Markers of ineffective erythropoiesis in non-transfusion dependent β-thalassaemia

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ABSTRACT
Non-transfused β-thalassaemia patients develop complications related to unsuppressed ineffective erythropoiesis (IE). Serum markers of IE would be useful for risk stratification and monitoring treatment. We studied β-thalassaemia trait (β-TT) and non-transfusion-dependent β-thalassaemia (β-NTDT) patients. Serum erythropoietin (EPO) and soluble transferrin receptor (sTfR) were correlated against markers of clinical severity (haemoglobin, LDH, retics, bilirubin, spleen size) and iron overload (ferritin, hepcidin, and MRI-T2* in NTDT patients).

Eleven β-NTDT and nine β-TT subjects were studied. β-NTDT patients had significantly higher markers of haemolysis and iron overload. In β-NTDT, liver iron ranged from mild to severe, but no cardiac loading was seen. EPO and sTfR were higher in patients with β-NTDT than β-TT, and correlated significantly with each other (ρ=0.630, p=0.003). Both markers were negatively correlated with haemoglobin (sTfR ρ=-0.540, p=0.014; EPO ρ=-0.807, p<0.001, and positively correlated with spleen size (sTfR ρ=0.783, p<0.001; EPO ρ=0.654, p=0.002) and markers of iron overload. There was a strong correlation between ferritin and hepcidin (ρ=0.720, p<0.001), and a relatively lower increment of hepcidin for the degree of iron overload in β-NTDT compared to β-TT.

EPO and sTfR appear to be reliable markers of erythropoiesis in non-transfused β-thalassaemia and correlate well with markers of disease severity. Their role in managing patients, predicting complications, and monitoring response to treatments aimed at reducing IE should be explored.

KEYWORDS:
Non-transfusion-dependent thalassaemia, β-thalassaemia intermedia, ineffective erythropoiesis

INTRODUCTION
β-thalassaemia is due to mutations in the β-globin gene which cause a reduction or absence of the synthesis of the β-globin chains. There is a wide range of clinical presentation within the β-thalassaemia syndromes. Those with anaemia too severe to be considered as minor, but who may be transfused occasionally are termed non-transfusion-dependent β-thalassaemia, (β-NTDT).[1] The imbalance in α/β-globin chain synthesis leads to excess free α-globin chains which form toxic aggregates.[2,3] This leads to early destruction of erythroid precursors in the marrow and defective mature erythrocytes, which is termed 'ineffective erythropoiesis' (IE).[4,5]

The three important pathogenetic features of IE are: accelerated erythroid differentiation; maturation blockade or arrest; and early death of erythroid precursors.[2,6] These result in reduced production of mature, functioning erythrocytes. In response to the chronic anaemia, there is a dramatic rise in erythropoietin (EPO) production.[2] Unfortunately, the marrow can only respond to this EPO signal by increasing the ineffective expansion of the erythroid compartment in the marrow, which in turn leads to bone deformities, osteoporosis and extramedullary erythropoiesis.[6]

Erythropoiesis is closely linked to iron metabolism, especially to its absorption from the gut. Normally, serum hepcidin values rise in iron sufficiency/overload and this down-regulates gut iron absorption.[7] However, in a mouse model of β-thalassaemia, bone marrow factors such as erythroferrone and GDF-11 increase in erythroid hyperplastic states, and inappropriately suppress hepcidin production,[7,8] facilitating iron absorption even in states of plentiful body iron. Hence, slowly-progressive iron overload, may occur in β-NTDT patients, albeit at an older age compared to thalassaemia major patients who are regularly transfused.[3]

The serum level of soluble transferrin receptor (sTfR) has been investigated as a marker of IE. The rate of synthesis of this receptor is related to cellular iron demands.[9] Kohgo et al., showed that sTfR levels paralleled peripheral reticulocyte counts, reflecting turnover of transferrin receptors in marrow erythrocyte precursor cells.[9] In β-NTDT, sTfR levels are elevated, whilst in β-thalassaemia major patients who are well transfused the levels are near-normal indicating effective suppression of marrow erythropoiesis.[10,11]

As β-NTDTs are not transfusion dependent, they were relatively neglected until recent clinical studies revealed that there is a spectrum within this group.[12] Those at the severe end of the spectrum overlap with thalassaemia major patients, and some of the β-NTDT patients eventually go on to regular transfusion because of the effects of chronic anaemia. Even those β-NTDT patients of intermediate severity may develop serious clinical complications due to IE and iron overload, so they require regular monitoring and careful management.[12]

Although there have been many reviews and laboratory studies on IE,[2,13] clinical studies correlating biomarkers of IE with clinical severity parameters are few. It would be useful to have markers of IE by which to assess severity, predict complications, and monitor new approaches to therapy, in β-NTDT.

