Methemoglobinemia and ascorbate deficiency in hemoglobin E β thalassemia: metabolic and clinical implications

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ABSTRACT

During investigations of the phenotypic diversity of hemoglobin E β thalassemia, the commonest form of severe thalassemia in many Asian countries, a patient was encountered with persistently high levels of methemoglobin associated with a left-shift in the oxygen dissociation curve, profound ascorbate deficiency and clinical features of scurvy; these abnormalities were corrected by treatment with vitamin C. Studies of erythropoietin production before and after treatment suggested that, as in an ascorbate-deficient murine model, the human hypoxia induction factor (HIF) pathway is not totally dependent on ascorbate levels. A follow-up study of 45 patients with HbE β thalassemia showed that methemoglobin levels were significantly raised and that there was also a significant reduction in plasma ascorbate levels, although not to those observed in the presenting patient. Haptoglobin levels were significantly reduced and the high frequency of the 2.2 haptoglobin genotype may place an additional pressure on ascorbate as a free-radical scavenger in this population. There was, in addition, a highly significant correlation between methemoglobin levels, splenectomy and factors that modify the degree of globin-chain imbalance. Since it is clear that methemoglobin levels are modified by several mechanisms, and because it may play a role in both adaptation to anemia and vascular damage, there is a strong case for its further study in other forms of thalassemia and sickle-cell anemia, particularly when splenic function is defective.
INTRODUCTION

Although low ascorbate levels have been observed in patients with different hemoglobinopathies\textsuperscript{1,2} there are very few reports of the clinical manifestations of scurvy in these conditions.\textsuperscript{3} During an analysis of the mechanisms for the broad phenotypic diversity of hemoglobin E (HbE) β thalassemia in Sri Lanka a patient was encountered with profound ascorbate deficiency and clinical features of scurvy who also had a high level of methemoglobin. This unusual combination of findings raised several important questions.

First, to what extent does ascorbate deficiency interfere with the hypoxia-sensing mechanism in humans, particularly with respect to erythropoietin response to anemia? The key players in this pathway are the prolylhydroxylase domain-containing enzymes (PHDs) that catalyze the prolyl-4-hydroxylation of the hypoxia-inducible factor (HIF) in the presence of oxygen and 2-oxoglutarate as co-substrates with iron and ascorbic acid as co-factors.\textsuperscript{4-6} Recent work in ascorbate-deficient mice suggests that other fail-safe mechanisms are involved in this reaction and that erythropoietin response is not altered in ascorbate deficiency\textsuperscript{7,8}; is this the case in humans? The second question raised by this unusual case is, because of the potential deleterious effects of methemoglobin on response to anemia\textsuperscript{9} and the vascular endothelium\textsuperscript{10}, how common are increased levels of methemoglobin in Hb E β thalassemia and related disorders, to what extent might this depend on ascorbate deficiency, and what other factors may be involved?

The results of these studies suggest that with respect to hypoxia recognition humans are able to compensate for ascorbate deficiency in the same way as the murine model. There is a highly significant increase in methemoglobin production in HbE β thalassemia and a significant reduction in plasma ascorbate levels although not to those observed in the patient whose findings initiated this study. There is, however, a highly significant relationship between the level of methemoglobin and
spleenectomy and also with the factors that modify globin-chain imbalance. A further complication in this population was the finding that the haptoglobin genotype was nearly all of the 2.2 variety which is less effective at hemoglobin binding\textsuperscript{11,12} and may place an additional pressure on ascorbate as a free radical scavenger.

Clearly, there are multiple factors involved in the increased level of methemoglobin production in this form of thalassemia and because of its potential effects on adaptation to anemia and vascular damage further studies of the mechanisms involved in its increased production require study in other forms of thalassemia and sickle-cell anemia.

