Oxidative stress and inflammation in iron-overloaded patients with β-thalassaemia or sickle cell disease

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Summary

Blood transfusion therapy is life-saving for patients with β-thalassaemia and sickle cell disease (SCD), but often results in severe iron overload. This pilot study examined whether the biomarkers of tissue injury or inflammation differ in these two diseases. Plasma malondialdehyde (MDA) was significantly increased 1.8-fold in thalassaemia relative to control patients. In contrast, MDA in SCD was not significantly different from controls. In multivariate analysis, the strongest predictors of elevated MDA were liver iron concentration ($P < 0.001$) and specific diagnosis ($P = 0.019$). A significant 2-fold elevation of non-transferrin bound iron (NTBI) was observed in thalassaemia relative to SCD. NTBI was not a significant predictor of high MDA in multivariate analysis. SCD patients showed a significant 2.2-fold elevation of the inflammatory marker interleukin (IL)-6 relative to controls, and a 3.6- and 1.7-fold increase in IL-5 and IL-10 relative to thalassaemia. Although α-tocopherol was significantly decreased by at least 32% in both thalassaemia and SCD, indicating ongoing oxidant stress and antioxidant consumption, γ-tocopherol, a nitric oxide-selective antioxidant, was increased 36% in SCD relative to thalassaemia. These results demonstrate that thalassaemia patients have increased MDA and circulating NTBI relative to SCD patients and lower levels of some cytokines and γ-tocopherol. This supports the hypothesis that the biology of SCD may show increased inflammation and increased levels of protective antioxidants compared with thalassaemia.

Keywords

iron overload; thalassaemia; sickle cell disease; oxidative stress; inflammation; cytokines; non-transferrin bound iron; malondialdehyde; lipid peroxidation; vitamin E

Although the prognosis for patients with thalassaemia and sickle cell disease (SCD) has greatly improved in recent decades with the use of modern newborn screening, prophylactic penicillin (and the availability of newer antibiotics), blood transfusions and iron chelation therapy, patients continue to be at high risk for iron overload and iron-induced toxicities. Iron-related cardiac disease remains the most common cause of death in thalassaemia (Zurlo *et al*, 1989; Borgna-Pignatti *et al*, 1998). In addition, over 70% of patients with thalassaemia suffer from primary or secondary amenorrhoea, hypogonadism, osteoporosis and other endocrine disorders (Bronspiegel-Weintrob *et al*, 1990). However, transfused patients with SCD have a

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conspicuous absence of iron overload-related organ injury (Finch *et al*, 1982; Wood *et al*, 2004). In pilot data from a recent study, Vichinsky *et al* 2005a confirmed this finding and demonstrated that the strongest predictors of organ injury in thalassaemia were duration of iron exposure and the specific diagnosis.

Biomarkers of oxidative damage are increased in both thalassaemia and SCD (Rachmilewitz *et al*, 1976; Repka & Hebbel, 1991). In spite of the iron overload in both diseases, oxidants originate from sources other than the iron loaded tissues. For example, in β-thalassaemia the excess unpaired α-haemoglobin chains denature and autoxidise, contributing to increased oxidants, ineffective erythropoiesis, haemolysis and shortened erythrocyte survival (Scott *et al*, 1993). Malondialdehyde (MDA), a product of lipid peroxidation and protein carbonyls, representing oxidation of the circulating proteins, are elevated in thalassaemia (Graziano *et al*, 1976; Snyder *et al*, 1981; Ramenghi *et al*, 1989; Shalev *et al*, 1995; Cighetti *et al*, 2002). On the other hand, SCD haemoglobin can bind to the RBC membrane and act as a Fenton reagent, increasing the generation of oxidants such as superoxide and hydroxyl radical (Repka & Hebbel, 1991). Further sources of oxidants unique to SCD vasculature include xanthine oxidase released from the liver, nitric oxide and secondary oxides of nitrogen (Aslan *et al*, 2001, 2003; Aslan & Freeman, 2004, Aslan *et al*, 2000) and endothelial NADPH oxidase (Wood *et al*, 2005). These increased oxidants and intravascular haemoglobin from haemolysis (Reiter *et al*, 2002) may contribute to the consumption of nitric oxide in SCD (Aslan *et al*, 2001). In turn, low levels of nitric oxide may result in haemodynamic instability (Aslan *et al*, 2001) and reduction of antioxidant capacity (Gladwin *et al*, 2003). Additionally, some studies have shown increased levels of plasma MDA in SCD (Jain *et al*, 1990; Sess *et al*, 1992; Sertac *et al*, 1997). However, it is unknown whether MDA levels differ in these two iron overload diseases that share similar chelation treatments.

To protect against oxidative damage, the body has endogenous defence mechanisms that are supported by dietary antioxidants. The lipid-soluble vitamin E (tocopherols) antioxidants, including α- and γ-tocopherol, are an important front line defence (Sess *et al*, 1992; Christen *et al*, 1997; Jiang *et al*, 2002; Jiang & Ames, 2003; Kassab-Chekir *et al*, 2003; Ruhl & Everhart, 2003). These antioxidants are better known as oxygen radical scavengers. However, γ-tocopherol, the primary form of vitamin E in the USA diet, has recently been shown to have a unique role in preventing oxidant damage (Christen *et al*, 1997; Jiang & Ames, 2003). In addition to functioning as an oxygen radical scavenger, γ-tocopherol scavenges reactive nitric oxide species and inhibits prostaglandin E_2-mediated inflammation (Jiang *et al*, 2000, 2001, 2002; Jiang & Ames, 2003). γ-Tocopherol, in contrast to α-tocopherol, is increased in response to inflammatory stimuli (Himmelfarb *et al*, 2003). It is unknown whether γ-tocopherol levels differ in these two diseases or contribute to differences in oxidative damage.

Sickle cell disease is well recognised as a chronic inflammatory disease (Belcher *et al*, 2000, 2003; Klings & Farber, 2001; Jison *et al*, 2004). Patients with SCD in crisis show elevated tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) compared with steady state SCD. In addition, IL-1, IL-6 and interferon-γ (IFN-γ) are elevated in steady state SCD compared with controls. It is unknown from these studies how iron overload will affect the levels of chronic inflammation in SCD. Elevations of the pro-inflammatory cytokines TNF-α and IL-2 have also been demonstrated in iron overloaded patients with thalassaemia (Del Vecchio *et al*, 2002). These cytokines returned to normal after treatment with the iron chelator deferiprone (L1) (Del Vecchio *et al*, 2002). Although it is true that oxidant stress can stimulate pro-inflammatory responses in both diseases, different patterns of cytokine and immuno-modulator expression have been described and therefore it is unknown whether the two diseases develop similar inflammatory responses.

The present pilot study examined whether biomarkers of oxidant injury (such as MDA), evidence for inflammation [C-reactive protein (CRP) and cytokines] and antioxidant levels (α- and γ-tocopherol) differ in SCD and thalassaemia.

