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Hemoglobin and hepcidin have good validity and utility for diagnosing iron deficiency anemia among pregnant women.

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Abstract

Background—Screening and diagnosis of iron deficiency anemia (IDA) is cumbersome as it may require testing for hemoglobin, ferritin, and an inflammatory biomarker.

Objective—The aim of this study was to compare the diagnostic capacity of hematologic biomarkers to detect IDA among pregnant women in Tanzania.

Methods—We pooled data from an iron supplementation trial of 1500 iron replete pregnant woman and a prospective cohort of 600 iron deficient pregnant women. Receiver operating

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Statement of authors' contributions

The paper was drafted by AIA and WF with contributions from all authors. WF, SA and ZP designed the study. AE, NG, AIA, RN, SA, ZP, CD, and WF participated in field implementation. AIA, CS, EH, DS, and WF contributed to statistical analyses. All authors contributed to the development of and approved the final version of the manuscript. WF had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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The clinical trial was registered at clinicaltrials.gov (NCT01119612).

characteristic curves (ROC) for hematologic biomarkers were used to assess the sensitivity, specificity, and area under the curve (AUC) for iron deficiency (ID) and iron deficiency anemia (IDA), crude, or corrected for inflammation. Regression models assessed the relationship of baseline biomarker categories (gestational age <27 weeks) and IDA at delivery.

Results—Hemoglobin had the largest AUC for crude ID (0.96) while hepcidin had the largest AUC for corrected ID (0.80). The optimal hepcidin cut-off for the diagnosis of corrected IDA based on maximal sensitivity and specificity was ≤1.6μg/L. An hepcidin cutoff of <4.3 μg/L had a sensitivity of 95% for regression-corrected ID. Among iron-replete women who did not receive iron, the association of baseline hemoglobin >110g/L with IDA at delivery (RR = 0.73; 95% CI: 0.47, 1.13) was attenuated. Baseline hepcidin>1.6μg/L was associated with reduced risk of anemia at delivery by 49% (95% CI: 27%, 45%).

Conclusion—Ascertaining hemoglobin and hepcidin levels may improve the targeting of iron supplementation programs in resource-limited countries, though hepcidin's high costs may limit its use.

Keywords

iron deficiency; anemia; biological markers; pregnancy outcomes; screening

Introduction

Anemia is a highly prevalent risk factor associated with the burden of multiple disease conditions globally[1]. Iron deficiency is assumed to be the most common cause of nutritional anemia in most parts of the world, and accounts for 37% of anemia among pregnant women[2, 3]. It predisposes to adverse pregnancy outcomes such as maternal mortality, operative delivery, preterm birth, and early infant mortality[4–7] and may also negatively influence cognitive function in the children[8]. A substantial proportion of cases of anemia in pregnancy respond to iron supplementation, and estimates ranging from 10 – 50% have been proposed[1, 9]. Coverage for iron supplementation in pregnancy is however poor – about 10% in Sub-Saharan Africa including Tanzania [10]. Screen-and-treat programs may provide a targeted, cost-effective approach to fill the gaps in coverage, and appropriate selection of biomarkers is critical to detect women who may most benefit from supplementation[5, 11].

Screening and definitive diagnosis of iron deficiency anemia (IDA) among pregnant women in developing county settings is currently cumbersome and requires the use of hemoglobin and ferritin, with C-reactive protein or other inflammatory markers, as concentrations of ferritin are significantly altered by inflammation[11–13]. Alternative biomarkers for iron deficiency and anemia, such as zinc protoporphyrin (ZPP), soluble transferrin receptor (sTfR) and hepcidin, are also influenced by inflammation to varying extents and require standardization prior to large scale use[13]. ZPP represents iron deficient erythropoiesis and the incorporation of zinc in place of iron into the heme moiety during biosynthesis[13]. Depletion of blood iron leads to increased circulating concentrations of a truncated version of the cellular transferrin receptor, sTfR[14]. Hepcidin is a peptide hormone that regulates iron absorption in the gastrointestinal tract, and increases with increasing body iron stores. Data on the diagnostic validity of these biomarkers for the diagnosis of IDA in low resource

settings is limited. Reference ranges for these biomarkers are also often based on healthy pregnant populations in western countries, limiting utility especially in malaria-endemic settings.