**PATIENTS AND METHODS**

**Patients**

The patient in whom the findings initiated this study was a 19-year-old female attending the National Thalassemia Centre, Kurenegala, Sri Lanka. She had presented aged 2 years with anemia and splenomegaly and later the diagnosis of Hb E \(\beta\) thalassemia was established. For the next seven years she received intermittent transfusion after which she underwent splenectomy based on a steady-state hemoglobin level of 5-6g/dl and a spleen that was enlarged to 19cm below the costal margin. For the next ten years, despite hemoglobin values in the 7-8g/dl range her growth and sexual maturation were delayed, although by the age of 19 years she had achieved mid-parental height and the menarche. Only when she reached the age of 17 years did the patient and her family disclose that she had had painful and enlarged gums and intermittent mucosal bleeding for several years. A detailed dietetic history at this time revealed that she ate no fruit of any form or vegetables, a diet which had persisted for several years. Screening for environmental factors that might induce methemoglobinemia was negative.
Based on subsequent findings in this patient and her family detailed studies were carried out on 45 patients attending the National Thalassemia Centre who were chosen at random from over 200 patients who were being followed with Hb E β thalassemia at the Centre. Clinical and hematologic data on this group of patients have been published previously, together with a detailed account of a classification system directed at defining the phenotypic variability of the disease. In short, a ‘mild’ phenotype covers those who never required transfusion, had stopped transfusion with no ill effects after several years follow up, or had required no further transfusion after splenectomy. ‘Severe’ phenotypes were defined as reliance on long-term transfusion. There were 25 of the former and 20 of the latter in this study.

In addition, methemoglobin values were estimated in 17 normal adult volunteers and 17 patients with different types of hemoglobinopathy at the Centre.

**Methods**

Venous blood was collected into heparin and EDTA from all study participants. Duplicate measurements of methemoglobin levels and $P_{50}$, using a Rapidpoint 405 analyser with an integral co-oximeter (Bayer) were made from the heparinized blood sample. This instrument incorporates a polychromator which allows the simultaneous measurements across the various fractions of haemoglobin in the range 473-671 nm. To confirm that methemoglobin levels were being accurately measured in this way blood samples from the propositus and a group of patients with HbE β thalassemia and normal controls were analyzed using the manual method of Evelyn and Malloy. There was close agreement between the methemoglobin values between the two methods used.

The samples were then centrifuged, the plasma removed and plasma ascorbate levels measured immediately using a ferric reducing ascorbate (FRASC) assay (procedure K671, BioVision, CA, USA.) In order to prevent plasma protein precipitation, the FRASC buffer was diluted 1 in 10 prior to use. Plasma haptoglobin
was measured using a commercial assay (procedure TP.801 Tridelta development Ltd, Co. Kildare, Ireland). Routine hematologic indices were measured in the EDTA sample (Coulter Electronics, UK). The sample was centrifuged, the plasma removed from the cells and both were stored at -20°C until shipped to Oxford on dry ice. Plasma erythropoietin and interleukin-8 levels were measured by enzyme-linked immunosorbent assay kit DEPOO, R & D Systems, UK and human 11.8 elisa kit M1918, Pelikine, The Netherlands). DNA was extracted from the cell pellet using Qiagen DNA blood mini kit (kit 51104, Qiagen, UK) and the haptoglobin genotype was determined by the polymerase chain reaction. Hemoglobin analysis, serum ferritin levels and hepatic iron concentrations by magnetic resonance imaging (MRI) followed previously reported methods.

To investigate the patient with a markedly raised methemoglobin concentration, further blood samples were collected from her and her immediate family and transferred into EDTA and ACD. EDTA samples were screened for glucose-6-phosphate dehydrogenase (G6PD) deficiency using a qualitative assay (procedure 400, Trinity Biotech, Ireland). Levels of reduced glutathione (GSH) were determined (kit 371757, Calbiochem, UK) and a red cell hemolysate, stabilized in EDTA-mercaptoethanol, was prepared and used for the measurement of cytochrome b₅ reductase (CYtb₅R) and glyceraldehyde phosphate dehydrogenase (GAPD). A red cell hemolysate was prepared from each ACD sample, G6PD activity was measured using a quantitative ultraviolet, kinetic assay (procedure 345-uv, Trinity Biotech, Co. Wicklow, Ireland). Glutathione reductase was measured using a quantitative manual method (kit GR2368, Randox, Co. Antrim, UK). Pyruvate kinase was measured according to the method described by Dacie and Lewis. Both cytochrome b₅ and cytochrome b₅ reductase genes and the HBA and HBB genes were sequenced.
A urine sample was collected from the patient and tested with Combur 10 diagnostic strips (Roche Diagnostics, UK) for the presence of nitrites and hemoglobin. The urine sediment was examined by microscopy for the presence of red blood cells.