MATERIALS AND METHODS
Subjects
This cross-sectional study was conducted between June and December 2017. A search for suitable patients was made from the University Malaya hospital electronic patient record system and a personal database, and by identifying haemoglobin electrophoresis results in the Haematology Unit laboratory that fulfilled our inclusion criteria. Patients who were included in the study were aged 12 years and above, with either β-thalassaemia trait (TT), or β-NTDT confirmed by standard laboratory criteria, i.e. high performance liquid chromatography and gel electrophoresis demonstrating β-thalassaemia intermedia by having abnormalities of both β-globin genes (E/β0, E/β+ or β+/β+) or mild to moderate HbE/β thalassaemia as defined by the Mahidol score for HbE/β thalassaemia severity.[14] The β-NTDT cases had to have received less than three units of transfused red blood cells in the previous 12 months, with the last transfusion (if any) occurring more than three months prior to recruitment. Exclusion criteria included: other forms of thalassemia (e.g. α-thalassaemias such as HbH disease, HbH with Constant Spring); sickle cell disease; concurrent iron deficiency; use of iron chelators; splenectomy; the presence of other haematological conditions such as immune thrombocytopenic purpura or autoimmune haemolytic anaemia. All subjects gave written informed consent to take part in this study.

Parameters measured
Haemoglobin (Hb), haemolytic markers (i.e. lactate dehydrogenase (LDH), bilirubin, reticulocytes), serum erythropoietin (EPO), soluble transferrin receptor (sTfR) and ferritin, were measured. Venous blood samples for sTfR and hepcidin were obtained and collected in 2 separate plain plastic tubes without additives. The tubes were kept at room temperature for 2 hours to allow the blood to clot, after which they were centrifuged, and the serum was then separated and kept frozen at -20C until the time of assay. The Hepcidin 25 (bioactive) HS ELISA (DRG Diagnostics, USA) and the N Latex sTfR assay (Siemens Healthcare Diagnostics Products, Germany) kits were used for the study.

The maximum diameter of both the liver and the spleen were measured either by ultrasound (in β-TT) or MRI (in β-NTDT). Liver and cardiac iron content were estimated by standard MRI T2* analysis, but only in patients with β-NTDT.

Statistical methods
Baseline demographics and patient characteristics were analysed using descriptive statistics. Correlations were assessed using Spearman's correlation coefficients (ρ) and scatter plots: strong, fair and weak correlations were defined as ρ >0.60, 0.30-0.60 and <0.30 respectively. The p-values were considered significant when <0.05. All statistical analyses were performed using SPSS for windows (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). Approval was obtained from the Research Ethics Committee of the University Malaya Medical Centre prior to commencement of the study.

RESULTS
Baseline demographics and patient characteristics
Twenty-three patients were initially recruited. Three patients were excluded from the study (one for iron deficiency; one for autoimmune haemolysis; and one for concomitant α-thalassaemia trait). Results of 20 patients were analysed. Eleven patients had β-NTDT (five with β-thalassaemia intermedia and six with HbE/β-thalassaemia) and nine had β-TT. Age, gender and transfusion requirements were similar between these two groups (Table I).

Table II shows the results for the markers of haemolysis, iron overload and erythropoiesis. As expected, Hb was lower, and HbF was higher, in the β-NTDT group. Markers of iron overload (ferritin and hepcidin) and erythropoiesis (EPO and sTfR) were markedly higher in β-NTDT than in β-TT. Nine β-NTDT patients underwent T2* MRI (two refused): in terms of liver iron loading, five showed mild (T2*>7.2msec, <5mg Fe/g), two moderate (3.3-7.2msec, 5-10mg Fe/g), and two severe (2.2-3.3msec, 10-15mg Fe/g). No iron loading of the heart was seen in any of these patients. Median liver and spleen sizes were bigger in β-NTDT patients.

Relationships between anaemia and erythropoietic markers
Looking at the whole study population (i.e. both groups) together, as a spectrum of IE, there was a strong negative correlation between EPO and Hb levels (ρ=-0.807, p<0.001). sTfR and Hb showed a weak but significant negative correlation (ρ=-0.540, p=0.014). sTfR and EPO showed a strong positive correlation with each other (ρ=0.630, p=0.003) (Figure 1).