The aim of the study was to assess the analytic and clinical validity of ZPP, sTfR and hepcidin in predicting anemia and iron deficiency among pregnant Tanzanian women. The findings from this study may guide the application of biomarkers for the screening and diagnosis of iron deficiency and anemia in pregnancy, especially in sub-Saharan Africa and malaria-endemic settings.

Methods

This analysis was based on a pooled dataset of a randomized controlled iron supplementation trial (RCT) and a prospective cohort study of pregnant women presenting at three antenatal clinics in Dar es-Salaam, Tanzania. In both studies, participants were eligible if they were 18 – 45 years old, HIV-negative, in their first or second pregnancy, presenting for antenatal care before 28 weeks' gestation, and planning to stay in Dar es Salaam until delivery. While participants recruited into the RCT were iron replete and not severely anemic (ferritin >12 μg/L and hemoglobin>85g/L; n=1500) at the time of screening, those recruited into the cohort were iron deficient (ferritin ≤12 μg/L; n=600). Participants in both studies were recruited between September 2010 and March 2013 and followed up monthly until delivery, and six weeks post-partum.

The primary aim of the RCT was to assess the efficacy of iron supplementation to prevent anemia and iron deficiency among iron-replete pregnant women in a malaria-endemic country, and evaluate the concomitant risk of placental malaria and other adverse maternal and neonatal outcomes[15]. Participants were therefore individually randomized in equal numbers to receive a daily oral dose of 60 mg iron (200mg of ferrous sulfate) or placebo (Tishcon Corp, New York, USA), identical in appearance and taste. The primary aim of the cohort study was to evaluate the magnitude and predictors of the response of hematologic biomarkers to iron supplementation among iron-deficient pregnant women. Participants in the cohort study therefore received a capsule containing 60mg iron (200mg of ferrous sulphate) and 0.25mg folic acid (Tishcon Corp, New York, USA) to be taken once daily from enrolment until delivery. Participants in both studies received malaria prophylaxis and antenatal care as per standard of care in Tanzania[16]. They were provided a month's supply of the capsules at each monthly visit and study staff collected used regimen bottles at each visit and counted remaining pills. The details of screening, treatment assignment and follow-up for the RCT[15] and the cohort study[5] have been described in greater detail elsewhere.

Enrollment into either study was done using rapid ferritin testing at the clinic (colloidal gold rapid assay, Glory Science Co. Ltd and Victory Medicine Inc., NY) and a confirmatory serum ferritin test (Cobas Integra; Roche Diagnostics) at the Muhimbili Research Laboratory using same day blood samples. Briefly, participants were immediately enrolled into the RCT if hemoglobin ≥110g/L (capillary blood testing using Hemocue) and serum ferritin >20μg/L (using rapid ferritin testing). Other participants were enrolled in the cohort study if serum ferritin <10μg/L (using rapid ferritin testing) and hemoglobin ≥85g/L. For

those with ferritin 10 – 20 μg/L, ferritin testing using Cobas Integra was the basis for further classification and study enrollment. They were enrolled in the RCT if serum ferritin using Cobas Integra was >12μg/L, and in the cohort study if ≤12μg/L. Participants with hemoglobin <85g/L were not enrolled in either study. The rapid ferritin test was a particle enhanced agglutination immunoassay that uses colloidal gold as the reporter reagent[17], and there was 95% agreement in the diagnosis of iron depletion at enrollment (ferritin ≤12μg/L) with the serum ferritin tests conducted at the laboratory using the Roche Cobas Integra.