**Statistical analysis**

Statistical analysis was performed using SPSS 16.0 for Windows (Release 16.0.1; Nov 15, 2007; SPSS Inc). Differences between median methemoglobin concentrations were assessed using the Mann-Whitney U test. Multiple regression analysis explored the relationship between methemoglobin and the potential predictor variables severity, transfusion status and splenectomy. A P value <0.05 was considered statistically significant.

**Ethical approval**

Approval for the research programme on HbE β thalassemia was obtained from the Ethical Committee of the College of Pediatricians, Colombo, Sri Lanka, and the Oxford Tropical Research Ethical Committee, Oxford, UK. This study was conducted in accordance with the Declaration of Helsinki.

**RESULTS**

The findings in the family that led to these studies are summarized in Table 1 and further biochemical data of the propositus, including findings before and after treatment, in Table 2. The propositus had a hemoglobin pattern typical of Hb E β thalassemia; sequencing of the *HBB* genes revealed the $\beta^E$ mutation on one chromosome and the severe $\beta$ thalassemia mutation, IVS1-5 (G-C), which is very common in the Sri Lankan population$^{16}$, on the other. Further sequencing of the *HBA* and *HBB* genes revealed no other abnormalities, excluding hemoglobin M mutations. The mother had findings typical of $\beta$ thalassemia trait, although complicated by iron deficiency anemia due to menorrhagia. The father had hemoglobin E trait while the two siblings were normal. Multiple estimations indicated
that the propositus had markedly raised levels of methemoglobin in the range of 10.7 – 13.6%. Her mother with β thalassemia trait had a slightly elevated methemoglobin level while the levels in other family members were normal. The cytochrome b5 reductase levels were raised in the propositus and normal in the other family members. Both cytochrome b5 and cytochrome b5 reductase genes from the propositus were sequenced and showed no abnormality. Glyceraldehyde phosphate dehydrogenase (GAPD) and glutathione reductase levels were within the normal range in the propositus and both her parents. Her P50 was significantly reduced compared with other family members, resulting in a marked left shift in the oxygen-dissociation curve compared with those of patients in the same population with HbE β thalassemia recently reported.21 Screening for glucose-6-phosphate dehydrogenase deficiency was negative in all family members.

To try to determine the reason for the extremely high methemoglobin level in the propositus further investigations were carried out as shown in Table 2. Her plasma ascorbate level was extremely low, while G6PD levels were elevated. Reduced glutathione levels (GSH) were at the lower limit of the normal range. Haptoglobin levels were slightly reduced and there was an increased level of interleukin 8. Treatment with vitamin C, 50mg alternate days, resulted in a dramatic fall in the methemoglobin level and an increase in the ascorbate level to above the lower range of normal. There was also a right-shift in the oxygen dissociation curve associated with a significant increase in the P50. There was no significant change in the relationship between hemoglobin and erythropoietin levels estimated on 5 samples before and after treatment.

To explore further the significance of the findings in this patient 45 patients with HbE β thalassemia were chosen at random from the 200 or more patients with this condition being followed in Kurenegala. Their division into strictly defined severity groups, as described in the Methods section, main clinical and hematologic findings and some of the genetic modifiers responsible for the variation in their phenotypic
severity have been reported previously.\textsuperscript{13,16} The major findings in these patients in relationship to the present study are summarized in Tables 3 and 4. As shown in Table 3 those with HbE $\beta$ thalassemia had a significant increase in methemoglobin compared with normal controls and univariate analysis showed that there was a highly significant increase in methemoglobin levels in those who had been splenectomized compared with those with intact spleens. As shown in Table 3, there was also a significant relationship between methemoglobin levels and phenotypic severity as judged by the findings in the mild and severe groups and mirrored by the transfusion requirements. In multiple regression analysis, only splenectomy was statistically significantly related to methemoglobin level (standardized $\beta=0.64$, $t=3.68$, $P<0.01$).

As shown in Table 4 the mean plasma ascorbate level in this group of patients was at the bottom limit of the normal range; in 10 cases it was subnormal. Although the number of cases available with matched plasma ascorbate and methemoglobin levels was too small to determine whether there was a significant relationship between the two at this level of plasma ascorbate, the mean level of methemoglobin in those in whom these measurements were available and who had all been splenectomized was 4.24\% and the mean level of plasma ascorbate was 23.1 nmol/ml, that is in the subnormal range.