Methods

Study design

Patients with thalassaemia or SCD and a history of receiving chronic transfusion therapy were recruited from the hematology clinic at the Children's Hospital and Research Center Oakland (CHRCO) at the time of a clinically indicated liver biopsy for evaluation of iron overload. Healthy controls were recruited from CHRCO staff and their families. The study received Institutional Review Board approval. Consent for adults and assent for children were obtained in accordance with the CHRCO Institutional Review Board guidelines. Twenty-eight chronically transfused haemoglobinopathy patients were studied (eight or more transfusions per year). Case subjects included 17 β-thalassaemia patients [7 male, age: 23.7 ± 8.6 years (mean ± standard deviation (SD), Table I)], 11 SCD patients [seven male, age: 12.7 ± 3.7 years (mean ± SD)] and nine healthy controls [six male, age: 28.2 ± 11.4 years (mean ± SD); serum ferritin <300 μg/l; eight Caucasian, one Asian]. Clinical information including demographics and history of transfusion, chelation, and therapy were obtained by interview and chart review.

Laboratory analyses

A blood sample (45 ml) was collected from subjects in the morning at least 2 h postprandial; subjects were advised to abstain from taking any medications (including chelation)/vitamin or mineral supplements for the previous 24 h. The study design specified that blood from transfused subjects be collected as long as possible after transfusion (i.e. in the period just proceeding the next transfusion). The mean days between transfusion and phlebotomy were 20.7 ± 10.4 d after the last transfusion. Blood was transported at room temperature to the analytical laboratory within 20 min of venepuncture for cell isolation and oxidation analysis. Serum, plasma and cells were separated by centrifugation.

Malondialdehyde (MDA)

Malondialdehyde was assayed in duplicate 250 μl plasma samples, using gas chromatography-mass spectrometry (GC–MS, model 5888; Agilent Technologies, Palo Alto, CA, USA), as previously described (Yeo *et al*, 1994, 1999). Two hundred micro molar diethylenetriaminepentaacetate and 2.5 mmol/l butylated hydroxytoluene (BHT) were added to prevent sample oxidation during analysis. Samples were spiked with 1 μmol/l $^2H_2$-MDA as an internal standard. To hydrolyse protein-bound MDA, 10 μl of 6.6 N H_2SO_4 was added for 10 min at room temperature. MDA was derivatised to pentafluorophenylhydrazine at room temperature for 1 h. Derivatised MDA was extracted with isooctane, and 70 μl was injected into the GC–MS for detection.

Vitamin E

Plasma α-tocopherol and γ-tocopherol were analysed as previously described (Jiang *et al*, 2002; Jiang & Ames, 2003). Briefly, tocopherols were extracted using a mixture of methanol/hexane (2:5, v/v) in the presence of 0.8 mmol/l BHT added to prevent oxidative losses during work-up. Tocopherols were separated on a 150×4.6 mm, 5 μm Supelcosil™ LC-18-DB column (Supelco, Bellefonte, PA, USA) and monitored by coulometric detection (Model Coulochem II; ESA Inc., Chelmsford, MA, USA) using a Model 5011 analytical cell (Jiang *et al*, 2002; Jiang & Ames, 2003).

Non-transferrin bound iron (NTBI)

Non-transferrin bound iron was determined by nitrilotriacetic acid (NTA) capture of NTBI and quantified by high performance liquid chromatography (HPLC) (Singh *et al*, 1990). This assay uses a large excess of a low affinity ligand NTA, which removes and complexes low molecular mass iron and iron non-specifically bound to serum proteins. Iron bound to transferrin, ferritin, desferrioxamine, and its metabolites is not accessed by the NTA ligand. The Fe–NTA complex present in the ultrafiltrate represents the NTBI and is then quantified using an HPLC procedure including on-column derivatisation with a high affinity iron chelator (3-hydroxy-1-propyl-2-methyl-pyridin-4-one). Using this method, normal individuals had negative NTBI values. A negative NTBI value, a common finding by several groups independent of the method used to measure NTBI (Singh *et al*, 1990; Porter *et al*, 1996; Bradley *et al*, 1997; Gosriwatana *et al*, 1999; Wickramasinghe *et al*, 1999; Jacobs *et al*, 2005), arises when the transferrin saturation in samples is less than 50% (Jacobs *et al*, 2005). Negative NTBI values can be caused when transferrin is less than 50% saturated and iron NTA loads rapidly onto transferrin. Hence, normal control samples, which contain around 30% saturated transferrin, bind iron bound to NTA and thus appear negative with respect to the experimental zero iron controls used with standards to generate calibration curves where no transferrin is present.

C-reactive protein

Plasma high sensitivity C-reactive protein (hsCRP) was determined in plasma samples by a nephelometric method utilising latex particles coated with (CRP) monoclonal antibodies by Quest Diagnostics Inc., San Juan Capistrano, CA, USA.

Cytokines

Plasma levels of 10 cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, granulocyte–macrophage colony-stimulating factor (GM-CSF), IFN-γ and TNF-α) were determined in samples by a multiplex antibody bead assay by BioSource International, Camarillo, CA, USA.

Liver iron

Tissue iron concentration was determined by inductively coupled plasma mass spectrometry (ICP-MS, Mayo, Rochester, NY, USA) as described previously (Vichinsky *et al*, 2005a). Liver biopsies were collected within an average of 15 d (mean 14.5 ± 20.5) of the phlebotomy for the study. Three samples were excluded from these analyses because they were collected more than 2 months from the blood sample.

Blood and liver function tests

Alanine transaminase (ALT) and ferritin were measured in the clinical lab at CHRCO. Serum ferritin concentrations were measured by a one step immunoassay (FERR Flex®; Dade Behring Inc., Newark, DE, USA). Serum ALT concentrations were measured on the Dimension® clinical chemistry system (Dade Behring Inc.). The manufacturer's reference range for the normal general population is 30–65 U/l ($n = 244$ adults). Ferritin and ALT are presented as the mean values obtained during the 6 month prior to the study phlebotomy. Hepatitis C RNA was identified by polymerase chain reaction.

Percentage transferrin saturation

This was determined on serum samples using the urea gel method of Evans and Williams (1978). Twenty-five micro litres of serum were treated with an excess of rivanol to remove most of the serum proteins excluding transferrin. After centrifugation, samples of the supernatants were run on polyacrylamide gels containing 6 mol/l urea. Under these partially denaturing conditions, the four different species of transferrin, namely apo-, diferric-, C- and

N-terminal monoferric, denatured to differing extents and were therefore separated on the gel, which enabled quantification using densitometry from which the per cent saturation was calculated.

Protein carbonyls

Oxidative damage to plasma proteins were assayed as previously described by measuring carbonyl groups on plasma proteins by reaction with 2,4-dinitrophenylhydrazine to form a hydrazine complex, which was measured spectrophotometrically (Houglum *et al*, 1990, 1997).

Diet

The Block Food Frequency Questionnaire (Block, 1998) was completed by a majority of subjects to obtain a semi-quantitative assessment of usual food intake over the previous 12 months. The Block Food Frequency Questionnaire has been validated in adult (Sinha *et al*, 1993) and paediatric populations (Block *et al*, 1995) and is used commonly in epidemiological trials of oxidative stress.

Statistics

Data are reported as mean ± SD. Comparisons among groups were made using one-way analysis of variation (ANOVA) analyses, where $P < 0.05$ was considered statistically significant. *Post hoc* tests were done by Tukey's analysis at a 5% procedure-wise error rate. As MDA and ferritin values were right skewed, the analyses on these two variables were performed on log-transformed data. Logistic regression was used to create multivariate models to describe relationships between oxidative injury (MDA, ALT) and factors that may influence their development (i.e. diagnosis, duration of chronic transfusion, liver iron concentration, NTBI or age). Other variables were also examined as potential confounders, such as serum ferritin and hepatitis C virus (HCV) and were found to be non-significant in the model.