Baseline samples were collected during the morning or afternoon hours in the clinic, and participants were not required to fast. Delivery samples were collected, as time allowed, immediately after delivery or within the first 48hrs postpartum. Enrolment and delivery blood samples were tested for a complete blood count (CBC testing using venous blood, AcT5 Diff AL, Beckman Coulter, FL, USA), serum ferritin (Cobas Integra), C-reactive protein (CRP, Roche Diagnostics) and α1-acid glycoprotein (AGP, Cobas Integra 400 Plus analyzer). In addition, a randomly selected subset of baseline and delivery samples (n=800) were tested for sTfR (Roche Diagnostics), hepcidin (EIA-5258, version 4.1, DRG International Inc., USA), and ZPP (measured by hematofluorometer). ZPP in whole blood was measured as previously described[5]. Washed red blood cells were used to exclude plasma bilirubin interference, if there was hemolysis. Participants with undetectable concentrations of biomarkers were assigned the lowest detectable concentrations. Further details of the testing for the biomarkers have been previously reported[5].

Anemia was defined as hemoglobin<110g/L[18]. Iron deficiency (ID) was defined using ferritin ≤15μg/L[19]. Serum ferritin concentration is frequently affected by the presence of inflammation or infection, potentially underestimating the prevalence of iron deficiency. Two alternative definitions for iron deficiency were therefore considered: the higher ferritin cutoff (hID) and the regression-correction approach (rID), previously described by Namaste and colleagues[20]. Briefly, the higher cutoff approach entails defining hID as ferritin ≤15 μg/L if inflammation was absent (CRP ≤8.2 mg/L or AGP ≤1 g/L), and ferritin <30 μg/L if inflammation was present (CRP >8.2 mg/L or AGP >1g/L)[5, 21–23]. The regression correction approach entails estimating the influence of inflammation (using CRP, AGP or both) on the ferritin concentration, and using the estimates obtained to correct the crude ferritin. The rID is then defined as ferritin ≤15μg/L. Iron deficiency anemia – using crude (IDA), higher cutoff (hIDA) or regression corrected (rIDA) approaches – was defined as iron deficiency in the presence of anemia.

The normal range for sTfR and hepcidin are 1.9 – 4.4mg/L and 13.3 – 54.4μg/L respectively, according to the manufacturers. Recent studies have however reported hepcidin cut-offs of 4.3μg/L for the diagnosis of IDA among pregnant Filipino women infected with *Schistosoma japonicum*, and 2.7 – 3.5μg/L among pregnant Gambian women, after applying the conversion factor proposed by Wray et al to allow comparability of the DRG and Bachem hepcidin ELISA assays[24–26]. In this analysis, ZPP > 70mmol/L, hepcidin ≤13.3μg/L, and sTfR >4.4mg/L were dichotomized to represent deficient iron status selected based on conventional cutoffs or manufacturers' normal ranges[27–30].

The median and interquartile range (IQR) of the concentration of each hematologic and inflammatory biomarker was examined by subgroup and in the overall sample at baseline. The baseline prevalence of deficient iron status using conventional cutoffs of individual biomarkers and corrected ferritin levels (hID and rID) were also examined. The baseline proportion of participants with elevated concentrations of inflammatory biomarkers (CRP >8.2 mg/L or AGP >1 g/L) by subgroup and overall were also assessed.

Sensitivity and specificity were estimated for ZPP (>70mmol/L), sTfR (>4.4mg/L) and hepcidin (≤13.3μg/L) as test biomarkers for anemia, ID, and IDA, using hemoglobin and ferritin, commonly used to diagnose ID and IDA, as gold standard comparators. Receiver operating characteristic (ROC) curves were plotted and the area under the ROC curves (AUC) along with their 95% confidence intervals were estimated. Since there is no widely accepted conventional cutoff for hepcidin, the Youden index, *J*, a summary characteristic of the ROC curve, which maximizes the sum of sensitivity and specificity, was calculated with hID and rID as the gold standard comparators [31, 32]. To evaluate the performance of the conventional and newly identified biomarker cutoffs, density distributions for biomarker concentrations were plotted to visually examine overlapping or gray areas in the concentration of the biomarkers among those with IDA at baseline and among those without.

