**Hepcidin detects iron deficiency in Sri Lankan adolescents with a high burden of haemoglobinopathy: a diagnostic test accuracy study**

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**Abstract**

*Background:* Anaemia affects over 800 million women and children globally. Measurement of hepcidin as an index of iron status shows promise, but its diagnostic performance where haemoglobinopathies are prevalent is unclear. We evaluated the performance of hepcidin as a diagnostic test of iron deficiency in adolescents across Sri Lanka.

*Methods:* We selected 2273 samples from a nationally representative cross-sectional study of 7526 secondary schoolchildren across Sri Lanka. We analysed associations between hepcidin and participant characteristics, iron indices, inflammatory markers and haemoglobinopathy states. We evaluated the diagnostic accuracy of hepcidin as a test for iron deficiency with estimation of the AUCROC, sensitivity/ specificity at each hepcidin cutoff, and calculation of the Youden Index to find the optimal threshold.

*Findings:* Hepcidin was associated with ferritin, sTfR and haemoglobin. The AUCROC for hepcidin as a test of iron deficiency was 0.78; hepcidin outperformed Hb and sTfR. The Youden index-predicted cutoff to detect iron deficiency (3.2ng/mL) was similar to thresholds previously identified to predict iron utilisation and identify deficiency in African populations. Neither age, sex, nor α- or β-thalassaemia trait affected diagnostic properties of hepcidin. Hepcidin pre-screening would prevent most iron-replete thalassaemia carriers from receiving iron whilst still ensuring most iron deficient children were supplemented.

*Interpretation:* The physiological relationship between hepcidin and iron status transcends specific populations. Measurement of hepcidin in individuals or populations could establish the need for iron interventions.

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**Introduction**

About 800 million women and children are anaemic worldwide, with the prevalence highest in Asia and sub-Saharan Africa.1 Reducing the burden of anaemia is a core 2025 global nutrition target. Iron supplementation in anaemic schoolchildren improves IQ scores,2 while in women, iron supplementation reduces symptomatic fatigue and improves physical exercise performance.3 These benefits form the basis for the rationale for public health programmes aimed at improving iron stores where the burden of anaemia is high. For example, India has introduced a programme of weekly iron supplementation for over 100 million adolescent boys and girls.4 In India, however, the prevalence of anaemia among adolescents is 56-90%,5 and universal interventions seem appropriate.

Elsewhere, anaemia may be less prevalent, and importantly, may be attributable to causes other than iron deficiency. In many parts of Asia, carriage of genetic disorders of haemoglobin contribute to the overall burden of anaemia. For example, in Cambodia only a minority of anaemia in schoolchildren was attributable to iron deficiency, with haemoglobinopathy being the dominant cause.6 There are established concerns regarding safety of universal iron programs due to risks, demonstrated in children, of malaria7 and alterations in intestinal microbiota,8 and emerging concerns about risk of iron overload especially in populations at high risk of haemoglobinopathy. Targeting of iron to those who are iron deficient and in need of iron in these complex populations may enable safer and more effective programs.

Hepcidin is the peptide hormone that regulates iron homeostasis.9 Plasma hepcidin directly mediates iron absorption and utilisation from the intestine, and is upregulated by iron stores and inflammation and suppressed by iron deficiency and increased erythropoiesis.10 Increased iron absorption due to hepcidin suppression in iron deficiency helps to overcome iron deficiency, but in patients with pathological hepcidin suppression, for example in thalassaemia, excess iron absorption results in iron loading. Hepcidin may be marginally suppressed in carriers of thalassaemia,11 resulting in increased iron absorption in this group and perhaps exposing them to risks of iron overload when subjected to prolonged iron supplementation.12

As the direct mediator of iron absorption, hepcidin has been evaluated as a potential indicator of iron status and iron absorption.13 Hepcidin predicts iron absorption in children and adults, has been shown to select a subgroup of African children whom might benefit from iron supplementation,14 and predicts iron deficiency in blood donors15 and in patients with complex medical conditions such as rheumatoid arthritis16. Hepcidin could provide a useful tool to screen individuals in populations where, while anaemia still presents an important public health problem, the prevalence may not be high enough to warrant universal interventions, or where multiple factors (for example thalassaemia carriage) contribute to the overall burden of anaemia.

Anaemia in Sri Lankan adolescents is a complex public health problem. The prevalence has been estimated to be about 20%;5 as such, this group may be a candidate for long term weekly iron supplementation. However, the Sri Lankan population also has a high concomitant burden of genetic haemoglobinopathy, including carriage of α-globin deletions (especially α-3.7), and ß-globin mutations (ß-thalassaemia and Haemoglobin-E).17 This complicates iron supplementation policies as iron would only benefit the minority with iron deficiency anaemia, provide no benefit and perhaps harm from increased risk of infection and gastrointestinal effects in the majority, and potentially promote iron overload in those who carry thalassaemia. This represents an important context to evaluate the diagnostic value of hepcidin as an index of iron deficiency and to consider any potential role in guiding iron interventions.