Relationships between clinical signs and erythropoietic markers
Strong, significant associations were noted between both markers of erythropoiesis, EPO and sTfR, and spleen size (ρ=0.654, p=0.002; and ρ=0.783, p<0.001, respectively). Moderate-to-strong correlations were also noted between liver size and both EPO and sTfR (ρ=0.574, p=0.008; and ρ=0.809, p<0.001, respectively).

Relationships between iron overload and erythropoietic markers
There was a significant positive correlation between hepcidin and ferritin (ρ=0.720, p<0.001). Although our data showed an increase in hepcidin with increasing ferritin, this is less pronounced in the NTDTs compared to the traits, as shown in Figure 2. EPO and sTfR strongly correlated with ferritin (EPO vs ferritin ρ=0.632, p=0.003; sTfR vs ferritin ρ=0.642, p=0.002). However the correlations of the two markers with hepcidin were weak and non-significant (EPO vs hepcidin ρ=0.167, p=0.482; sTfR vs hepcidin ρ=0.370, p=0.108).

DISCUSSION
Anaemia and markers of erythropoiesis
This is the first study comparing markers of erythropoiesis in

Table I: Patient demographics and characteristics

Characteristic	All (n=20)	β-TT (n=9)	β-NTDT (n=11)	p-value
Age, years.				
Median (range)	31.5 (14-66)	37 (14-66)	29 (16-62)	0.710
Ethnicity				
Malay	14 (70%)	4 (44.4%)	10 (90.9%)	0.024
Chinese	6 (30%)	5 (55.6%)	1 (9.1%)	
Gender				
Male	4 (20%)	1 (11.1%)	3 (27.3%)	0.369
Female	16 (80%)	8 (88.9%)	8 (72.7%)	
Ever Transfused?				
Yes	10 (50%)	3 (33.3%)*	7 (63.6%)	0.178
No	10 (50%)	6 (66.7%)	4 (36.4%)	

βTT: β-thalassaemia trait.
β-NTDT: non-transfusion-dependent β-thalassaemia.
*Isolated episodes of transfusion in β-TT: 2 during pregnancies and 1 during intercurrent illness.

Table II: Haemoglobin, and markers of haemolysis, erythropoiesis and iron overload

Parameter Median (range)	All subjects (n=20)	β-TT (n=9)	β-NTDT (n=11)	p-value (β-TT vs β-NTDT)
Hb (g/L)	91.0 (62.0-140.0)	104.0 (91.0-140.0)	80.0 (62.0-131.0)	0.004
RBC (x10^{12}/L)	5.1 (3.8-7.3)	5.2 (4.3-6.6)	4.5 (3.8-7.3)	0.295
MCV (fL)	61.5 (52.0-71.0)	65.0 (61.0-71.0)	65.0 (52.0-69.0)	0.002
MCH (pg)	19.3 (15.5-22.2)	20.4 (19.1-22.2)	17.9 (15.5-20.2)	0.001
MCHC (g/L)	312.5 (290.0-327.0)	315.0 (305.0-327.0)	308.0 (290-326)	0.230
HbF (%)	4.7 (0.4-43.7)	1.6 (0.4-12.3)	7.0 (1.8-43.7)	0.007
Reticulocyte count (x10^9/L)	132.1 (59.3-239.0)	95.9 (78.3-173.0)	163.0 (59.3-239.0)	0.031
LDH (U/L)	177 (121-564)	163 (128-207)	219 (121-564)	0.041
Total Bilirubin (μmol/L)	26 (8-65)	13 (8-49)	40 (18-65)	0.007
Hepcidin (ng/mL)	21.9 (4.9-76.1)	15.5 (4.9-38.1)	22.4 (12.2-76.1)	0.112
Ferritin (μg/L)	383.2 (25-4218)	101.7 (25.0-445.0)	495.5 (160-4218.0)	0.001
Erythropoietin (mg/L)	34.4 (5.0-97.7)	13.9 (5.0-45.0)	45.9 (14.5-97.7)	0.007
sTfR (mg/L)	3.7 (0.1-13.1)	1.8 (0.1-3.4)	5.8 (3.1-13.1)	<0.001
MRI T2*liver (msec)♯	9.5 (0.8-15.9)	Not performed	9.5 (0.8-15.9)	
Liver size (cm)	16.1 (11.0-23.5)	14.4 (11.0-16.0)	19.6 (15.4-23.5)	<0.001
Spleen size (cm)	11.6 (7.9-21.2)	10.0 (7.9-12.8)	16.2 (10.0-21.2)	0.001

