile range and whiskers show the range except for outlying values ≥1.5 box lengths from the upper and lower edge of the box which are shown as open circles.
Reference lines - -- - -- - -- represent the upper limit of normal range
Reference lines – – – – – – represent the lower limit of normal range

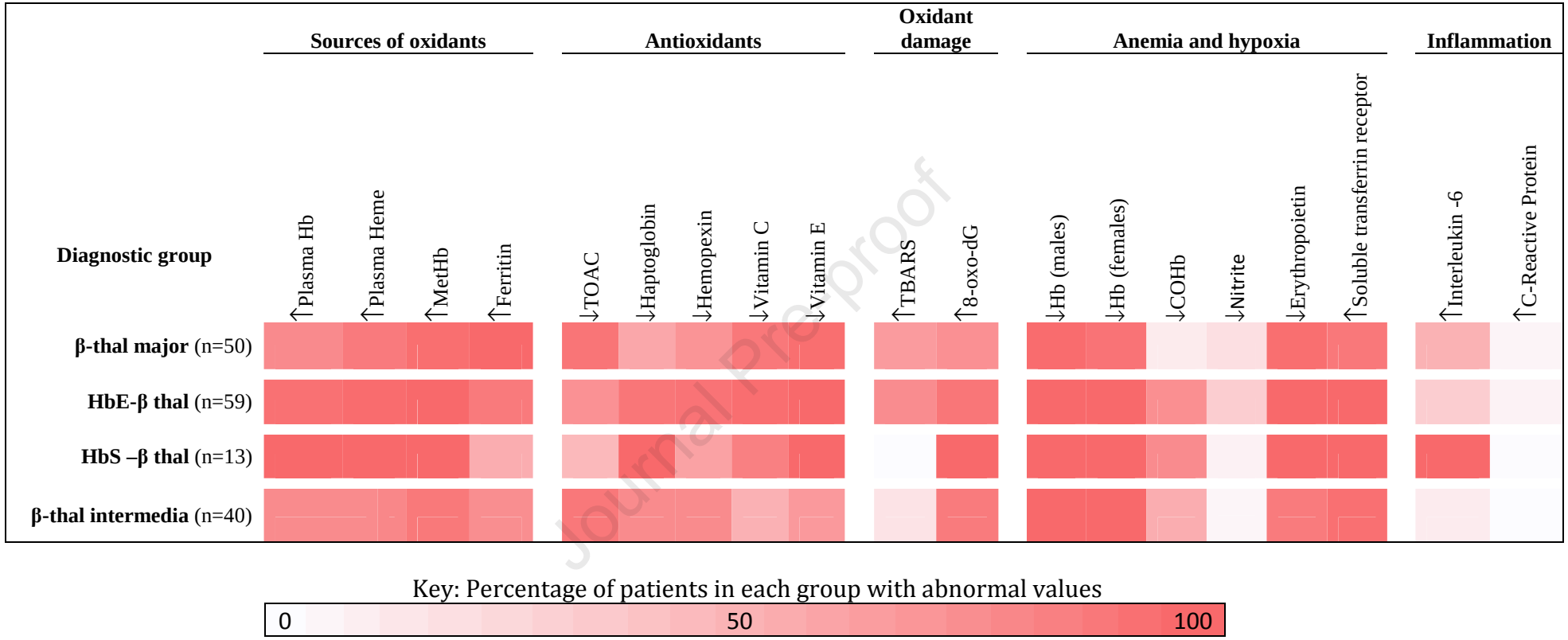
Figure 3. Antioxidants according to diagnostic group. Horizontal lines show the median value, box length is the interquartile range and whiskers show the range except for outlying values 1.5 to 3, or >3, box lengths from the upper and lower edge of the box which are shown as open circles and stars respectively.
Reference lines - -- - -- - -- represent the upper limit of normal range
Reference lines – – – – – – represent the lower limit of normal range

Figure 4. Oxidant damage according to diagnostic group.
Horizontal lines show the median value, box length is the interquartile range and whiskers show the range except for outlying values 1.5 to 3, or >3, box lengths from the upper and lower edge of the box which are shown as open circles and stars respectively.
Reference lines - -- - -- - -- represent the upper limit of normal range
Reference lines – – – – – – represent the lower limit of normal range