 In a nationally representative study of Sri Lankan secondary school children we collected data from a sample enriched for individuals at risk of iron deficiency or thalassaemia carriage. We investigated the relationship between hepcidin and iron status, with interactions for thalassaemia, then evaluated the properties of hepcidin as a diagnostic test of iron deficiency in this population and identified the optimal diagnostic threshold for hepcidin.

**Methods**

*Study design*

We obtained samples from a nationally representative cross-sectional study designed to assess the frequency and distribution of haemoglobin variants across Sri Lanka. From each of 25 districts we purposefully selected approximately three schools (72 in total, including 5 temporary schools for displaced students from the North) that were geographically spaced and representative of the population.18

*Laboratory Measurements*

We collected a 5mL blood sample from each student and transferred it into EDTA and plain tubes. We measured Hb and red cell indices (Coulter Counter, Beckman Coulter UK) and identified Hb variants by High Performance Liquid Chromatography using the ß-thalassaemia short program (BioRad, India). DNA was tested for α-globin gene deletions (3.7 and 4.2) by multiplex PCR.19 We measured serum ferritin and transferrin receptor (sTfR) by ELISA (IBL International and R&D systems, respectively), and inflammation (hsCRP, Architect C8000, Abbott Laboratories). The sTfR-ferritin (sTfR-F) index was calculated as sTfR/log10(ferritin).

We quantified serum hepcidin by competitive ELISA (hepcidin-25 human bioactive ELISA and hepcidin-25 High Sensitivity ELISA, DRG). The high sensitivity ELISA was introduced by DRG during the course of the study; we calculated an adjustment factor to ensure comparability based on data provided by DRG. We validated the adjustment by ensuring mean hepcidin levels were not different between control groups assayed using both kits, in whom mean ferritin, sTfR and CRP were also similar. The lower limit of detection (LOD), estimated as being the hepcidin value corresponding to 3SDs below the mean 0ng/mL hepcidin standard OD450, was calculated to be 0.78ng/mL. Samples that gave readings less than the LOD were reported as LOD/2 (0.39ng/mL). Inter-plate coefficients of variation (CV%) for the mean OD450nm on the ‘old’ DRG kit: high and low concentration controls (provided by kit) were 13.8 and 12.2 respectively (n=56). Inter-plate CV% for the mean OD450nm on the new HS-DRG kit for high and low controls (included with the assay) were 9.0% and 10.6% respectively (n=9).

Twenty-eight additional samples were also run in parallel using the DRG hepcidin-25 bioactive ELISA and the Bachem Hepcidin-25 (human) Enzyme Immunoassay (run as previously described)14 to allow comparison of results between methods.

*Statistics*

Data were analysed using Stata 11 (StataCorp., College Station, TX, USA). Hepcidin, ferritin, sTfR, CRP and sTfR-F index data were skewed and variables were log10 transformed for analysis. Arithmetic or geometric means were calculated where appropriate along with the 2.5th to 97.5th centile range.

Using linear regression, we estimated associations between hepcidin, participant characteristics, inflammatory markers and iron indices. Statistical significance was defined as P<0.05 and β coefficients were estimated by analysis of variables standardised such that their variances were 1. We performed stepwise multiple regression, and retained only those variables with P<0.05. Regression diagnostics were performed and the optimal model selected.

We used a gold standard definition of iron deficiency, termed “combined definition”, previously deployed in sub-Saharan African children: serum ferritin<15ng/mL, or <30ng/mL in the presence of CRP>5mg/L, and sTfR/log10ferritin (sTfR-F) index>2.14 This definition combines the current WHO definition of iron deficiency using ferritin (adjusted for inflammation),20 along with cutoffs of the sTfR-F index.,21 This ‘combined definition’ ensures that individuals with ferritin concentrations between 15 and 30ng/mL and inflammation are only considered iron deficient if they also demonstrate tissue iron depletion.14 In order to permit comparison of sTfR with hepcidin, we used a second gold standard: ‘ferritin alone’, reflecting current WHO guidelines: ferritin<15ng/mL, or <30ng/mL in the presence of inflammation (CRP>5mg/L).20

Receiver operating characteristic (ROC) curves were graphed and area under the curve (AUCROC) values calculated. We compared the AUCROC of hepcidin to detect iron deficiency between individuals with/ without carriage of haemoglobinopathy. Sensitivities and specificities were calculated for each cutoff of hepcidin and the Youden Index [(sensitivity + specificity) – 1] estimated. The ROC curves and Youden Index were inspected to determine the point at which the sensitivity and specificity were both maximised.