(β-TT β-thalassemia trait, β-NTDT β-non-transfusion-dependent thalassemia,
Hb haemoglobin, RBC red blood cell count, MCV mean cell volume, MCH Mean corpuscular haemoglobin. MCHC mean corpuscular haemoglobin concentration,
HbF fetal haemoglobin, LDH lactate dehydrogenase, sTfR soluble transferrin receptor.)
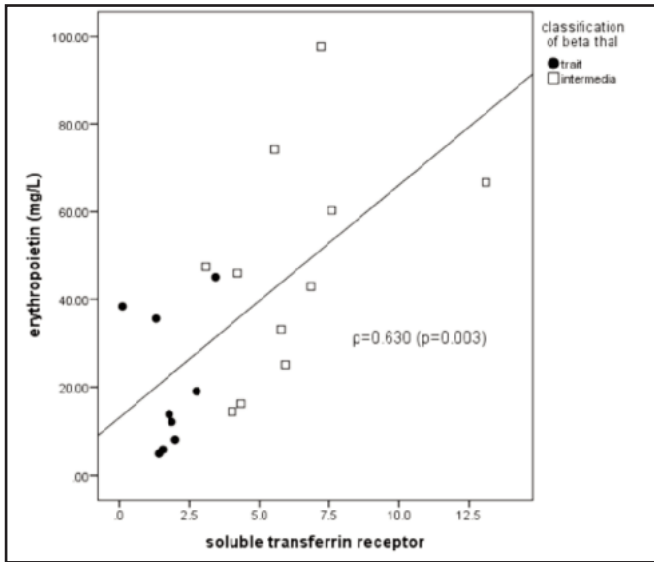♯ MRI T2* hepatic loading (n=9, as 2 patients refused to undergo MRI).
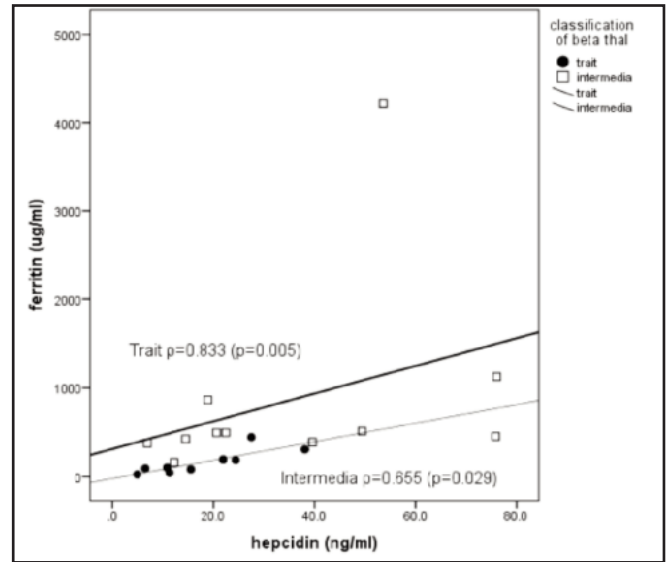
Fig. 1: Correlation between EPO and sTfR.

Fig. 2: Correlation between hepcidin and ferritin (for βTT and β-NTDT separately).

β-NTDT and β-TT patients. There have been studies performed on ineffective erythropoiesis in NTDTs, but generally with a mixture of β and α thalassaemia patients.[13] We made co-existing α-thalassaemia an exclusion criterion as the reduction of α chains might ameliorate the degree of ineffective erythropoiesis. Patients involved in our study had not had a blood transfusion during the preceding three months, and were not on iron chelators, eliminating the confounding effects these might have on erythropoiesis and markers of iron overload.

Anaemia was more pronounced in the NTDTs, but with some overlap in range with the traits. HbF levels were higher in the intermedias, as expected.[6] Markers of haemolysis (lactate dehydrogenase, reticulocyte count and total bilirubin) were higher in NTDTs reflecting the high red cell turnover.