Results

Demographics

The demographics of the study populations are shown in Table I. No significant differences were seen in sex, height, weight, body mass index, chelation or percentage of HCV infection, among the study groups. Duration of chronic transfusion was significantly longer in thalassaemia patients ($P < 0.001$) and they were significantly older than the SCD group ($P = 0.002$). Splenectomy ($P = 0.025$) was more common in thalassaemia patients, but there was no significant difference in chelation treatment (deferioxamine, $P = 0.062$) compared with SCD patients.

The caloric intake was not different among the three groups (approximately 2000 ± 1000 kcal) and no difference was observed in total fat, protein, carbohydrate, calcium, iron, vitamin A, C, E and D intake between groups (including supplements).

Comparison of iron burden

Parameters of iron burden are shown in Table II. Although both thalassaemia and SCD groups had evidence of severe haemosiderosis by quantitative liver iron measurements, these were higher in SCD than thalassaemia patients in this study population (15.0 ± 5.6 vs. 9.2 ± 7.2 mg/g dry tissue weight, $P = 0.033$, Table II). Serum ferritin and transferrin saturation showed similar high elevation in both thalassaemia and SCD patients relative to controls. Despite higher tissue iron burden, SCD patients had significantly lower NTBI than thalassaemia patients (Table II).

Oxidative injury and inflammation

In thalassaemia patients, MDA levels were significantly higher than controls. This difference was not observed for SCD patients (MDA values were log-transformed prior to analysis; Table III). Further analysis of MDA comparing it with liver iron concentration in both disease patient groups showed a positive correlation between liver iron concentration and plasma MDA (Fig 1; thalassaemia, $R^2 = 0.695$, $P = 0.003$ and SCD, $R^2 = 0.644$, $P = 0.032$). Plasma α-tocopherol was significantly reduced in both thalassaemia and SCD patients relative to controls. In contrast, γ-tocopherol levels were significantly higher in SCD compared with thalassaemia patients as well as controls [Table III, the control group was in the range found for normal healthy controls used in other studies (Himmelfarb *et al*, 2003)]. The dietary questionnaire found no difference in intake of γ- or α-tocopherol. Protein carbonyls, a measure of oxidative damage to amino acid side chains, were not significantly changed in either group in comparison to controls.

Multivariate analysis using log MDA as the dependent variable representing oxidant injury was employed to examine the pattern of several of the following relationships simultaneously (Table IV). This analysis examined as co-variants (i.e. predictors): diagnosis, liver iron concentration, duration of chronic transfusion and age. Two significant predictors of MDA were found, diagnosis of thalassaemia and quantitative liver iron ($P < 0.001$ and =0.019 respectively). Age was not a significant predictor in multivariate analysis (Table IV). ALT, another marker of tissue injury was significantly increased (1.7-fold, $P = 0.020$) in the thalassaemia group relative to the SCD group (Table III). Further multivariate analysis, with ALT as the dependent variable, and liver iron concentration and diagnosis as independent variables in the model, found that diagnosis of thalassaemia was a predictor of higher ALT ($P = 0.025$, data not shown).

Markers of inflammation including hsCRP and 10 cytokines were compared with each of the groups (Table III). A 2.5-fold higher hsCRP level was observed in SCD compared with thalassaemia patients, which showed a trend toward statistical significance ($P = 0.061$; Table III). The inflammatory cytokines IL-5, IL-6 and IL-10 were highest in SCD patients, with IL-6 and IL-10 being significantly different from controls, while IL-5 and IL-10 were also significantly elevated in SCD compared with thalassaemia patients. There was no significant difference between the levels of IL-5, IL-6 and IL-10 in thalassaemia and control patients (Table III). There were no significant differences among groups for the other cytokines examined (GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-8, and TNF-α, data not shown).

Discussion

Emerging clinical data suggest that iron overloaded SCD patients are at less risk for organ injury than patients with thalassaemia. Wood *et al* 2004 recently reported increased cardiac iron deposition in chronically transfused haemoglobinopathy patients. Decreased cardiac function only occurred in thalassaemia patients, not in iron overloaded SCD patients (Wood *et al*, 2004). In a recent study involving 30 thalassaemia and 43 SCD patients with severe haemosiderosis, we confirmed that greater cardiac and endocrine disease occurred in the thalassaemia patients (Vichinsky *et al*, 2005a). Although the specific role of iron overload in mediating injury is the primary focus of this paper, the unique pathophysiologies of SCD and thalassaemia certainly play a critical role in promoting the observed pathology. Specifically, in SCD the tissue injury related to sickling of RBCs and in thalassaemia the surplus of α-globin chains and intramedullary ineffective erythropoiesis are certainly important factors. Also important is the enhanced gastrointestinal iron absorption seen thalassaemia patients that may initiate different iron trafficking compared with SCD. Clearly, disease diagnosis is important; in fact, multivariate analysis showed disease diagnosis of thalassaemia to be a significant predictor of MDA and this variable may be closely related to these distinct underlying

fundamental pathophysiologies. Furthermore, concerning the ethnicity difference between SCD and thalassaemia; we cannot discount ethnicity being a factor. However, while this is true, the incidence and prevalence of iron-induced organ injury was not influenced by the ethnicity of the patients in multiple large thalassaemia trials (Cunningham *et al*, 2004; Vichinsky *et al*, 2005b). In order to investigate the relationship of iron overload and specific disease to injury, we examined biomarkers of oxidative stress, inflammation and tissue injury. However, while each class of biomarker is traditionally thought to represent one of these processes, it is important to note that they are not distinct entities and have considerable interaction, i.e. oxidative stress can initiate tissue injury and/or inflammation.

Plasma malondialdehyde was studied as a marker of tissue injury and oxidative stress. MDA is a well-recognised biomarker of lipid peroxidation, but its measurement has been limited by the specificity of the methodology. The present study utilises GC–MS, a technique that avoids problems of oxidation during processing (as may be seen in thiobarbituric acid reactive substance analysis) and specifically measures MDA (Liu *et al*, 1997). Another factor we have taken into account is artifactual oxidation during the measurement of MDA because of the high NTBI in our samples. We took great care to prevent any possible enhanced MDA during the preparation of our samples by adding chelators and antioxidants. Regression analysis revealed that higher liver iron concentrations are associated with more plasma MDA in both disease groups. Further multivariate analysis identified specific disease (thalassaemia) and liver iron concentration as significant predictors of high MDA. In animal models, high liver iron levels induce elevation of lipid peroxides and oxidants (Knutson *et al*, 2000; Walter *et al*, 2002) presumably through iron-initiated Fenton chemistry. Increased lipid peroxidation markers have previously been observed in thalassaemia patients (Livrea *et al*, 1998; Cighetti *et al*, 2002; Laksmitawati *et al*, 2003). However, MDA studies in SCD have been largely focused on *in vitro* studies of sickle-cell erythrocyte membranes (Buchanan & Holtkamp, 1981; Hebbel & Miller, 1984; Jain & Shohet, 1984; Jain *et al*, 1989, 1990; Sertac *et al*, 1997). This is the first study to compare oxidative injury with iron overloaded SCD and thalassaemia patients.