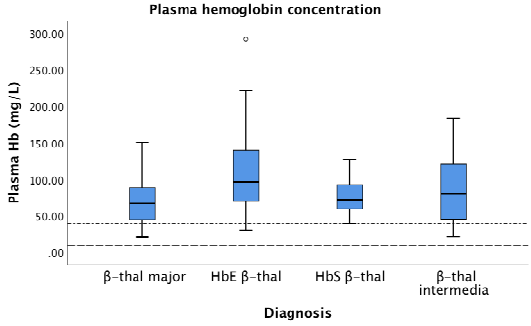
Figure 5. Anemia, hypoxia and erythropoiesis according to diagnostic group.
Horizontal lines show the median value, box length is the interquartile range and whiskers show the range except for outlying values 1.5 to 3, or >3, box lengths from the upper and lower edge of the box which are shown as open circles and stars respectively.
Reference lines - -- - -- - -- represent the upper limit of normal range
Reference lines – – – – – – represent the lower limit of normal range

Figure 6. Inflammation according to diagnostic group.
Horizontal lines show the median value, box length is the interquartile range and whiskers show the range except for outlying values 1.5 to 3, or >3, box lengths from the upper and lower edge of the box which are shown as open circles and stars respectively.

Reference lines - -- - -- - -- represent the upper limit of normal range

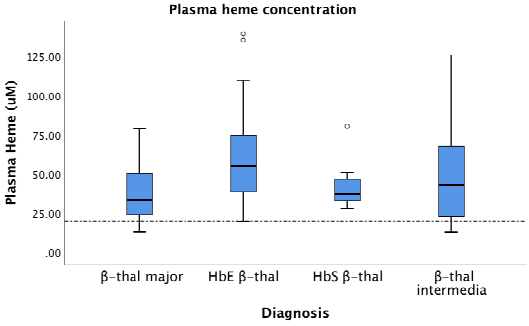


2a.



X^2 =10.88, P=0.012

2b.



X^2 =10.88, P=0.012

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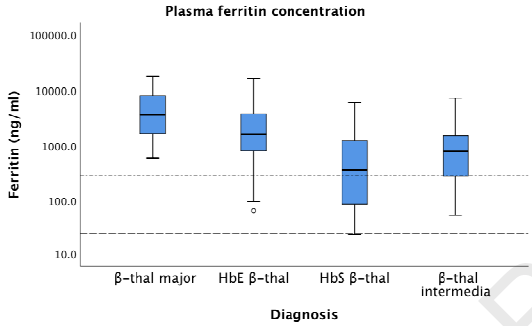

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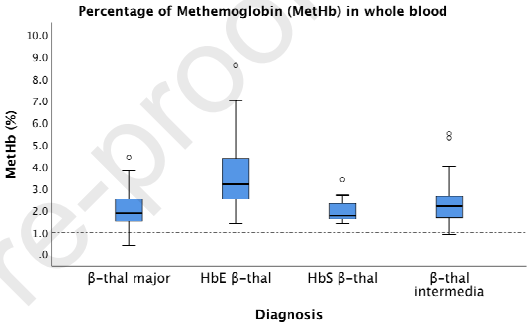
2d.



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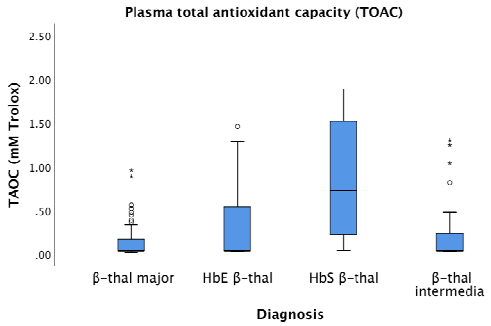
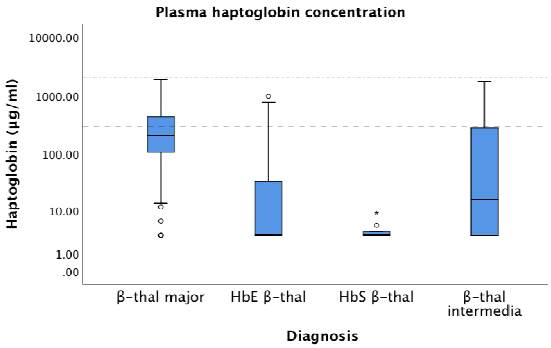
3a.