The influence of the biomarker cut-offs on important clinical outcomes (anemia, hIDA and rIDA) was evaluated by obtaining relative risks from multivariate-adjusted log-binomial regression[33]. In a few instances, the model did not converge and log-Poisson models, which provide consistent but not fully efficient estimates of the relative risk and its confidence intervals were used[34]. Potential confounders were selected in the manner described by Hosmer and Lemeshow[35]. Briefly, baseline sociodemographic, nutritional, and hematologic variables that were significant at $p<0.25$ in univariate models for the clinical outcomes (anemia, hIDA and rIDA) were considered for inclusion. Selected variables were included in multivariate models and variables that were not significant at $p<0.05$ were excluded. Variables that caused >20% change in any of the beta estimates were added back into the model, along with variables that have been previously established in the literature to be important predictors[36–42].

Variables included were age (18 – 25, 26 – 35 and >36 years), gestational age at enrollment (weeks), years of formal education (0 – 7 years, 8 – 11 years and ≥12 years), number of household assets(0 – 1, 2 – 3 and 4 – 5), consumption of meat (<75g, ≥75g per week), season of enrolment [December – March (dry), April – May (rain), June – September (harvest) and October – November (post-harvest)], multiple gestation (yes, no) and clinic attended were also considered.

The sample size for the RCT (N=1,500) was calculated to detect a ≥35% higher effect of iron supplements on the risk of placental malaria, at 80% statistical power, assuming a prevalence of 20% and 10% loss to follow-up. The sample size for the cohort study (N=600) was calculated to detect an 11% change in ferritin concentration at 80% statistical power, assuming a mean of 20 μg/L and SD of 10 μg/L[5]. Both studies would also have sufficient power to detect changes in biomarker concentrations up to 8% and 12% for sTfR and hepcidin, respectively. None of the covariates was missing >5% of observations. P-values

were two-sided, and significance was set at <0.05. Statistical analyses were conducted with SAS v9.2 (SAS Institute Inc.) – code available on request. Values presented in the text are medians (IQRs), means (±SD), means (95% CI), means (±SE) and relative risks.

Participants gave written informed consent at enrolment. Ethical approval for the study was obtained from the institutional review boards of the Harvard T.H. Chan School of Public Health and Muhimbili University of Health and Allied Sciences, and regulatory approval from the Tanzanian National Institute for Medical Research (NIMR), and the Tanzanian Food and Drug Administration (TFDA). The clinical trial was registered at clinicaltrials.gov (NCT01119612).

Results

This analysis included 2,100 HIV-negative pregnant women presenting to antenatal clinics in Dar es Salaam. Thirty percent were iron deficient and received iron. Of the 70% who were iron-replete, 35% received iron while 35% received placebo The mean age (±SD) of the women was 24 years (±4). Women were enrolled at a mean gestational age of 19weeks (±4), and 47% received iron supplementation for more than 90 days before delivery. Fifty-seven percent of women were primigravida and use of malaria prevention measures was common, especially bednets (Table 1). Among iron replete individuals, those who received iron had similar baseline characteristics as those who received placebo due to success of randomization[15]. There were modest differences in the baseline characteristics of the iron deficient group compared to the iron replete groups. The mean gestational age at enrolment was higher in the iron deficient group (20 weeks compared to 18 weeks, *p*-value <0.0001). Participants in the iron deficient group also consumed less meat (72% compared to 81% had ≥75g per week, *p*-value <0.0001).

Table 2 shows the baseline concentrations of the biomarkers across treatment groups. Thirty-nine percent (39%) were anemic (hemoglobin<110g/L), 36% were iron deficient (ferritin ≤15μg/L) and 97% had low hepcidin concentrations (≤13.3μg/L). The prevalence of iron deficiency varied, depending on the biomarker, cutoff or definition. Elevated inflammatory biomarker levels were lower in the iron-deficient groups, but high overall.

We plotted receiver operating characteristic (ROC) curves for ID, hID, rID, IDA, hIDA and rIDA, and compared the AUCs for the biomarkers (Figure 1 and Table 3). Hemoglobin had the largest AUC for crude ID (AUC = 0.96; (0.95, 0.97)) while hepcidin had the largest AUC for hID (AUC = 0.83; (0.80, 0.86)) and rID (AUC = 0.80; (0.76, 0.83). Hepcidin and ZPP had similar AUCs for IDA, hIDA and rIDA.