Overall, the level of haptoglobin was subnormal in the patients with HbE $\beta$ thalassemia, although no case of absent haptoglobin was encountered. The majority of the patients had the 2.2 haptoglobin genotype. There was a wide range of hepatic iron concentrations which were not significantly related to the methemoglobin level. There was also a highly significant elevation of IL-8 levels in this patient population; levels were elevated (>10 pg/ml) in 21 cases, some having very high levels (Table 4).
The findings in the other hemoglobin disorders studied (Table 5) suggest further studies of patients with β thalassemia major or intermedia and sickle cell anemia and related conditions are indicated. The rare sickle cell disorders in Sri Lanka all have the Asian haplotype which is associated with a relatively mild phenotype.

DISCUSSION

There seems little doubt that the high level of methemoglobin in the patient whose findings initiated these studies resulted from profound ascorbate deficiency. It has been estimated that ascorbate is responsible for approximately 16% of methemoglobin reduction in red cells, the remainder relying on several enzymes, notably cytochrome b5 reductase, glyceraldehyde-3-PO4 dehydrogenase and glutathione reductase. The levels of these enzymes were all elevated or normal in the propositus and her family members and structural studies of the cytochrome b5 reductase gene of the propositus were normal. Sequencing of the HBA and HBB genes excluded HbM. Furthermore, the administration of cautious doses of ascorbate, because of the possible deleterious effects of rapid iron mobilisation, rapidly reversed the methemoglobin levels into the low-normal range. Before treatment the propositus also had a low P50 and a marked left shift in her oxygen dissociation curve, a finding that has been previously observed in association with increased methemoglobin levels. The ferric (Fe+++ ) hemes are unable to reversibly bind oxygen and they increase the oxygen affinity of the associated ferrous hemes (Fe++) in the hemoglobin tetramer, causing a left shift in the oxygen dissociation curve. There was a significant increase in the P50 with a right shift in the oxygen dissociation curve following treatment with ascorbate. In short, this reflects a more effective adaptation to anemia, as recently described in patients with HbE β thalassemia.
The finding that multiple estimations of the erythropoietin response to a particular hemoglobin level did not change in the propositus before and after treatment with ascorbate is of particular interest with regard to response to hypoxia. These findings are similar to those reported recently in an ascorbate-deficient mouse model. An increased erythropoietin response to anemia depends on the oxygen-sensing properties of the prolylhydroxylase domain-containing enzymes (PHDs) that catalize the prolyl-4-hydroxylation of the hypoxia-inducible factor (HIF) and require oxygen and 2-oxoglutarate as co-substrates with iron and ascorbic acid as co-factors. The erythropoietin response to anemia in the murine model was normal despite profound ascorbate deficiency. A major compensatory mechanism appeared to be the action of reduced glutathione, the levels of which remained normal or elevated in the propositus in the present study. These observations suggest that the relationship between ascorbate and hypoxia response in humans and mice are similar.

What are the broader issues resulting from these findings? In particular, since the results of the studies in this unusual patient provide clear evidence that ascorbate deficiency can induce methemoglobinemia in HbE β thalassemia, how common are raised methemoglobin levels in this condition, and are the levels related mainly to ascorbate or are other factors involved? There have been relatively few reports of the levels of methemoglobin in the inherited hemoglobin disorders. An early study of a few cases of HbE β thalassemia in northern India suggested that methemoglobin levels might be elevated in this condition, and raised levels have been reported in some cases of inherited unstable hemoglobins and sickle cell anemia. In the present study there was a significant elevation of methemoglobin in a group of patients with HbE β thalassemia whose mean level of plasma ascorbate was at the lower limit of normal; 10 cases showed subnormal levels. However, no cases were encountered with a reduction to the level found in the propositus in this study and the extent to which ascorbate deficiency may be responsible for the modest elevation in methemoglobin in these patients requires further study. The most striking finding,
however, was the highly significant relationship between splenectomy and methemoglobin levels together with the effect of phenotypic severity, including blood transfusion status. Since the main factors underlying phenotypic variability in this group of patients identified so far are the co-inheritance of α thalassemia or relatively high levels of HbF, both of which modify the degree of globin-chain imbalance, it seems likely that splenic function and the degree of excess α-chain synthesis play a major role in determining the level of methemoglobin, at least in HbE β thalassemia.