Duration of chronic transfusion and NTBI seem to be likely candidates as predictors of MDA, because duration of transfusion indicates the time the iron stress has been endured and NTBI estimates the possible availability of free iron. However, neither was a predictor of MDA in multivariate analysis. Concerning duration of chronic transfusion, elevated MDA is probably a real-time marker of oxidative injury (Kadiiska *et al*, 2005a,b) and indicative of current liver iron concentration. Duration of chronic transfusion correlates better with cumulative tissue injury (Vichinsky *et al*, 2005a). We suggest that this may explain why duration of transfusion is not a predictor of MDA in multivariate analysis. NTBI is also not a significant predictor of MDA. This is surprising, because with high levels of both plasma MDA and NTBI, it is the potential free iron in NTBI that could be initiating Fenton chemistry *in vivo*, which induces the lipid peroxidation that gives increased MDA. However, this is not the case. We suggest that the increased levels of plasma MDA in thalassaemia may result from several mechanisms. First, plasma MDA could be enhanced in thalassaemia 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-haemoglobin chains (Scott *et al*, 1993). The excess α-chains in thalassemic red blood cells are unstable and prone to denaturation and oxidation (Scott *et al*, 1993) and, in the present work, may have contributed more to enhanced plasma MDA than the SCD haemoglobin, which is also capable of producing oxidants (Repka & Hebbel, 1991). The α-chains in thalassemic red blood cells can autoxidise, release haem and generate superoxide (Scott *et al*, 1993), which can then increase lipid peroxidation and thus enhance MDA levels. Furthermore, plasma MDA may be increased in thalassaemia because of peroxidation of tissues that leak MDA into the plasma. For example, plasma ALT, another marker of tissue injury, was also increased in thalassaemia relative to SCD and is indicative of liver dysfunction and leakage of liver metabolites into the plasma. Parallel to ALT, plasma MDA may also rise partly

as a result of possible liver lipid peroxidation and leakage into the plasma. It is an attractive suggestion that MDA may leak from the liver because of its strong correlation to liver iron concentration in multivariate analysis.

We also examined inflammation, a known regulator of iron transport and storage, as another possible explanation for the observed differences in MDA levels, and to determine the important inflammatory differences that may exist between these two diseases. SCD is characterised by microvascular hypoxia-reperfusion leading to inflammation. Human and animal models have found elevated TNF-α, IL-6, IL-10 and hsCRP as markers of inflammation in SCD (Hedo *et al*, 1993; Singhal *et al*, 1993; Bank *et al*, 1998; Bourantas *et al*, 1998; Dale & Alberio, 1998; Sess *et al*, 1998; Belcher *et al*, 2000, 2003; Makis *et al*, 2000; Barbeau *et al*, 2001; Ludwiczek *et al*, 2003; Banerjee & Kuypers, 2004). Our results support these observations. Inflammation (Fillet *et al*, 1989) and cytokines (IL-10) stimulate the uptake and retention of iron into monocytes and reticuloendothelial (RE) cells (Ludwiczek *et al*, 2003). Recently, hepcidin that is increased in inflammation, has been identified as a critical participant in this cellular regulation of iron storage (Papanikolaou *et al*, 2005). In transfused thalassaemia or SCD patients the hepicidin level may vary depending on the severity of anaemia as suggested in a recent study (Kearney *et al*, 2005). Increased cytokines (such as IL-10) may explain the lower NTBI levels in SCD patients as compared with thalassaemia patients (Ludwiczek *et al*, 2003). We and others suggest that the NTBI of SCD patients may be taken up by the RE system, sparing the parenchymal tissues of injury (Pippard, 1994). In contrast, the high NTBI in thalassaemia may also contribute to the increased cardiac iron and organ dysfunction observed in thalassaemia patients.

Antioxidant capacity is an important determinant of tissue injury, especially in patients with increased oxidant stress. We have found significant differences in antioxidant capacity in thalassaemia and SCD patients in terms of plasma tocopherol levels. Plasma α-tocopherol was decreased in both thalassaemia and SCD patients in ours and other studies (Rachmilewitz *et al*, 1976; Sess *et al*, 1998; De Luca *et al*, 1999; Kassab-Chekir *et al*, 2003). Our observed changes in α-tocopherol were not because of variation in dietary intake and are probably related to its consumption as a scavenger of oxidants. These low levels probably contribute to increased MDA levels. In contrast to the decreased α-tocopherol, γ-tocopherol levels were increased in both groups, but levels were significantly higher in the SCD patients. Inflammation has been shown to decrease degradation of γ-tocopherol through inhibition of a cytochrome P450 3A–dependent process (Parker *et al*, 2000; Sontag & Parker, 2002) and may explain our observed difference. We suggest that the higher levels of γ-tocopherol in SCD may be important in reducing tissue injury related to inflammation and oxidant stress.

Conclusions

Malondialdehyde levels were higher in thalassaemia compared with SCD (conclusions summarised in Table V). The strongest predictors of elevated MDA, by multivariate analysis, were diagnosis and liver iron concentration. In contrast, several inflammatory markers appeared higher in SCD. This inflammation in SCD may generate increased levels of γ-tocopherol, leading to decreased tissue peroxidation and injury. Inflammation may also be contributing to the lower NTBI levels observed in SCD, further protecting against tissue injury by removing a source of circulating labile iron. In summary, we suggest that the unique inflammatory physiology of SCD may protect against organ injury by restricting iron to protected areas within the RE system (Fillet *et al*, 1989; Ludwiczek *et al*, 2003) and maintaining protective antioxidants.

References

Aslan M, Freeman BA. Oxidant-mediated impairment of nitric oxide signaling in sickle cell disease – mechanisms and consequences. Cellular and Molecular Biology 2004;50:95–105. [PubMed: 15040433]

Aslan M, Thornley-Brown D, Freeman BA. Reactive species in sickle cell disease. Annals of the New York Academy of Sciences 2000;899:375–391. [PubMed: 10863554]

Aslan M, Ryan TM, Adler B, Townes TM, Parks DA, Thompson JA, Tousson A, Gladwin MT, Patel RP, Tarpey MM, Batinic-Haberle I, White CR, Freeman BA. Oxygen radical inhibition of nitric oxide-dependent vascular function in sickle cell disease. Proceedings of the National Academy of Sciences of the Unites States of America 2001;98:15215–15220.