*Ethics*

Approval for this research program was obtained from the Ethical Committee of the University of Kelaniya, Colombo, Sri Lanka; and the Oxford Tropical Research Ethical Committee, Oxford, United Kingdom. Permission for enrolment into the study was obtained from each of the schools and families involved.

*Role of the funding source*

The funding source had no role in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the paper for publication. The corresponding authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

**Results**

From a total of 7526 students we analysed samples from 2273 children aged 12-19 years: 587 with normal red cell indices and 1686 with low red cell indices (MCV<80fL and/or MCH<27pg), enriching our dataset for haemoglobinopathies. The flow of participants is detailed in Figure 1.

Participant demographics and iron indices are summarised in Table 1. The prevalence of anaemia within this selected study group was 19.8%, and using the combined definition iron deficiency was seen in 19.2% and was more prevalent in girls (27.6%) than boys (10.4%) (P<0.001). Only 3.5% of children had inflammation as defined by CRP>5ng/mL. Geometric mean hepcidin for the overall group was 3.86ng/mL [95% confidence interval 1.01-16.39]; in iron replete individuals it was 4.44ng/mL [4.30-4.59] compared with 2.27ng/mL [2.14-2.40] in iron deficient children (P<0.001) and 2.81ng/mL [2.62-3.01] in anaemic children.

Of our sample, 5.2% had β-thalassaemia trait, 21.6% α+ thalassaemia trait (heterozygous), 0.9% were α+ homozygous or compound heterozygous thalassaemia, and 1.2% HbE trait. Hepcidin was suppressed in non-iron deficient β-thalassaemia trait carriers (4.1ng/mL) compared with non-iron deficient children without haemoglobinopathy (5.2ng/mL, P<0.001) (Supplementary Table 1). This effect was also seen in the non-iron deficient α-thalassaemia carriers compared to the non-iron deficient samples without haemoglobinopathy (4.8ng/mL vs 5.3ng/mL, P=0.02) (Supplementary Figure 1).

*Associations with hepcidin*

We evaluated associations of hepcidin with demographic characteristics and erythropoietic, iron, and inflammatory indices (Table 2). By univariate linear regression, hepcidin was associated with age, sex, Hb, ferritin, CRP, sTfR and sTfR-F index. By stepwise multiple regression analysis, hepcidin was associated with ferritin, sTfR, Hb and CRP with an adjusted R2=0.259 (Table 2). Hepcidin was not associated with age, sex or carriage of haemoglobinopathy after adjustment, indicating that these factors mediate their effect on hepcidin via iron stores and erythropoiesis.

*Hepcidin as a diagnostic test of iron deficiency*

We generated ROC curves and measured the AUCROC for hepcidin as a test for iron deficiency with the combined definition (Figure 2A). The AUCROC was 0.78 [0.76-0.81]. We next compared the performance of hepcidin as a diagnostic test of iron deficiency to other indices of iron status (defined by the ‘ferritin alone’). Hepcidin (AUCROC 0.75 [0.73-0.77]) performed significantly better than either Hb (AUCROC 0.68 [0.65-0.70], p<0.001) or sTfR (AUCROC 0.66 [0.64-0.69], P<0.001) (Figure 2B). The diagnostic test accuracy of hepcidin was not different when AUCROC were compared by sex, or anaemia or carriage of ß-thalassaemia, α-thalassaemia and HbE (Figure 2C) (Table 3).

We next estimated the diagnostic properties of several potential hepcidin thresholds (Table 4). Hepcidin<3.2ng/mL achieved the maximum Youden index (0.46), and correctly classified 71.3% of samples, with a sensitivity of 75.6% and a specificity of 70.2%. Alternative thresholds could be selected which yielded different sensitivity and specificity (Table 4). At the same hepcidin cutoff, 72.1% of the β-thalassaemia carriers were correctly classified, with a sensitivity of 76.2% and specificity of 71.3%.

To compare this threshold to previous data, we analysed the hepcidin concentrations of twenty-eight samples using both the DRG and Bachem assays (Supplementary Figure 2). Although absolute hepcidin values differ between the two assays, there was a linear correlation (R2=0.92) between results returned by the two assays. We previously identified an optimal hepcidin threshold, using the Bachem assay, of 5.5ng/mL (based on the Youden index) in African pre-school children.14 This threshold corresponds to a hepcidin value of 3.1ng/mL with the DRG assay, which is similar to the cutoff identified in the Sri Lankan population.