What is the source of the increased methemoglobin? As in other forms of β thalassemia excess α-chains are produced in HbE thalassemia with the production of red cell inclusions; despite the mild instability of HbE βE chains are not found in these precipitates. One of the major degradation products of excess α-chains are hemichromes which bind to the red cell membrane and promote sequestering of band 3. As they form they go through reversible and irreversible phases during which methemoglobin is produced as an intermediate. It is possible, therefore, that abnormal red cells exposed to this mechanism are recognized and sequestered in the spleen and hence the level of methemoglobin is increased after splenectomy. Since, like other forms of thalassemia there is a significant hemolytic component in HbE β thalassemia, it follows that the circulation will be continually exposed to increased levels of methemoglobin.

Another potential source of methemoglobin, in this case in plasma, is the further oxidation of hemoglobin released during hemolysis, the fail-safe mechanism in this case again is binding by haptoglobin. In the present study the haptoglobin levels were reduced in the patients with HbE β thalassemia although only to a minor degree. However, molecular analysis showed that in almost every case the haptoglobins were of the 2.2 variety which has been shown to be less effective than the 1.1 variety with respect to hemoglobin binding and which occurs commonly in some Asian countries. Recent studies suggest that because of its reduced binding
properties it may put greater pressure on the utilisation of ascorbate as a free radical scavenger and, indeed, may be associated with increased frequency of the clinical manifestations of ascorbate deficiency.\textsuperscript{11,12,35}

Methemoglobin is a significant activator of endothelial cells by stimulation of E-selectin, IL-6 and IL-8 production.\textsuperscript{10} It is of interest therefore that the IL-8 levels in this series of patients with HbE β thalassemia were considerably raised. Because of increasing evidence for vascular complications in other forms of thalassemia intermedia\textsuperscript{36} and in sickle cell disease, and because of the results of the small pilot study shown in Table 5, further investigation of the potential pathological role of methemoglobin is indicated, particularly in conditions with reduced splenic function or in which splenectomy is commonly practised.
ACKNOWLEDGEMENTS

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AUTHORSHIP

Contribution. AA and CF carried out the laboratory studies. SA carried out the statistical analysis. APrem, A Per, DB, TP and NO collected and analyzed the clinical data on the patients, and AA and DJW designed the study and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no conflict of interest.

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References


32. Wickramasinghe SN, Lee MJ. Observations on the relationship between γ-globin chain content and globin chain precipitation in thalassaemic