Aslan M, Ryan TM, Townes TM, Coward L, Kirk MC, Barnes S, Alexander CB, Rosenfeld SS, Freeman BA. Nitric oxide-dependent generation of reactive species in sickle cell disease. Actin tyrosine induces defective cytoskeletal polymerization. Journal of Biological Chemistry 2003;278:4194–4204. [PubMed: 12401783]

Banerjee T, Kuypers FA. Reactive oxygen species and phosphatidylserine externalization in murine sickle red cells. British Journal of Haematology 2004;124:391–402. [PubMed: 14717789]

Bank N, Kiroycheva M, Ahmed F, Anthony GM, Fabry ME, Nagel RL, Singhal PC. Peroxynitrite formation and apoptosis in transgenic sickle cell mouse kidneys. Kidney International 1998;54:1520–1528. [PubMed: 9844128]

Barbeau P, Woods KF, Ramsey LT, Litaker MS, Pollock DM, Pollock JS, Callahan LA, Kutlar A, Mensah GA, Gutin B. Exercise in sickle cell anemia: effect on inflammatory and vasoactive mediators. Endothelium 2001;8:147–155. [PubMed: 11572476]

Belcher JD, Marker PH, Weber JP, Hebbel RP, Vercellotti GM. Activated monocytes in sickle cell disease: potential role in the activation of vascular endothelium and vaso-occlusion. Blood 2000;96:2451–2459. [PubMed: 11001897]

Belcher JD, Bryant CJ, Nguyen J, Bowlin PR, Kielbik MC, Bischof JC, Hebbel RP, Vercellotti GM. Transgenic sickle mice have vascular inflammation. Blood 2003;101:3953–3959. [PubMed: 12543857]

Block G. Invited commentary: comparison of the Block and the Willett food frequency questionnaires. American Journal of Epidemiology 1998;148:1160–1161. [PubMed: 9867260]discussion, 1162–1165

Block G, Norris JC, Mandel RM, DiSogra C. Sources of energy and six nutrients in diets of low-income Hispanic-American women and their children: quantitative data from HHANES, 1982–1984. Journal of the American Dietetic Association 1995;95:195–208. [PubMed: 7852686]

Borgna-Pignatti C, Rugolotto S, De Stefano P, Piga A, Di Gregorio F, Gamberini MR, Sabato V, Melevendi C, Cappellini MD, Verlato G. Survival and disease complications in thalassemia major. Annals of the New York Academy of Sciences 1998;850:227–231. [PubMed: 9668544]

Bourantas KL, Dalekos GN, Makis A, Chaidos A, Tsiara S, Mavridis A. Acute phase proteins and interleukins in steady state sickle cell disease. European Journal of Haematology 1998;61:49–54. [PubMed: 9688292]

Bradley SJ, Gosriwitana I, Srichairatanakool S, Hider RC, Porter JB. Non-transferrin-bound iron induced by myeloablative chemotherapy. British Journal of Haematology 1997;99:337–343. [PubMed: 9375751]

Bronspiegel-Weintrob N, Olivieri NF, Tyler B, Andrews DF, Freedman MH, Holland FJ. Effect of age at the start of iron chelation therapy on gonadal function in beta-thalassaemia major. New England Journal of Medicine 1990;323:713–719. [PubMed: 2388669]

Buchanan GR, Holtkamp CA. Platelet aggregation, malondialdehyde generation and production time in children with sickle cell anaemia. Thrombosis and Haemostasis 1981;46:690–693. [PubMed: 7330819]

Christen S, Woodall AA, Shigenaga MK, Southwell-Keely PT, Duncan MW, Ames BN. Gamma-tocopherol traps mutagenic electrophiles such as NO(X) and complements alphatocopherol: physiological implications. Proceedings of the National Academy of Sciences of the United States of America 1997;94:3217–3222. [PubMed: 9096373]

Cighetti G, Duca L, Bortone L, Sala S, Nava I, Fiorelli G, Cappellini MD. Oxidative status and malondialdehyde in beta-thalassaemia patients. European Journal of Clinical Investigation 2002;32:55–60. [PubMed: 11886433]

Cunningham MJ, MacKlin EA, Neufeld EJ, Cohen AR. Complications of beta-thalassemia major in North America. Blood 2004;104:34–39. [PubMed: 14988152]

Dale GL, Alberio L. Is there a correlation between raised erythropoietin and thrombotic events in sickle-cell anaemia? Lancet 1998;352:566–567. [PubMed: 9716076]

De Luca C, Filosa A, Grandinetti M, Maggio F, Lamba M, Passi S. Blood antioxidant status and urinary levels of catecholamine metabolites in beta-thalassaemia. Free Radical Research 1999;30:453–462. [PubMed: 10400457]

Del Vecchio GC, Schettini F, Piacente L, De Santis A, Giordano P, De Mattia D. Effects of deferiprone on immune status and cytokine pattern in thalassaemia major. Acta Haematologica 2002;108:144–149. [PubMed: 12373086]

Evans RW, Williams J. Studies of the binding of different iron donors to human serum transferrin and isolation of ironbinding fragments from the N- and C-terminal regions of the protein. Biochemical Journal 1978;173:543–552. [PubMed: 100104]

Fillet G, Beguin Y, Baldelli L. Model of reticuloendothelial iron metabolism in humans: abnormal behavior in idiopathic hemochromatosis and in inflammation. Blood 1989;74:844–851. [PubMed: 2502204]

Finch CA, Lee MY, Leonard JM. Continuous RBC transfusions in a patient with sickle cell disease. Archives of Internal Medicine 1982;142:279–282. [PubMed: 7059256]

Gladwin MT, Schechter AN, Ognibene FP, Coles WA, Reiter CD, Schenke WH, Csako G, Waclawiw MA, Panza JA, Cannon RO III. Divergent nitric oxide bioavailability in men and women with sickle cell disease. Circulation 2003;107:271–278. [PubMed: 12538427]

Gosriwatana I, Loreal O, Lu S, Brissot P, Porter J, Hider RC. Quantification of non-transferrin-bound iron in the presence of unsaturated transferrin. Analytical Biochemistry 1999;273:212–220. [PubMed: 10469492]

Graziano JH, Miller DR, Grady RW, Cerami A. Inhibition of membrane peroxidation in thalassaemic erythrocytes by 2,3-dihydroxybenzoic acid. British Journal of Haematology 1976;32:351–356. [PubMed: 1252369]

Hebbel RP, Miller WJ. Phagocytosis of sickle erythrocytes: immunologic and oxidative determinants of hemolytic anemia. Blood 1984;64:733–741. [PubMed: 6466875]

Hedo CC, Aken'ova YA, Okpala IE, Durojaiye AO, Salimonu LS. Acute phase reactants and severity of homozygous sickle cell disease. Journal of Internal Medicine 1993;233:467–470. [PubMed: 7684769]

Himmelfarb J, Kane J, McMonagle E, Zaltas E, Bobzin S, Boddupalli S, Phinney S, Miller G. Alpha and gamma tocopherol metabolism in healthy subjects and patients with endstage renal disease. Kidney International 2003;64:978–991. [PubMed: 12911548]

Houglum K, Filip M, Witztum JL, Chojkier M. Malondialdehyde and 4-hydroxynonenal protein adducts in plasma and liver of rats with iron overload. Journal of Clinical Investigation 1990;86:1991–1998. [PubMed: 2123889]

Houglum K, Ramm GA, Crawford DH, Witztum JL, Powell LW, Chojkier M. Excess iron induces hepatic oxidative stress and transforming growth factor beta 1 in genetic hemochromatosis. Hepatology 1997;26:605–610. [PubMed: 9303489]

Jacobs EM, Hendriks JC, van Tits BL, Evans PJ, Breuer W, Liu DY, Jansen EH, Jauhiainen K, Sturm B, Porter JB, Scheiber-Mojdehkar B, von Bonsdorff L, Cabantchik ZI, Hider RC, Swinkels DW. Results of an international round robin for the quantification of serum non-transferrin-bound iron: need for

defining standardization and a clinically relevant isoform. Analytical Biochemistry 2005;341:241–250. [PubMed: 15907869]