The sensitivity and specificity of ID for hemoglobin<110g/L were 100% and 85% respectively. The sensitivity and specificity of hID for hepcidin≤13.3μg/L were 100% and 7% respectively. An hepcidin cutoff of <4.3 μg/L had a sensitivity of 95% for regression-corrected ID. The hepcidin cutoff of ≤1.8μg/L maximized the sensitivity plus specificity for detecting hID (84% sensitivity and 71% specificity) and rID (83% sensitivity and 67% specificity). The hepcidin cutoff of ≤1.6μg/L maximized the sensitivity plus specificity for

detecting hIDA (84% sensitivity and 62% specificity) and rIDA (83% sensitivity and 60% specificity).

We compared the density distributions of hepcidin, sTfR and ZPP by hIDA at baseline (Supplementary figure 1, 2 and 3) and observed substantial overlap in the distributions of the biomarkers among those with hIDA and among those without.

Among pregnant women who did not receive iron supplements during pregnancy, the prospective risks of clinical outcomes were estimated in relation to the conventional (hemoglobin >110g/L, ZPP <70mmol/L, sTfR >4.4mg/L, hepcidin >13.3 μg/L) and the alternative cutoffs for hepcidin (>1.6μg/L, >1.8μg/L and >4.3μg/L) of the biomarkers at baseline (Table 4). Baseline hemoglobin>110g/L was associated with a reduction in the risk of anemia by 41% (95% CI: 30%, 51%) and hIDA by 44% (95% CI: 19%, 62%). The association with rIDA (RR = 0.73; 95% CI: 0.47, 1.13) was attenuated but the direction was preserved. Baseline hepcidin>1.6μg/L was associated with a reduction in the risk of anemia and hIDA at delivery by 49% (95% CI: 27%, 45%) and 50% (95% CI: 3%, 74%) respectively. Baseline hepcidin>1.8μg/L was associated with a reduction in the risk of anemia and hIDA at delivery by 42% (95% CI: 19%, 59%) and 49% (95% CI: 1%, 74%) respectively. Baseline hepcidin>4.3μg/L was associated with a reduction in the risk of anemia at delivery by 39% (95% CI: 5%, 61%). All hepcidin cutoffs considered were associated with substantial reductions in the risk of regression-corrected IDA at delivery, although the confidence intervals were wide.

Discussion

We evaluated the clinical utility and validity of three hematologic biomarkers compared to anemia, iron deficiency and iron deficiency anemia among pregnant Tanzanian women and identified possible new cut-offs for use among pregnant women in Tanzania and similar settings. We observed that baseline hemoglobin and hepcidin have good analytic validity and may adequately predict the risk of anemia, iron deficiency and iron deficiency anemia at delivery.

We compared the analytic validity of four hematologic biomarkers among HIV-negative Tanzanian pregnant women and found hepcidin and hemoglobin to be the most predictive for iron deficiency and iron deficiency anemia. ZPP testing is cheap, simple, and less influenced by inflammation than the other biomarkers and ZPP may be suitable for large scale use if concerns regarding training and supplies are addressed[43]. For instance, ZPP requires whole blood to measure, imposing time and storage constraints for testing. Further, the concentration of ZPP may be elevated due to thalassemia[42, 44], which has prevalence estimated at between 2.3% and 15% in sub-Saharan Africa, depending on variant[45, 46]. The validity of ZPP to diagnose IDA was fair, similar to hepcidin's, but the challenges with testing highlighted above are inherent to the nature of the test.

Soluble transferrin receptor concentration represents functional iron deficiency and is mostly driven by erythropoietic activity in the bone marrow, making it potentially useful for evaluating response to iron supplementation[14]. Its utility as an iron status biomarker is

however limited by conditions where altered erythropoiesis and iron deficiency coexist – such as pregnancy and hemolytic anemia[14, 44]. The validity of sTfR in our population of pregnant women in a malaria-endemic setting was poor. Estimating the sTfR-ferritin ratio could potentially surmount some of these challenges, but we were unable to evaluate its utility since ferritin was used in defining the comparators in the study.