<table>
<thead>
<tr>
<th>Variable (units)</th>
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<th>Variable (units)</th>
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<tbody>
<tr>
<td>Variable (units)</td>
<td>Hb (g/dl)</td>
<td>MCV (fl)</td>
<td>MCH (pg)</td>
<td>Hb F (%)</td>
<td>Hb A₂ (%)</td>
<td>Hb E (%)</td>
<td>Met Hb (%)</td>
<td>P₅₀ (mmHg)</td>
<td>Cytb,R (U/gHb)</td>
<td>GAPD (IU/gHb)</td>
<td>Glutathione Reductase (IU/gHb)</td>
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<tr>
<td>Propositus</td>
<td>6.4</td>
<td>74.3</td>
<td>21.0</td>
<td>18.7</td>
<td>-</td>
<td>76.7</td>
<td>10.7-13.6</td>
<td>21.3-23.6</td>
<td>50.7</td>
<td>301.5</td>
<td>9.81</td>
</tr>
<tr>
<td>Mother</td>
<td>10.7</td>
<td>54.9</td>
<td>15.8</td>
<td>&lt;1</td>
<td>4.2</td>
<td>-</td>
<td>1.9</td>
<td>26.5</td>
<td>28.9</td>
<td>271.0</td>
<td>13.0</td>
</tr>
<tr>
<td>Father</td>
<td>14.2</td>
<td>78.0</td>
<td>23.9</td>
<td>&lt;1</td>
<td>-</td>
<td>24.9</td>
<td>0.7</td>
<td>25.2</td>
<td>17.7</td>
<td>230.0</td>
<td>5.5</td>
</tr>
<tr>
<td>Brother</td>
<td>14.6</td>
<td>81.5</td>
<td>28.5</td>
<td>&lt;1</td>
<td>2.6</td>
<td>-</td>
<td>0.5</td>
<td>25.2</td>
<td>14.6</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Sister</td>
<td>13.0</td>
<td>84.5</td>
<td>29.6</td>
<td>&lt;1</td>
<td>2.6</td>
<td>-</td>
<td>0.4</td>
<td>25.3</td>
<td>21.8</td>
<td>-</td>
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Table 1: Hematologic and related studies in the propositus, before treatment with Vitamin C, and her relatives.
<table>
<thead>
<tr>
<th>Variable (units)</th>
<th>Ascorbate (nmol/ml)</th>
<th>Met Hb (%)</th>
<th>P50 (mmHg)</th>
<th>Mean Hb (g/dl)</th>
<th>Mean Epo (IU/ml)</th>
<th>G6PD (U/gHb)</th>
<th>Pyruvate Kinase (IU/gHb)</th>
<th>GSH (μmol/gHb)</th>
<th>Haptoglobin (g/dl)</th>
<th>Hepatic Iron (mg/g dw)</th>
<th>IL-8 (pg/ml)</th>
<th>Urinary nitrite screen</th>
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<tbody>
<tr>
<td>Pre-treatment</td>
<td>2.1-6.7</td>
<td>10.7-13.6</td>
<td>21.3-23.6</td>
<td>6.2</td>
<td>94.3</td>
<td>21.1</td>
<td>7.7</td>
<td>6.18</td>
<td>0.26</td>
<td>2.9</td>
<td>179.6</td>
<td>Negative</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>30.2-36.6</td>
<td>0.85</td>
<td>26.9</td>
<td>7.0</td>
<td>76.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2: Further biochemical and related analyses of the propositus including, in some cases, data obtained before and after treatment with Vitamin C. The hemoglobin and erythropoietin (Epo) values are the means of 5 estimations.
<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>Median Met Hb (%)</th>
<th>Interquartile Range</th>
<th>Range</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal controls</td>
<td>17</td>
<td>0.3</td>
<td>0.25-0.4</td>
<td>0.1-0.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hb E β thalassaemia (all cases)</td>
<td>45</td>
<td>2.7</td>
<td>1.9-3.65</td>
<td>0.9-6.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hb E β thalassaemia (splenectomized)</td>
<td>20</td>
<td>3.7</td>
<td>3.1-4.2</td>
<td>0.9-6.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hb E β thalassaemia (spleen intact)</td>
<td>25</td>
<td>2.3</td>
<td>1.5-2.8</td>
<td>0.9-4.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hb E β thalassaemia (mild)</td>
<td>25</td>
<td>2.45</td>
<td>1.9-3.6</td>
<td>0.9-4.8</td>
<td>0.084</td>
</tr>
<tr>
<td>Hb E β thalassaemia (severe)</td>
<td>20</td>
<td>3.1</td>
<td>1.9-3.9</td>
<td>0.9-6.3</td>
<td>0.001</td>
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<tr>
<td>Hb E β thalassaemia (0-20 blood transfusions)</td>
<td>21</td>
<td>2.5</td>
<td>1.85-3.1</td>
<td>0.9-3.6</td>
<td>0.001</td>
</tr>
<tr>
<td>Hb E β thalassaemia (&gt;20 blood transfusions)</td>
<td>18</td>
<td>3.65</td>
<td>3.4-4.5</td>
<td>1.6-6.3</td>
<td>0.018</td>
</tr>
<tr>
<td>Hb E β thalassaemia (mild, spleen intact)</td>
<td>13</td>
<td>2.2</td>
<td>1.75-2.5</td>
<td>0.9-3.6</td>
<td>0.012</td>
</tr>
<tr>
<td>Hb E β thalassaemia (severe, spleen intact)</td>
<td>12</td>
<td>3.6</td>
<td>2.65-4.05</td>
<td>0.9-5.0</td>
<td>0.032</td>
</tr>
<tr>
<td>Hb E β thalassaemia (severe, splenectomized)</td>
<td>12</td>
<td>2.65</td>
<td>1.38-3.18</td>
<td>0.9-4.8</td>
<td>0.002</td>
</tr>
<tr>
<td>Hb E β thalassaemia (mild, spleen intact)</td>
<td>13</td>
<td>2.2</td>
<td>1.75-2.5</td>
<td>0.9-3.6</td>
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</tr>
<tr>
<td>Hb E β thalassaemia (severe, spleen intact)</td>
<td>12</td>
<td>2.65</td>
<td>1.38-3.18</td>
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<td>Hb E β thalassaemia (mild, splenectomized)</td>
<td>12</td>
<td>3.6</td>
<td>2.65-4.05</td>
<td>0.9-5.0</td>
<td>0.012</td>
</tr>
<tr>
<td>Hb E β thalassaemia (severe, splenectomized)</td>
<td>8</td>
<td>4.0</td>
<td>3.7-4.45</td>
<td>3.5-6.3</td>
<td>0.012</td>
</tr>
</tbody>
</table>