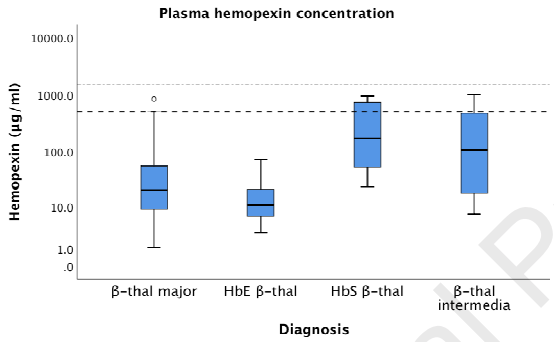
X^2=9.95, P=0.019

3b.



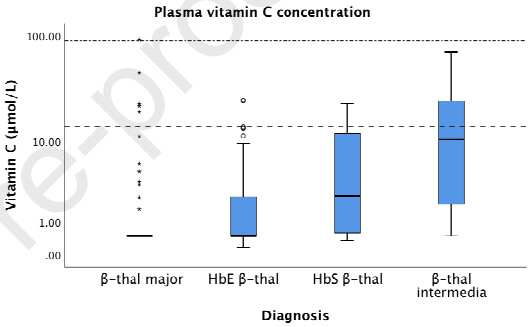
X^2=46.96, P<0.001

3c.



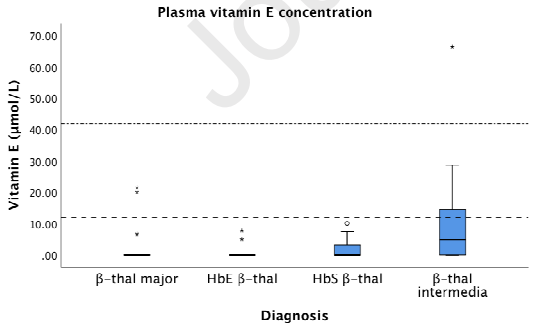
X^2=38.10, P<0.001

3d.



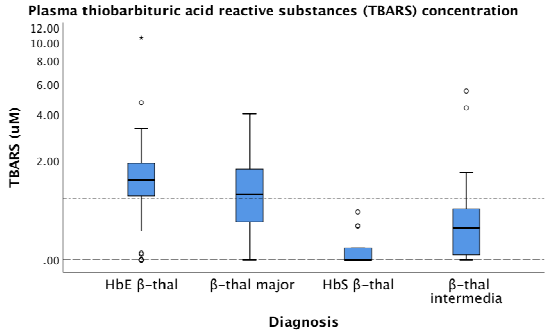
X^2=33.74, P<0.002

3e.



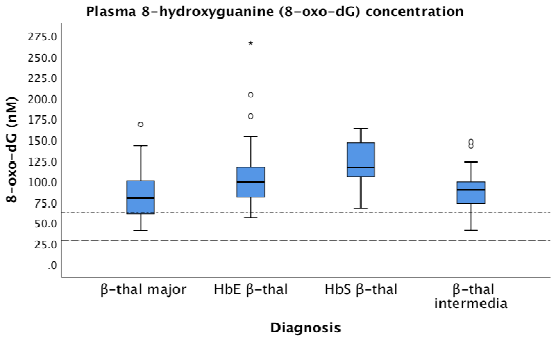
X^2=47.10, P<0.001

4a.



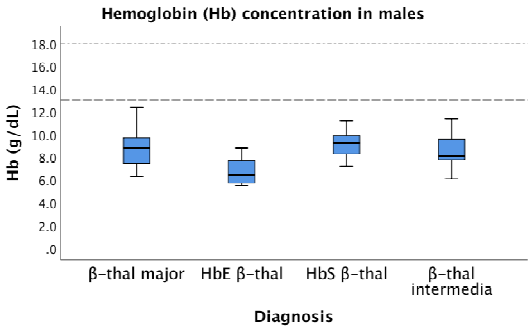
X^2=53.70, P<0.001

4b.



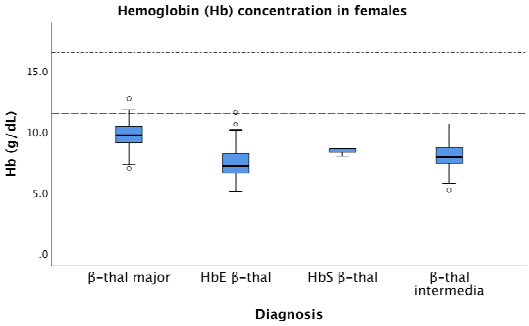
X^2=13.15, P=0.004

5a.