In our sample, hepcidin represented an excellent alternative biomarker for assessing iron deficiency. It is a key determinant of dietary iron absorption in the gastrointestinal tract and iron redistribution in the reticuloendothelial system[47]. Hepcidin binds ferroportin on the surface of enterocytes, liver cells, macrophages and placental cells. This leads to ferroportin degradation and is a key step regulating the bioavailability of iron[44, 48]. Inflammation also upregulates hepcidin synthesis via the action of interleukin-6, leading to reduced iron bioavailability and iron-restricted erythropoiesis[44, 48]. Hepcidin may therefore be able to distinguish between anemia of inflammation and iron deficiency anemia[49]. Hepcidin had the highest AUC for iron deficiency in our study, and participants with hepcidin>1.6μg/L at enrolment had a 49% lower risk of anemia at delivery. Recent studies among pregnant women in the Gambia and the Philippines also reported that hepcidin had superior diagnostic accuracy for iron deficiency, compared to other biomarkers [24, 50]. The AUCs for hepcidin in our study were high regardless of adjustment for CRP, suggesting equally good validity in the presence of inflammation, and potentially rendering assessment of inflammatory biomarkers unnecessary.

A re-evaluation of conventional biomarker cut-offs and their possible replacement, if unsatisfactory, is good epidemiologic practice, as cutoffs may have been selected arbitrarily and may not necessarily reflect physiologic function[32]. Furthermore, pregnant women are typically excluded from studies to determine reference intervals for normal population[32]. Hepcidin concentrations at enrolment in our study were relatively low. More than 95% of the participants were classified as deficient as determined by the conventional cut-off for hepcidin (13.3μg/L), despite only 29% being iron deficient as determined by serum ferritin testing. This significant discrepancy suggests a need to re-evaluate the hepcidin cut-off for use in this and similar populations. We identified an alternative cutoff of 1.6μg/L for hepcidin based on the maximal Youden Index for iron deficiency anemia, tested with DRG's ELISA (corresponding to 2.8μg/L for Bachem's ELISA). The Youden index gives equal weight to sensitivity and specificity, and the optimal cut-off is that at which the Youden Index is greatest[32]. Using the Youden Index approach among pregnant Gambian women, cut-offs ranging from 0.5 – 2μg/L have been suggested for Bachem hepcidin (corresponding to 0.9 – 3.5μg/L for DRG hepcidin) between 14- and 30-weeks gestation. A higher hepcidin cutoff was identified among pregnant Filipino women infected with *S. japonicum* – 4.3μg/L and 6.1μg/L(using DRG tests), depending on whether testing was done among anemic and non-anemic women, or among anemic women only (corresponding to 7.6 and 10.8μg/L for Bachem's ELISA), and defining iron deficiency using ferritin <30μg/L [50]. The use of appropriate cut-offs for hepcidin may enable improved identification of participants with iron deficiency, especially if unique clinical and population characteristics are considered.

Using the Youden Index is limited by the fact that it does not account for the prevalence of the condition in the population of interest, the risk of adverse outcomes related to not

receiving treatment and the costs associated with testing. From an economic point of view, the optimal sensitivity and specificity depends on the costs of the tests, the prevalence of IDA and the costs of remedying it. In our population, the prevalence of iron deficiency is high and the risk of adverse birth outcomes among iron deficient patients who do not receive iron supplementation far outweigh the cost of providing iron supplementation to patients who are not iron deficient[5, 15]. Providing iron supplements to pregnant women has been shown to be safe[15, 51] and compliance to iron supplements provided is poor. Therefore, a highly sensitive test and cut-off could be prioritized, at the expense of specificity, to identify as many women at risk of IDA as possible, and may minimize the total costs associated with testing. In this study, an hepcidin cutoff of <4.3 μg/L had a sensitivity of 95% for regression-corrected ID. Lowering the hepcidin cutoff to ≤1.8μg/L still yielded good sensitivity (83%), and patients whose baseline hepcidin concentration exceeded 1.8μg/L had a 42% lower risk of anemia at delivery, among those who did not receive iron supplements. We propose that targeted iron supplementation could be implemented in this and similar settings using hepcidin <4.3μg/L among pregnant women with anemia. Selecting biomarkers and cutoffs based on a consideration of the total costs of testing, the clinical utility of the tests and the statistical measures of performance of the tests will improve screening and diagnosis of anemia and iron deficiency.