Jain SK, Shohet SB. A novel phospholipid in irreversibly sickled cells: evidence for *in vivo* peroxidative membrane damage in sickle cell disease. Blood 1984;63:362–367. [PubMed: 6692040]

Jain SK, Ross JD, Levy GJ, Little RL, Duett J. The accumulation of malonyldialdehyde, an end product of membrane lipid peroxidation, can cause potassium leak in normal and sickle red blood cells. Biochemical Medicine and Metabolic Biology 1989;42:60–65. [PubMed: 2775562]

Jain SK, Ross JD, Levy GJ, Duett J. The effect of malonyldialdehyde on viscosity of normal and sickle red blood cells. Biochemical Medicine and Metabolic Biology 1990;44:37–41. [PubMed: 2390288]

Jiang Q, Ames BN. Gamma-tocopherol, but not alphatocopherol, decreases proinflammatory eicosanoids and inflammation damage in rats. FASEB Journal 2003;17:816–822. [PubMed: 12724340]

Jiang Q, Elson-Schwab I, Courtemanche C, Ames BN. Gamma-tocopherol and its major metabolite, in contrast to alphatocopherol, inhibit cyclooxygenase activity in macrophages and epithelial cells. Proceedings of the National Academy of Sciences of the United States of America 2000;97:11494–11499. [PubMed: 11005841]

Jiang Q, Christen S, Shigenaga MK, Ames BN. Gammatocopherol, the major form of vitamin E in the US diet, deserves more attention. American Journal of Clinical Nutrition 2001;74:714–722. [PubMed: 11722951]

Jiang Q, Lykkesfeldt J, Shigenaga MK, Shigeno ET, Christen S, Ames BN. Gamma-tocopherol supplementation inhibits protein nitration and ascorbate oxidation in rats with inflammation. Free Radical Biology and Medicine 2002;33:1534–1542. [PubMed: 12446211]

Jison ML, Munson PJ, Barb JJ, Suffredini AF, Talwar S, Logun C, Raghavachari N, Beigel JH, Shelhamer JH, Danner RL, Gladwin MT. Blood mononuclear cell gene expression profiles characterize the oxidant, hemolytic, and inflammatory stress of sickle cell disease. Blood 2004;104:270–280. [PubMed: 15031206]

Kadiiska MB, Gladen BC, Baird DD, Germolec D, Graham LB, Parker CE, Nyska A, Wachsman JT, Ames BN, Basu S, Brot N, Fitzgerald GA, Floyd RA, George M, Heinecke JW, Hatch GE, Hensley K, Lawson JA, Marnett LJ, Morrow JD, Murray DM, Plastaras J, Roberts LJ II, Rokach J, Shigenaga MK, Sohal RS, Sun J, Tice RR, Van Thiel DH, Wellner D, Walter PB, Tomer KB, Mason RP, Barrett JC. Biomarkers of oxidative stress study II: are oxidation products of lipids, proteins, and DNA markers of CCl4 poisoning? Free Radical Biology and Medicine 2005a;38:698–710. [PubMed: 15721980]

Kadiiska MB, Gladen BC, Baird DD, Graham LB, Parker CE, Ames BN, Basu S, FitzGerald GA, Lawson JA, Marnett LJ, Morrow JD, Murray DM, Plastaras J, Roberts LJ 2nd, Rokach J, Shigenaga MK, Sun J, Walter PB, Tomer KB, Barrett JC, Mason RP. Biomarkers of oxidative stress study: III. Effects of the nonsteroidal anti-inflammatory agents indomethacin and meclofenamic acid on measurements of oxidative products of lipids in CCl4 poisoning. Free Radical Biology and Medicine 2005b;38:711–718. [PubMed: 15721981]

Kassab-Chekir A, Laradi S, Ferchichi S, Haj Khelil A, Feki M, Amri F, Selmi H, Bejaoui M, Miled A. Oxidant, antioxidant status and metabolic data in patients with beta-thalassemia. Clinica Chimica Acta 2003;338:79–86.

Kearney SL, Nemeth E, Neufeld EJ, Thapa D, Ganz T, Weinstein DA, Cunningham MJ. Urinary hepcidin in congenital chronic anemias. Pediatric Blood & Cancer. 200510.1002/pbc.20616in press

Klings ES, Farber HW. Role of free radicals in the pathogenesis of acute chest syndrome in sickle cell disease. Respiratory Research 2001;2:280–285. [PubMed: 11686897]

Knutson MD, Walter PB, Ames BN, Viteri FE. Both iron deficiency and daily iron supplements increase lipid peroxidation in rats. Journal of Nutrition 2000;130:621–628. [PubMed: 10702595]

Laksmitawati DR, Handayani S, Udyaningsih-Freisleben SK, Kurniati V, Adhiyanto C, Hidayat J, Kusnandar S, Dillon HS, Munthe BG, Wirawan R, Soegianto RR, Ramelan W, Freisleben HJ. Iron status and oxidative stress in beta-thalassaemia patients in Jakarta. Biofactors 2003;19:53–62. [PubMed: 14757977]

Liu J, Yeo HC, Doniger SJ, Ames BN. Assay of aldehydes from lipid peroxidation: gas chromatography-mass spectrometry compared to thiobarbituric acid. Analytical Biochemistry 1997;245:161–166. [PubMed: 9056207]

Livrea MA, Tesoriere L, Maggio A, D'Arpa D, Pintaudi AM, Pedone E. Oxidative modification of low-density lipoprotein and atherogenetic risk in beta-thalassemia. Blood 1998;92:3936–3942. [PubMed: 9808587]

Ludwiczek S, Aigner E, Theurl I, Weiss G. Cytokine-mediated regulation of iron transport in human monocytic cells. Blood 2003;101:4148–4154. [PubMed: 12522003]

Makis AC, Hatzimichael EC, Mavridis A, Bourantas KL. Alpha-2-macroglobulin and interleukin-6 levels in steady-state sickle cell disease patients. Acta Haematologica 2000;104:164–168. [PubMed: 11279305]

Papanikolaou G, Tzilianos M, Christakis JI, Bogdanos D, Tsimirika K, MacFarlane J, Goldberg YP, Sakellaropoulos N, Ganz T, Nemeth E. Hepcidin in iron overload disorders. Blood 2005;105:4103–4105. [PubMed: 15671438]

Parker RS, Sontag TJ, Swanson JE. Cytochrome P4503A-dependent metabolism of tocopherols and inhibition by sesamin. Biochemical and Biophysical Research Communications 2000;277:531–534. [PubMed: 11061988]

Pippard, MJ. Secondary iron overload. In: Brock, JH.; Halliday, JW.; Pippard, MJ., editors. Iron Metabolism in Health and Disease. W.B. Saunders Ltd; London: 1994. p. 272-300.