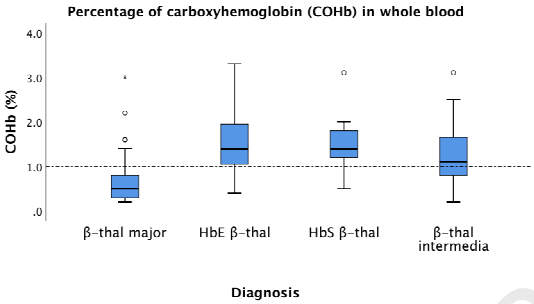
X^2=19.64, P<0.001

5b.



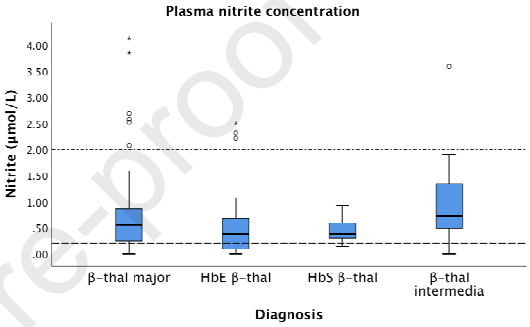
X^2=30.62, P<0.001

5c.



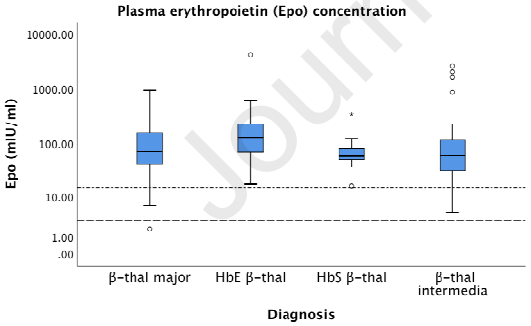
X^2=47.11, P<0.001

5d.



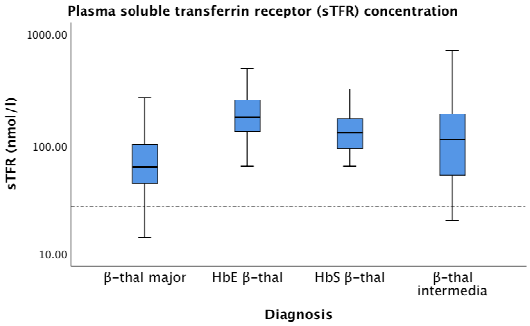
X^2=10.53, P=0.015

5e.



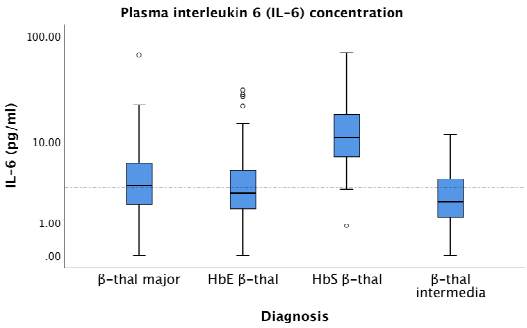
X^2=13.30, P=0.004

5f.



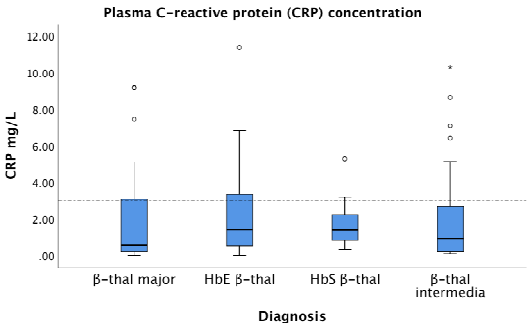
X^2=44.06, P<0.001

6a.



X^2=16.50, P=0.001

6b.



X^2=3.45, P=0.327

Highlights

- Oxidative stress and damage were common in the β-thalassemia syndromes in Sri Lanka
- Oxidative stress and damage varied both between and within thalassemia syndromes
- Oxidative stress and damage tended to be worse in patients with HbE β-thalassemia
- Oxidative stress and damage were associated with splenectomy and chelation therapy
- Poor diets likely further exacerbate antioxidant deficiency