There are important limitations to consider. Hepcidin levels were low in our study, partly due to recent malaria infection, gestational age at enrolment, erythropoietic drive and the high prevalence of iron deficiency, although the erythropoietic drive is likely to be most important [52–54]. Serum hepcidin concentrations are often undetectable or low (≤1μg/L) in iron deficiency[52]. Further, pregnant women in our setting typically present to antenatal clinics in their 2nd trimester when hepcidin concentrations may have decreased to encourage iron bioavailability for accumulation in the placenta and transfer to the fetus[55]. The high cost of test kits and the lack of standardized assays for hepcidin represent a significant barrier to its use in developing countries, but its cost can be expected to reduce with greater uptake. A suitable reference material that would enable the harmonization of hepcidin assays has been recently identified[56]. The development of a bedside strip for hepcidin testing would simplify testing substantially and may thereby increase uptake of iron supplements. Although urinary hepcidin testing represents a less cumbersome alternative to blood testing, and urinary hepcidin concentration is strongly correlated with serum concentrations, additional testing of creatinine may be required and values may be influenced by renal function[52].

Although our study included iron deficient and iron replete participants, the sample does not perfectly represent the general population of HIV-uninfected women attending antenatal clinics in Tanzania. These findings may also not be generalizable to HIV-infected pregnant women and others at risk of chronic inflammation, which alters iron pathophysiology and biomarker responses[57]. Since 65% of our study participants were enrolled before 20 weeks and gestational age was adjusted for in our analysis, our results are unlikely to have been modified by hemodilution. Rapid resolution of hemodilution and the metabolic response to obstetric hemorrhage in the immediate postpartum period may affect iron status measurement[58]. Iron status measurements are therefore likely to differ considerably depending on the timing of blood sample collection during this period. Any measurement

error in our assessment of delivery iron status measures are however likely to be random, and unrelated to the baseline biomarker levels.

Alternative definitions of iron deficiency that are currently used in practice (ID, hID) or proposed for use (rID) were the comparators of 'gold standards' in this study. Serum ferritin was used in defining these comparators, and we were therefore unable to consider its validity and utility. Studies that evaluate alternative serum ferritin cutoffs using hepcidin testing and risk of important clinical outcomes may be helpful in this regard. This approach is not ideal, since the gold standard for the evaluation of iron status is stainable bone marrow iron. Assessing bone marrow iron requires invasive procedures, and few studies among healthy pregnant women in low and middle income countries have ever evaluated the validity of iron biomarkers using bone marrow iron as the comparator[59]. The sensitivity of serum ferritin to stainable bone marrow iron is modest[60, 61]. The evidence with respect to the sensitivity and specificity of ferritin also needs to be updated, using modern techniques and equipment, and in diverse geographic and socioeconomic settings[60].

Conclusion

Hemoglobin and hepcidin have excellent analytic validity for screening and diagnosis of iron deficiency anemia, and good predictive utility for the prospective risk of iron deficiency anemia at delivery among Tanzanian pregnant women. Ascertaining hemoglobin and hepcidin levels may improve the targeting of iron supplementation programs in resource-limited countries to reduce the global burden of anemia and iron deficiency, though hepcidin's high costs and need for standardization may limit its use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in text:

AGP	α1-acid glycoprotein
AI	Anemia of inflammation
BMI	Body Mass Index
CBC	Complete blood count
hID	iron deficiency based on the higher cutoff approach

hIDA	iron deficiency anemia based on the higher cutoff approach
CI	Confidence Interval
CRP	C-reactive protein
ELISA	Enzyme Linked Immunosorbent Assay
HIV	Human Immunodeficiency Virus
ID	Iron deficiency
IDA	Iron deficiency anemia
IQR	Interquartile range
RCT	Randomized controlled trial
rIDA	regression-corrected IDA
RR	Relative risk
SD	Standard deviation
SE	Standard error
sTfR	Soluble Transferrin receptor
ZPP	Zinc protoporphyrin