Porter JB, Abeysinghe RD, Marshall L, Hider RC, Singh S. Kinetics of removal and reappearance of non-transferrin-bound plasma iron with deferoxamine therapy. Blood 1996;88:705–713. [PubMed: 8695819]

Rachmilewitz EA, Shohet SB, Lubin BH. Lipid membrane peroxidation in beta-thalassaemia major. Blood 1976;47:495–505. [PubMed: 1252619]

Ramenghi U, David O, Dianzani I, Sacchetti L, Biasi F, Chiarpotto E, Poli G. Analysis of the carbonyl compounds produced in beta thalassaemic erythrocytes by oxidative stress. Haematologica 1989;74:531–535. [PubMed: 2628233]

Reiter CD, Wang X, Tanus-Santos JE, Hogg N, Cannon RO Jr, Schechter AN, Gladwin MT. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. Nature Medicine 2002;8:1383–1389.

Repka T, Hebbel RP. Hydroxyl radical formation by sickle erythrocyte membranes: role of pathologic iron deposits and cyto-plasmic reducing agents. Blood 1991;78:2753–2758. [PubMed: 1668610]

Ruhl CE, Everhart JE. Relation of elevated serum alanine aminotransferase activity with iron and antioxidant levels in the United States. Gastroenterology 2003;124:1821–1829. [PubMed: 12806616]

Scott MD, van den Berg JJ, Repka T, Rouyer-Fessard P, Hebbel RP, Beuzard Y, Lubin BH. Effect of excess alpha-hemoglobin chains on cellular and membrane oxidation in model beta-thalassemic erythrocytes. Journal of Clinical Investigation 1993;91:1706–1712. [PubMed: 7682576]

Sertac A, Bingol F, Aydin S, Uslu A. Peroxidative damage in sickle-cell erythrocyte ghosts: protective effect of allopurinol. General Pharmacology 1997;28:427–428. [PubMed: 9068985]

Sess D, Carbonneau MA, Thomas MJ, Dumon MF, Peuchant E, Perromat A, Le Bras M, Clerc M. First observations on the main plasma parameters of oxidative stress in homozygous sickle cell disease. Bulletin de la Societe de Pathologie Exotique 1992;85:174–179. [PubMed: 1327359]

Sess ED, Carbonneau MA, Meite M, Peuchant E, Dumont MF, Receveur MC, Thomas MJ, Perromat A, Sangare A, Le Bras M, Clerc M. Markers of lipid peroxidation, inflammatory proteins and plasma tocopherols in homozygotic and heterozygotic sickle cell anemia. Bulletin de la Societe de Pathologie Exotique 1998;91:238–241. [PubMed: 9773200]

Shalev O, Repka T, Goldfarb A, Grinberg L, Abrahamov A, Olivieri NF, Rachmilewitz EA, Hebbel RP. Deferiprone (L1) chelates pathologic iron deposits from membranes of intact thalassemic and sickle red blood cells both in vitro and in vivo. Blood 1995;86:2008–2013. [PubMed: 7655028]

Singh S, Hider RC, Porter JB. A direct method for quantification of non-transferrin-bound iron. Analytical Biochemistry 1990;186:320–323. [PubMed: 2363505]

Singhal A, Doherty JF, Raynes JG, McAdam KP, Thomas PW, Serjeant BE, Serjeant GR. Is there an acute-phase response in steady-state sickle cell disease? Lancet 1993;341:651–653. [PubMed: 7680738]

Sinha R, Patterson BH, Mangels AR, Levander OA, Gibson T, Taylor PR, Block G. Determinants of plasma vitamin E in healthy males. Cancer Epidemiology, Biomarkers and Prevention 1993;2:473–479.

Snyder LM, Sauberman N, Condara H, Dolan J, Jacobs J, Szymanski I, Fortier NL. Red cell membrane response to hydrogen peroxide-sensitivity in hereditary xerocytosis and in other abnormal red cells. British Journal of Haematology 1981;48:435–444. [PubMed: 7259992]

Sontag TJ, Parker RS. Cytochrome P450 omega-hydroxylase pathway of tocopherol catabolism. Novel mechanism of regulation of vitamin E status. Journal of Biological Chemistry 2002;277:25290–25296. [PubMed: 11997390]

Vichinsky EP, Buetensky E, Fung E, Hudes M, Theil E, Ferrell L, Williams R, Louie L, Lee P, Harmatz P. A review comparing organ dysfunction in transfused patients with SCD or B thalassemia. American Journal of Hematology 2005a;80:70–74. [PubMed: 16138345]

Vichinsky EP, MacKlin EA, Waye JS, Lorey F, Olivieri NF. Changes in the epidemiology of thalassaemia in North America: a new minority disease. Pediatrics 2005b;116:e818–e825. [PubMed: 16291734]

Walter PB, Knutson MD, Paler-Martinez A, Lee S, Xu Y, Viteri FE, Ames BN. Iron deficiency and iron excess damage mitochondria and mitochondrial DNA in rats. Proceedings of the National Academy of Sciences of the United States of America 2002;99:2264–2269. [PubMed: 11854522]

Wickramasinghe SN, Thein SL, Srichairatanakool S, Porter JB. Determinants of iron status and bilirubin levels in congenital dyserythropoietic anaemia type I. British Journal of Haematology 1999;107:522–525. [PubMed: 10583252]

Wood JC, Tyszka JM, Carson S, Nelson MD, Coates TD. Myocardial iron loading in transfusion-dependent thalassemia and sickle cell disease. Blood 2004;103:1934–1936. [PubMed: 14630822]

Wood KC, Hebbel RP, Granger DN. Endothelial cell NADPH oxidase mediates the cerebral microvascular dysfunction in sickle cell transgenic mice. FASEB J 2005;19:989–991. [PubMed: 15923406]

Yeo HC, Helbock HJ, Chyu DW, Ames BN. Assay of malondialdehyde in biological fluids by gas chromatography-mass spectrometry. Analytical Biochemistry 1994;220:391–396. [PubMed: 7978283]

Yeo HC, Liu J, Helbock HJ, Ames BN. Assay of malondialdehyde and other alkanals in biological fluids by gas chromatography-mass spectrometry. Methods in Enzymology 1999;300:70–78. [PubMed: 9919511]

Zurlo MG, De Stefano P, Borgna-Pignatti C, Di Palma A, Piga A, Melevendi C, Di Gregorio F, Burattini MG, Terzoli S. Survival and causes of death in thalassaemia major. Lancet 1989;2:27–30. [PubMed: 2567801]
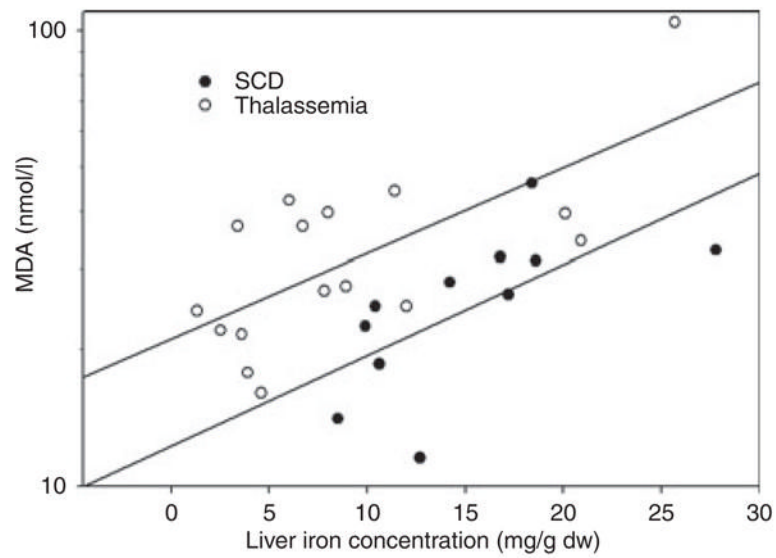
Fig 1.
Regression analysis comparing plasma malondialdehyde (MDA) (nmol/l, log scale) with liver iron concentration [mg/g dry weight (dw)]. Regressions for thalassaemia (top; $R = 0.695$, $P = 0.003$) and sickle cell disease (bottom; $R = 0.644$, $P = 0.032$), show that, in both diseases, higher liver iron concentrations are associated with more plasma MDA.