Finally, we sought to establish the clinical implications if iron treatment was stratified in this population using hepcidin, compared with universal distribution of iron or distribution predicated on detection of anaemia. As shown in Table 5, provision of iron only to individuals with hepcidin<3.2ng/mL prevented about 60% of individuals from receiving iron whilst ensuring at least three quarters of iron deficient individuals received iron. Importantly, if Hb screening alone had been undertaken, directing iron on the basis of anaemia would result in two thirds of iron-replete ß-thalassaemia carriers (in whom iron supplementation may result in loading) receiving iron, whereas directing iron based on hepcidin could reduce this to only 29% of carriers.

**Discussion**

In a large cross-sectional study of adolescents across Sri Lanka, we assessed regulation of serum hepcidin concentrations and the value of this biomarker as an index of iron deficiency. At the population level, associations with hepcidin represent known biologic regulation of the gene by iron, erythropoiesis and inflammation. Performance of hepcidin as an index of iron deficiency in this population was not perturbed by carriage of thalassaemia. Importantly, the threshold for hepcidin we identified was similar to previously reported cutoffs identified in studies in Africa and elsewhere in Asia.

Our data confirm at the population level the known mechanistic regulation of hepcidin, which thereby controls iron absorption and recycling in a manner that represents the net output of multiple integrated signals.22 Population level associations between hepcidin and iron stores and inflammation have likewise been demonstrated in developed contexts.23 As the direct mediator of iron absorption and recycling, hepcidin provides direct insight into iron physiology, particularly utilisation of oral iron. Hepcidin can be used to predict iron uptake and utilisation,24,25 as well as to distinguish iron deficient children within a population with a high burden of anaemia and mixed iron deficiency and inflammation/infection.14

This is the largest diagnostic test accuracy study of hepcidin yet performed, measuring hepcidin in almost 2300 children along with gold standards and potential inflammatory and genetic influences on hepcidin levels. Several studies have previously evaluated the performance of hepcidin as an index of iron deficiency, in both population and clinical contexts. Optimal thresholds for hepcidin to detect iron deficiency appear similar across different populations and study designs, indicating biologic suppression of hepcidin in iron deficiency.14,26 Current work is in progress to harmonise hepcidin measurements across the range of different kits and platforms presently available.27 A threshold commutable to the value identified in this study is therefore likely to be an appropriate candidate for diagnosis of iron deficiency.

Compared with studies in Africa, we found a lower AUCROC for hepcidin to diagnose iron deficiency in this study. Unlike the African population, anaemia and inflammation were uncommon in this study; as such, distinction in hepcidin between cases with iron deficiency (which produces hepcidin suppression) and cases with inflammation and iron loading (with elevations in hepcidin) may have been less discrete. The prevalence of iron deficiency based on our definition was relatively low in this study; this is reflected here by low positive predictive value for low hepcidin levels, which is typically the case when evaluating tests in populations where the disease of interest is uncommon. Hepcidin was a more accurate diagnostic test for iron deficiency than haemoglobin, as we also observed in Africa. This reflects an emerging realisation that in the public health, anaemia and iron deficiency are not synonymous, and often only a minority of cases of anaemia are attributable to iron deficiency;1 the corollary is that many cases of iron deficiency exist without overt anaemia. These data indicate that haemoglobin levels should not be used alone to identify iron deficiency.

Carriers of thalassaemia have mildly increased erythropoiesis (reflected by elevations in sTfR without low ferritin) due to modest ineffective erythropoiesis with an imbalance of globin chains. Elevations in erythropoiesis and mild anaemia likely cause an increase in the expression of the bone-marrow derived hormone erythroferrone, which acts to suppress expression of hepcidin.11 This represents a milder phenotype to the condition seen in homozygote or compound heterozygote thalassaemia conditions.28 We were concerned that suppression of hepcidin in patients carrying thalassaemia could impair its utility to detect iron deficiency in the same population. This distinction is important as carriers of thalassaemia may have increased iron absorption due hepcidin suppression. Reassuringly, our data indicated that the AUCROC for hepcidin to detect iron deficiency was not different between thalassaemia carriers and controls, and the optima hepcidinl threshold was similar in carriers and non-carriers. Furthermore, unlike haemoglobin, hepcidin measurement can help direct iron away from iron-replete thalassaemia carriers for whom iron supplementation may be harmful.

 Guided by the Youden Index, a hepcidin cutoff of 3.2ng/mL had the highest simultaneous specificity and sensitivity. However, this threshold represents a tradeoff between sensitivity and specificity and selection of the appropriate cutoff ultimately depends on clinical or public health scenario to which it will be applied, based on the risk of misclassifying an iron replete individual as iron deficient or vice versa. In this study, our reference (gold standard) was a combination of ferritin and soluble transferrin receptor. These assays reflect bone marrow iron stores and tissue iron need. Although, thresholds used to define iron deficiency with these indices remain unclear and subject to ongoing re-consideration,29 it would not have been feasible to measure bone marrow iron in this large cross-sectional population study. The definitions of iron deficiency we used here are widely accepted and have been previously deployed in studies evaluating hepcidin as a diagnostic test.