**Table 3:** Analysis of methemoglobin levels in 45 patients with HbE β thalassemia with a breakdown of cases into splenectomized and non-splenectomized, low and high transfusion rates, and mild and severe phenotypes as defined in the text.
<table>
<thead>
<tr>
<th>Variable (units) [normal range]</th>
<th>n</th>
<th>Median</th>
<th>Interquartile Range</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methemoglobin (%) [0.1-0.6]</td>
<td>45</td>
<td>2.70</td>
<td>1.9-3.65</td>
<td>0.9-6.3</td>
</tr>
<tr>
<td>Plasma Ascorbate (nmol/ml) [28-84]</td>
<td>20</td>
<td>28.23</td>
<td>21.03-31.68</td>
<td>12.6-56.3</td>
</tr>
<tr>
<td>IL-8 (pg/ml) [&lt;10]</td>
<td>45</td>
<td>7.97</td>
<td>3.47-172.8</td>
<td>0.39-2210</td>
</tr>
<tr>
<td>Hepatic Iron (mg/g dw) [0.6-1.2]</td>
<td>33</td>
<td>6.0</td>
<td>2.95-10.75</td>
<td>1.0-33.0</td>
</tr>
</tbody>
</table>

Haptoglobin (g/l) [0.3-2.0]  
- All cases 38 0.24 0.20-0.28 0.16-0.42  
- Haptoglobin genotype 1:1 0 - - -  
- Haptoglobin genotype 2:1 8 0.27 0.26-0.33 0.22-0.33  
- Haptoglobin genotype 2:2 25 0.23 0.19-0.26 0.16-0.41

**Table 4**: Additional data from the 45 patients with HbE β thalassemia including ascorbate and iron status and levels of IL-8 and haptoglobin, including the different genetic forms of the latter.
<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No.</th>
<th>Methemoglobin %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (median; range)</td>
<td>17</td>
<td>0.3; 0.1-0.6</td>
</tr>
<tr>
<td>β thalassemia intermedia</td>
<td>4</td>
<td>1.4, 2.9, 4.7, 4.8</td>
</tr>
<tr>
<td>Hb E/δβ thalassemia</td>
<td>1</td>
<td>2.4</td>
</tr>
<tr>
<td>δβ/β thalassemia</td>
<td>3</td>
<td>2.0, 2.8, 2.8</td>
</tr>
<tr>
<td>Hb SS disease</td>
<td>2</td>
<td>0.3, 1.2</td>
</tr>
<tr>
<td>Hb S/β thalassemia</td>
<td>1</td>
<td>1.8</td>
</tr>
<tr>
<td>Hb SD disease</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>Hb SE disease</td>
<td>1</td>
<td>1.4</td>
</tr>
<tr>
<td>β thalassemia trait</td>
<td>2</td>
<td>0.4, 0.5</td>
</tr>
<tr>
<td>Hb E trait</td>
<td>2</td>
<td>0.3, 0.3</td>
</tr>
</tbody>
</table>

**Table 5:** Miscellaneous methemoglobin levels in conditions related to HbE β thalassemia obtained from patients attending the same clinic.
Methemoglobinemia and ascorbate deficiency in hemoglobin E β thalassemia: metabolic and clinical implications

Angela Allen, Christopher Fisher, Anuja Premawardhena, Dayananda Bandara, Ashok Perera, Stephen Allen, Timothy St. Pierre, Nancy Olivieri and David Weatherall

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