Table I

Demographics.

Parameter	Thalassaemia	SCD	Control	P value
Age (years)	23.6 ± 8.6 (17) (a)	12.7 ± 3.7 (11) (b)	26.5 ± 12.3 (9) (a)	0.002[*]
Height (cm)	154.2 ± 9.1 (16) (a)	150.4 ± 23.8 (10) (a)	164.8 ± 21.8 (9) (a)	0.207[*]
Weight (kg)	52.3 ± 8.9 (17) (a)	45.4 ± 20.2 (11) (a)	62.2 ± 26.1 (9) (a)	0.125[*]
BMI (kg/m^2)	22.3 ± 4.7 (16) (a)	19.1 ± 3.8 (10) (a)	21.7 ± 4.6 (9) (a)	0.201[*]
Sex M/F	7/10 (a)	7/4 (a)	4/5 (a)	0.489[†]
Splenectomy, n	12/17 (67%) (a)	3/11 (27%) (b)	—	0.025[†]
Chelation, n[¶]	16/17 (94%) (a)	7/11 (64%) (a)	—	0.062[‡]
Transfusion, years	16.1 ± 8.6 (17) (a)	4.6 ± 2.9 (11) (b)	—	<0.001[§]
HCV, n	5/17 (29%) (a)	0 (0%) (a)	—	0.125[‡]

Values are means ± SD. Number of patients analysed are shown in parentheses (*n*) to the right of the SD. Within a row groups sharing the same lowercase letter are not significantly different from each other (5% procedure wise error rate). BMI, body mass index; HCV, hepatitis C virus; SCD, sickle cell disease.

[*] The *P* value was obtained from an ANOVA.

[†] The *P* value was obtained from a chi-square test.

[‡] The *P* value was obtained from a Fisher's exact test.

[§] The *P* value was obtained from a two-sample *t*-test.

[¶] Chelation was by deferoxamine.

Table II

Measurements of iron status.

Parameter	Thalassaemia	SCD	Control	ANOVA*P* value
Liver iron (mg/g dw)	9.2 ± 7.2 (16) (b)	15.0 ± 5.6 (11) (a)		0.033
Ferritin (μg/l)	1868 ± 1042 (17) (a)	2515 ± 1153 (11) (a)	65.0 ± 70.9 (9)(b)	<0.001
Transferrin saturation (%)	82.5 ± 21.7 (6) (a)	64.5 ± 23.3 (4) (a,b)	40.2 ± 13.1 (5) (b)	0.014
NTBI (μmol/l)	4.0 ± 1.6 (15) (a)	1.9 ± 2.1 (11) (b)	-1.0 ± 0.4 (9) (c)	<0.001

Values are means ± SD. Number of patients analysed are shown in parentheses (n) to the right of the SD. Within a row, groups not sharing the same letter are significantly different from each other (5% procedure wise error rate).

dw, dry weight; NTBI, non-transferrin bound iron; SCD, sickle cell disease.

Table III

Measurements of oxidative injury and inflammation.

Parameter	Thalassaemia	SCD	Control	ANOVA P value
MDA, nmol/l[*]	35.3 ± 20.0 (17) (a)	26.1 ± 9.7 (11) (a,b)	19.3 ± 13.4 (9) (b)	0.006
α-tocopherol, μmol/l	14.8 ± 4.6 (17) (b)	15.7 ± 4.1 (11) (b)	22.7 ± 3.4 (9) (a)	<0.001
γ-tocopherol, μmol/l	3.3 ± 2.1 (17) (b)	5.5 ± 1.4 (11) (a)	1.8 ± 0.7 (9) (c)	<0.001
Protein carbonyls, nmol/mg	0.7 ± 0.3 (16) (a)	0.6 ± 0.1 (11) (a)	0.6 ± 0.1 (9) (a)	0.454
ALT, U/l	57.5 ± 31.1 (17) (a)	36.1 ± 11.0 (11) (b)	34.9 ± 7.7 (9) (b)	0.020
hsCRP, mg/l	1.0 ± 1.2 (16) (a)	2.7 ± 3.2 (10) (a)	0.9 ± 0.7 (9) (a)	0.061
Cytokines: pg/ml				
IL-5	0.3 ± 0.3 (13) (b)	1.0 ± 0.6 (10) (a)	0.6 ± 0.6 (8) (a,b)	0.010
IL-6	3.1 ± 1.7 (15) (a,b)	4.5 ± 2.2 (11) (a)	2.0 ± 1.0 (8) (b)	0.014
IL-10	2.1 ± 1.2 (17) (b)	3.5 ± 1.6 (11) (a)	1.8 ± 1.0 (9) (b)	0.007

Values are means ± SD. Number of patients analysed are shown in parentheses (*n*) to the right of the SD. Within a row, groups not sharing the same letter are significantly different from each other (5% procedure wise error rate).

[*] MDA values were log-transformed prior to analysis.

ALT, alanine transaminase; IL, interleukin; hsCRP, high sensitivity C-reactive protein; MDA, malondialdehyde; SCD, sickle cell disease.

Table IV

Oxidant injury analysis model using two groups (thalassaemia and SCD).

	Bivariate (crude)	Multivariate (adjusted)		
	P-value	*P*-value	Regression coefficient	95% confidence intervals
MDA (dependent variable, nmol/l) R^2 for model =0.55, $n = 26$				
Diagnosis [thalassaemia (reference) *versus* SCD]	0.185	0.019	−0.481	−0.870, −0.091
Liver iron concentration (mg/g)	0.012	<0.001	+0.045	0.024, 0.067
Duration of chronic transfusion (years)	0.199	0.641	+0.005	−0.016, 0.026
Age (years)	0.041	0.697	+0.004	−0.017, 0.025

MDA values were log transformed prior to analysis.

MDA, malondialdehyde; SCD, sickle cell disease.

Table V

Model of transfusional iron overload in thalassaemia and SCD (shown are the relative differences).

Thalassaemia	SCD
General tissue changes: More organ dysfunction	
Changes in specific biomarkers: More MDA (adjusted), ALT, NTBI	More IL-5 and IL-10 More γ-tocopherol
Hypothesis: Transfusional iron load stored in the liver, heart and endocrine organs. Tissue iron load leads to dysfunction.	Transfusional iron load is taken up by activated RE cells keeping iron away from vital organs. γ-tocopherol is higher which may protect.

ALT, alanine transaminase; MDA, malondialdehyde; NTBI, non-transferrin bound iron; RE, reticuloendothelial; SCD, sickle cell disease.