 In Sri Lankan adolescent children, the prevalence of anaemia was below the threshold for which a routine universal iron intervention programme (for example daily or weekly iron supplementation) would be considered. Indeed, because this study was enriched with patients likely to carry haemoglobinopathies, the population prevalence of anaemia and iron deficiency in Sri Lanka is even lower still. Importantly, as anaemia is a poor index of iron status, it is also a poor guide for appropriate allocation of iron interventions in this setting. Strategies for optimally directing iron interventions should ensure iron is preferentially distributed to children whom need it but not to those who are iron replete (or infected) and who would not benefit or indeed in whom it might be harmful. Measurement of hepcidin may help classify individuals as ‘ready to receive iron’ for optimal stratification in iron distribution programs.

**Panel: Research in Context**

*Evidence before this study*

We searched Pubmed on July 30th 2016 for articles that evaluated hepcidin as an index of iron deficiency (i.e. reporting AUCROC, sensitivity/ specificity/ positive or negative predictive value/ likelihood ratio) in a population or outpatient setting, using the following search: hepcidin AND (iron/deficiency [MeSH] OR "iron deficiency" [TIAB] OR diagnostic test [MeSH] OR diagnosis [TIAB] OR sensitivity and specificity [MeSH]). Of 840 titles retrieved, 6 studies recruiting a total of 1853 patients were identified.

Choi *et al* (n=59) reported an AUCROC=0.85 for hepcidin to detect iron deficiency in Korean children 5 months to 17 years; a cutoff of ≤6.895ng/mL (using the Bachem assay) had a sensitivity of 79.2% and specificity of 82.8%.26 In pre-selected Egyptian children (n=100), urinary hepcidin had an AUCROC of 0.84, 0.94 and 1.00 to detect mild and moderate ID, and IDA, respectively.30 In non-anaemic female blood donors had an AUCROC of 0.89 to detect iron deficiency (n=261).15 Wolff et al studied healthy adult volunteers (n=33) and found that plasma and urine hepcidin had an AUCROC to detect iron deficiency (defined as ferritin<30ug/L) of 0.94 for plasma and AUCROC =0.93.31 Jonker *et al* compared hepcidin with bone marrow iron stores in 87 non-inflamed Malawian children undergoing elective surgery, and found that ferritin and sTfR outperformed hepcidin as an index of iron deficiency. Utilising similar gold standard definitions of iron deficiency, we studied 1313 Gambian and Tanzanian pre-school children, and observed that the AUCROC for hepcidin to identify iron deficiency was 0.85, with a threshold using the Bachem ELISA of 5.5ng/mL to distinguish iron deficiency across the overall population and among anaemic children.9,26

*Added value of this study*

In a large survey of Sri Lanka children we have measured hepcidin in more subjects than the total of previous studies combined. Ours is the largest diagnostic test accuracy study for hepcidin as a test of iron deficiency and the first such study in South Asia, and the first in a setting where haemoglobinopathy is endemic. We found that hepcidin can reliably detect iron deficiency and potentially prevent carriers of thalassaemia from receiving iron.

*Implications of all the available evidence*

Congruence between our findings and previous data suggest hepcidin measurement will be of value in testing for iron deficiency in both developed- and developing-world settings, and indicate potential thresholds to be used as cutoffs.

**Contributors**

DW, HD, SP, AMP and AP conceived the study. AP, LP, RR, GG and LR undertook the fieldwork. KW, AA, AEA, CF, EE and CW performed laboratory analysis. KW, SP, AA, DW and HD analysed data. KW, SP, HD drafted the manuscript. All authors contributed to interpretation of the data and approved the final manuscript.

**Declaration of interests**

We declare no competing interests

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**Table 1. Summary of patient characteristics and iron indices.**