Patients with β-NTDT had higher sTfR and EPO levels, reflecting the increased rate of (ineffective) erythropoiesis (Table II). Thalassaemic marrow may have five-to-six times the number of erythroid precursors compared to healthy marrow, but with a 15-fold increase in apoptotic rate.[15] Two major cytokines control erythropoiesis, stem cell factor (SCF) and erythropoietin (EPO).[2] EPO not only controls the rate of erythroid precursor proliferation, it prevents apoptosis. The negative correlation between EPO and haemoglobin in our study is consistent with EPO production responding to anaemia but failing to produce adequate red cell production (i.e., IE). The significant negative correlation between sTfR and Hb in our study is consistent with ineffective erythroid hyperplasia being proportional to the degree of anaemia, as found in previous studies.[13]

The response of thalassaemic marrow to EPO is insufficient, resulting in erythroid hyperplasia and expansion of the bone marrow without an adequate increase in production and release of mature erythrocytes into the peripheral blood.[4] Direct measurement of bone marrow expansion in thalassaemia (e.g., by radiological means) has not yet been

explored. However, it has been shown that sTfR strongly correlates with bone marrow expansion in β-thalassaemia intermedia.[16] Further evidence for this relationship is shown by the positive correlation between spleen and liver size with sTfR levels in our study.

Iron overload and erythropoiesis
Despite not having regular transfusions, NTDT patients are often found to have significant iron overload, especially in the liver.[6,12] Hepcidin is the main regulator of iron absorption and its level is usually increased in iron overload, downregulating iron absorption.[2] However, hepcidin is partially suppressed by ineffective erythropoiesis, allowing intestinal iron absorption and increased release of recycled iron from the reticuloendothelial system, causing clinical iron overload.[6,12]

In this study, we chose hepcidin and ferritin as biochemical markers for iron overload. A significant positive correlation was seen between the two markers, with a lesser degree of increase in hepcidin was seen in NTDT compared to TT (Figure 2). This is consistent with relative hepcidin suppression in NTDT which could contribute to an inappropriate failure to suppress iron absorption and hence, higher ferritin levels in NTDTs compared to traits.

Although EPO and sTfR correlated well with ferritin, the same degree of correlation was not found with hepcidin. Hepcidin levels respond to competing stimuli: they are increased in response to iron overload but reduced by mediators related to erythropoiesis such as erythroferrone and GDF-15.[18] The lower degree of correlation is likely due to hepcidin being an indirect marker of iron overload. Hepcidin has been shown to be better as a marker of iron deficiency rather than that of iron overload.[17]

It is important to note that iron overload is strongly influenced by transfusion history. It would be best if there had been a complete, lifelong transfusion record for our

patients. Unfortunately this was not available as these patients were transfused only intermittently and some had had transfusions at other hospitals.

The relationship between ineffective erythropoiesis and iron overload is further demonstrated by the positive correlation between both EPO and sTfR with iron loading as measured by serum ferritin. It is interesting to note that even in NTDTs there is a wide spectrum of severity of iron overload in the liver, ranging from mild to very severe. This lends further support to the heterogeneity of NTDT and the need to stratify them further, into different classes of severity.

Clinical signs and biomarkers of erythropoiesis
Expansion of erythropoiesis in the bone marrow is associated with localised bone deformities and osteoporosis, but it also causes extramedullary haematopoiesis, e.g. proliferation and homing of these precursors to the spleen and liver causing hepatosplenomegaly.[6] Haematopoietic tissue can expand further into other areas causing debilitating clinical consequences, for example paraplegia if this occurs in the spinal canal. Our study has shown that there is a correlation between both sTfR and EPO with both spleen and liver size. The stronger correlation with sTfR suggests it is the most reliable, currently available, marker of bone marrow expansion and extramedullary haematopoiesis. Further studies are ongoing to examine the relationship between these markers of erythropoiesis and clinical complications such as osteoporosis.

CONCLUSION
In conclusion, our study demonstrates a clear correlation of the markers of erythropoiesis, sTfR and EPO, with extramedullary haematopoiesis (as shown by hepatosplenomegaly), and with iron overload. There is potential to use these two markers in the clinical setting to identify β-NTDT patients at higher risk of complications. These findings merit further assessment in a larger prospective study to establish whether they could be used to risk stratify patients with β-NTDT. Such markers could also be used to monitor the effectiveness of different forms of treatment, especially those specifically targeted at reducing IE, such as the TGF-β ligand scavengers luspatercept and sotatercept.[18]

DISCLOSURE OF INTEREST
The authors declare no conflicts of interest.

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