|  |  |
| --- | --- |
| **Variable** |  |
| **Age, Mean [2.5, 97.5 centile] (yrs)** | 15.9 [14, 19] |
| **Female, n/N (%)** | 1166/2273 (51.2) |
| **Haemoglobinopathy** |
| **β-thalassaemia trait, n/N (%)** | 118/2263 (5.2) |
| **α-thalassaemia trait, n/N (%)** | 474/2198 (21.6) |
| **α-homozygous or compound heterozygous thalassaemia, n/N (%)** | 20/2198 (0.9) |
| **HbE, n/N (%)** | 28/2273 (1.2) |
| **Iron parameters** |
| **Variable** | **Mean [2.5, 97.5 centile]** | **Abnormal n/N (%)2**  |
| **Hepcidin (ng/mL)** | 3.861 [1.01, 16.39] |  |
| **Ferritin (ng/mL)** | 24.11 [2.76, 106.57] | 585/2210 (26.5) |
| **CRP (mg/L)** | 0.421 [0.07, 7.12] | 74/2117 (3.5) |
| **Hb (g/dL)** | 13.58 [10.4, 17.1] | 439/2217 (19.8) |
| **sTfR (mg/L)** | 1.991 [1.04, 5.13] | 884/2255 (39.4) |
| **sTfR-F index** | 1.521 [0.63, 6.29] | 563/2203 (25.6) |
| **Iron Deficiency (combined definition)3, n/N (%)** |  | 422/2203 (19.2) |

1Geometric mean

2 Ferritin<15ng/mL; CRP>5mg/L; Hb<12g/dL in girls & in boys 12-14 yrs, Hb<13g/dL in boys 14-19 yrs; sTfR>2.074mg/L; sTfR-F>2

3 Ferritin<15ng/mL, or ferritin<30ng/mL if CRP>5mg/L, & sTfR-F>2

**Table 2. Associations between hepcidin and participant characteristics and iron indices**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Variable** | **Regression Coefficient** | **95% CI** | **P**  | **β coefficient1** |
| **Univariate linear regression for log10(hepcidin)** |
| **Age (yrs)** | 0.048 | 0.025, 0.070 | <0.001 | 0.090 |
| **Sex2** | -0.24 | -0.30, -0.18 | <0.001  | -0.16  |
| **Hb (g/dL)** | 0.13 | 0.11, 0.15 | <0.001  | 0.30  |
| **log10(ferritin)** | 0.37 | 0.34, 0.40 | <0.001  | 0.46  |
| **log10(sTfR)** | -0.50 | -0.58, -0.43 | <0.001  | -0.27  |
| **log10(sTfR-F)** | -0.52 | -0.56, -0.47 | <0.001  | -0.43  |
| **log10(CRP)**  | 0.15 | 0.12, 0.18 | <0.001  | 0.23  |
| **β-thalassaemia trait**  | -0.02 | -0.16, 0.12 | 0.76  | -0.006  |
| **HbE trait**  | 0.15 | -0.12, 0.43 | 0.27 | 0.023  |
| **α-thalassaemia trait**  | 0.12 | 0.044, 0.19 | 0.002 | 0.066  |
| **α-homozygous or compound heterozygous thalassaemia** | 0.49 | 0.16, 0.81 | 0.003 | 0.063  |
| **Multiple linear regression model for log10(hepcidin)** |
| **Log(ferritin)**  | 0.27 | 0.24, 0.30 | <0.001  | 0.34  |
| **Log(sTfR)**  | -0.25 | -0.32, -0.17 | <0.001  | -0.13  |
| **Hb (g/dL)** | 0.05 | 0.04, 0.07 | <0.001  | 0.13  |
| **Log(CRP)**  | 0.10 | 0.07, 0.12 | <0.001  | 0.15 |

All variables were included in the multiple regression analysis. Only variables with P<0.05 were included in the final fitted model.

1Variables standardised to have a variance of 1, allowing comparison of regression coefficients between variables.

2 Coded variable: Male = 0; Female = 1

**Table 3. Area under ROC curves comparing performance of hepcidin as an index of iron deficiency in children of different sex, anaemia or haemoglobinopathy status**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Condition  | Observations  | AUCROC | 95% CI | P  |
| No β-thalassaemia trait | 2078  | 0.79 | 0.76, 0.81 | 0.90 |
| β-thalassaemia trait  | 115  | 0.78 | 0.65, 0.90 |
| No α-thalassaemia1 | 1648  | 0.78 | 0.76, 0.81 | 0.78  |
| α-thalassaemia | 482  | 0.77 | 0.71, 0.84 |
| No haemoglobinopathy2 | 1543  | 0.78 | 0.76, 0.81 | 0.58  |
| All haemoglobinopathy | 583  | 0.77 | 0.71, 0.82 |
| Male  | 1075  | 0.76 | 0.72, 0.81 | 0.77 |
| Female  | 1114  | 0.77 | 0.74, 0.80 |
| Non-anaemic3  | 1732  | 0.77 | 0.74, 0.80 | 0.50  |
| Anaemic  | 415  | 0.75 | 0.71, 0.80 |

1 3.7 heterozygous (n=416), 4.2 heterozygous (n=46), 3.7 homozygous (n=14), compound heterozygous (n=6)

2 β-thalassaemia trait (n=115), α-thalassaemia (n as above1), HbE trait (n=26), S thalassaemia trait (n=5)

3 Hb<12g/dL in girls & in boys 12-14 yrs, Hb<13g/dL in boys 14-19 yrs

**Table 4. Properties of different cutoffs of hepcidin as a diagnostic test of iron deficiency in this population**1

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Cutoff (ng/mL) | Sensitivity | 95% CI | Specificity | 95% CI | Positive predictive value | 95% CI | Negative predictive value | 95% CI | Positive likelihood ratio (%) | Negative likelihood ratio (%) | Correctly classified (%) | Youden index  |
| <2.0 | 43.4  | 38.6, 48.2 | 88.7 | 87.2, 90.1 | 47.7 | 42.6, 52.8 | 86.9 | 85.2, 88.4 | 3.84 | 0.64 | 80.0 | 0.320 |
| <2.5 | 60.0  | 55.1, 64.7 | 81.4  | 79.5, 83.1 | 43.2  | 39.2, 47.4 | 89.6  | 88.0, 91.0 | 3.22 | 0.49 | 77.3 | 0.413 |
| <3.0 | 72.3  | 67.7, 76.5 | 72.9  | 70.8, 75.0 | 38.8  | 35.3, 42.3 | 91.7 | 90.2, 93.1 | 2.67 | 0.38 | 72.8 | 0.452 |
| <3.12 | 74.2  | 69.7, 78.3 | 71.5  | 69.4, 73.6 | 38.2  | 34.8, 41.6 | 92.1  | 90.6, 93.5 | 2.61 | 0.36 | 72.0 | 0.458 |
| <3.2 | 75.6 | 71.2, 79.6 | 70.2  | 68.1, 72.4 | 37.6  | 34.3, 40.9 | 92.4  | 90.8, 93.7 | 2.54 | 0.35 | 71.3 | 0.459 |
| <3.3 | 77.0  | 72.7, 80.9 | 68.6  | 66.3, 70.7 | 36.7  | 33.5, 40.0 | 92.6  | 91.1, 94.0 | 2.45 | 0.34 | 70.2 | 0.453 |
| <3.5 | 79.1  | 75.0, 82.9 | 65.4  | 63.2, 67.6 | 35.2  | 32.1, 38.3 | 93.0  | 91.4, 94.3 | 2.29 | 0.32 | 68.0 | 0.446 |
| <4.0 | 84.6  | 80.8, 87.9 | 57.6  | 55.2, 59.9 | 32.1  | 29.3 34.9 | 94.0  | 92.5, 95.4 | 1.99 | 0.27 | 62.7 | 0.421 |
| <4.5 | 87.9  | 84.4, 90.9 | 49.9 | 47.5, 52.2 | 29.4  | 26.9, 31.9 | 94.6  | 92.9, 95.9 | 1.75 | 0.24 | 57.1 | 0.378 |

1 Iron deficiency defined as ferritin<15ng/mL, or <30ng/mL if CRP>5mg/L, and sTfR-F>2

2 A hepcidin concentration of 3.1ng/mL reported by the DRG hepcidin 25 (bioactive) ELISA corresponds to a hepcidin concentration of 5.5ng/mL measured using the Bachem hepcidin-25 (human) EIA kit (see Supplemental Figure 2)

Sensitivity (correctly identified positives), Specificity (correctly identified negatives), Positive predictive value (proportion of true positives), Negative predictive value (proportion of true negatives), Positive likelihood ratio (sensitivity / (specificity – 1), Negative likelihood ratio ((1-sensitivity) / specificity) and Youden index (sensitivity + specificity – 1) are shown. The maximum Youden index was 0.463 at a hepcidin cutoff of 3.24ng/mL.

**Table 5: Proportions of study population subgroups receiving iron based on policies of iron supplementation to all children, to anaemic children or to children with low hepcidin levels**

|  |  |  |  |
| --- | --- | --- | --- |
| **Subgroup of study population** | **Universal iron distribution** | **Iron administered if anaemic 2** | **Iron administered if** **hepcidin<3.2ng/mL** |
| Overall population | 100% | 439/2217 (19.8%) | 899/2273 (39.6%) |
| Iron deficient1 | 100% | 148/413 (35.8%) | 319/422 (75.6%) |
| Non-iron deficient1 | 100% | 267/1734 (15.4%) | 530/1781 (29.8%) |
| ß-thalassaemia trait | 100% | 74/105 (70.5%) | 45/118 (38.1%) |
| α-thalassaemia trait | 100% | 87/492 (17.7%) | 170/494 (34.4%) |
| ß-trait - iron replete | 100% | 55/82 (67.0%) | 27/94 (28.7%) |
| α-thalassaemia trait - iron replete  | 100% | 56/417 (13.4%) | 119/419 (28.4%) |
| ß-thalassaemia trait - Iron deficient 1 | 100% | 16/20 (80.0%) | 16/21 (76.2%) |
| α-thalassaemia trait - Iron deficient1 | 100% | 25/63 (40.0%) | 43/63 (68.3%) |

1Ferritin<15ng/mL, or ferritin<30ng/mL if CRP>5mg/L, & sTfR-F>2

2Hb<12g/dL in girls & in boys 12-14 yrs, Hb<13g/dL in boys 14-19 yrs

**Figure Legend**

**Figure 1. Flow of participants through the study**

**Figure 2. ROC curves for hepcidin to identify iron deficiency**

(**A**) Hepcidin compared to ferritin<15ng/mL, or <30ng/mL if CRP>5mg/L, and sTfR-F index>2 (AUCROC 0.78) (n=2203) (**B**) using ferritin<15ng/mL as the gold standard, comparing hepcidin (AUCROC 0.75) to sTfR (AUCROC 0.66, P<0.001) and Hb (AUCROC 0.68, P<0.001) (n=2149) (**C**) ROC curve comparing the performance of hepcidin at distinguishing iron deficiency in samples without α- or β-thalassaemia trait (AUCROC 0.79, n=1620) with samples with α- (AUCROC 0.77, n=468) and with β-thalassaemia trait (AUCROC 0.75, n=101) (not significant between any group). (**D**) Youden indices [(sensitivity + specificity)-1] at each cutoff of hepcidin. (Inset) Youden indices in the range of 1.8 to 4.5ng/mL. The maximal Youden index was 0.463 and occurred at a hepcidin cut-off of 3.24ng/mL (sensitivity, 76.8%; specificity, 69.6%).

ROC = Receiver Operating Characteristic

**Supplementary Material**

**Supplementary Table 1. Iron indices in non-iron deficient2 samples with and without β-thalassaemia trait, and with and without α-thalassaemia trait.**

|  |  |  |
| --- | --- | --- |
| Variable | β-thalassaemia | α-thalassaemia |
| **Without****n=514** | **2.5, 97.5 centile** | **With****n=92** | **2.5, 97.5 centile** | **P**  | **Without****n=478** | **2.5, 97.5 centile** | **With****n=419** | **2.5, 97.5 centile** | **P** |
| Hepcidin1  | 5.2 | 1.9, 16.1 | 4.1 | 1.5, 16.6 | <0.001 | 5.3 | 1.9, 15.3 | 4.8 | 1.1, 22.3 | 0.02 |
| Ferritin1  | 36.9 | 10.4, 144.7 | 37.4 | 13.3, 168.0 | 0.86  | 36.7 | 10.4, 144.5 | 33.2 | 10.3, 105.6 | 0.02 |
| sTfR1  | 1.8 | 1.0, 5.3 | 2.6 | 1.4, 5.7 | <0.001  | 1.8 | 1.0, 5.1 | 1.7 | 1.1, 3.3 | 0.008 |
| sTfR-F1  | 1.2 | 0.6, 3.0 | 1.7 | 0.8, 3.8 | <0.001  | 1.2 | 0.6, 3.0 | 1.2 | 0.6, 2.2 | 0.4 |
| Hb (g/dL) | 14.7 | 12.2, 18.0 | 11.9 | 9.9, 16.6 | <0.001  | 14.7 | 12.2, 18.0 | 13.8 | 11.2, 16.8 | <0.001 |
| Hep:ferritin1  | 0.14 | 0.05, 0.6 | 0.11 | 0.03, 0.4 | 0.0011  | 0.14 | 0.05, 0.6 | 0.14 | 0.3, 0.7 | 0.97 |

1 Geometric mean

2 ID defined as ferritin<15ng/mL, or <30ng/mL if CRP>5mg/l, and sTfR-F>2

**Supplementary Figure Legend**

**Supplementary Figure 1.** Hepcidin was significantly lower in both non-iron deficient β-thalassaemia samples (4.1ng/mL, n=92, p<0.001) and non-iron deficient α-thalassaemia samples (4.8ng/mL, n=419, p=0.02) compared to non-iron deficient samples without haemoglobinopathy (5.2ng/mL, n=514). Red lines represent the geometric mean and 95% CI.

**Supplementary Figure 2.** Correlation between hepcidin concentrations measured using the DRG hepcidin-25 (bioactive) ELISA and Bachem hepcidin-25 (human) EIA kit. R2=0.92, coefficient 0.266 [95% CI 0.235-0.297]. A hepcidin concentration of 5.5ng/mL measured using the Bachem assay is equivalent to a hepcidin concentration of 3.1ng/mL measured using the DRG assay.