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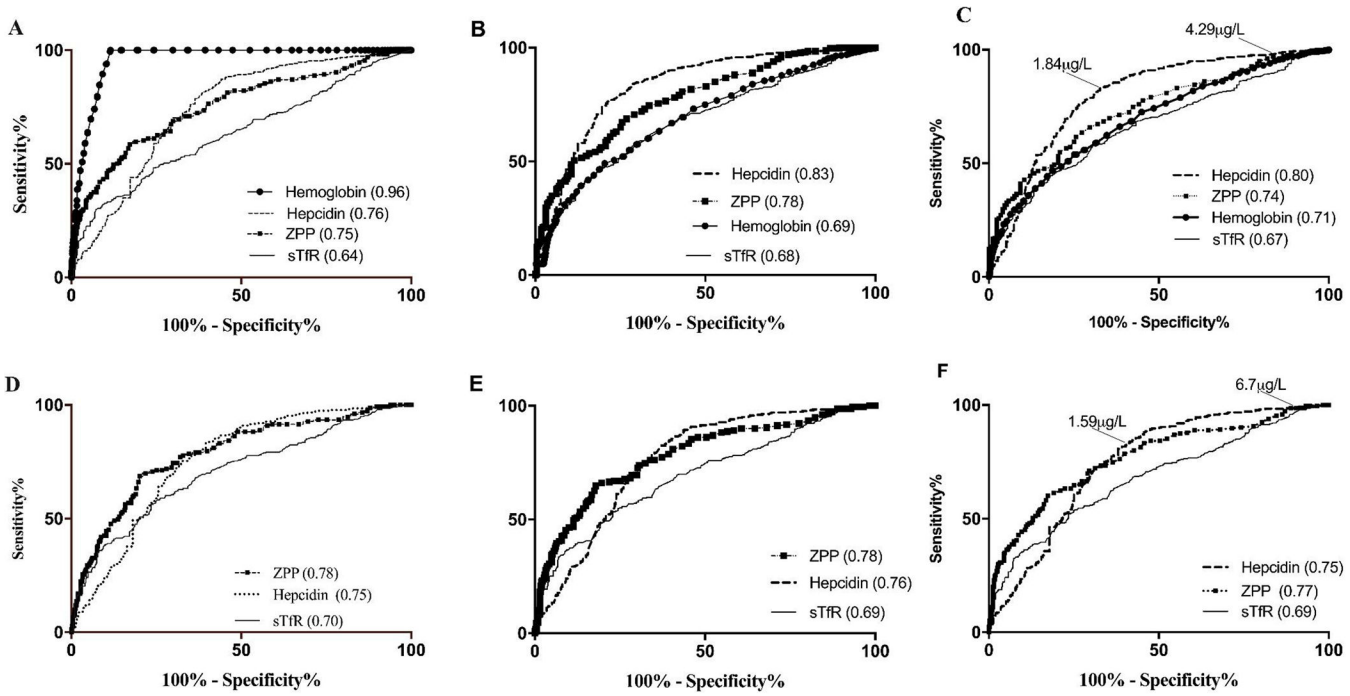

Fig 1.

Receiver operating characteristic (ROC) curves for baseline hematologic biomarkers among pregnant Tanzanian women to diagnose (A) iron deficiency, (B) iron deficiency based on the higher cutoff approach (hID), (C) regression-corrected ID, (D) iron deficiency anemia, (E) iron deficiency anemia based on the higher cutoff approach (hIDA), and (F) regression-corrected iron deficiency anemia

Circles in (C) depict sensitivity and specificity of hepcidin to diagnose hID at different cut-offs. Values in parentheses after biomarker names indicate the area under the ROC curves (AUC). [c] Cutoff is conventional; [j] Cut-off identified using Youden Index

Table 1:

Basic socio-demographic and clinical characteristics of Tanzanian pregnant women at initiation of antenatal care, n=2,100[1]

Characteristics	Iron replete, received iron, N=750	Iron replete, received placebo, N=750	Iron deficient, received iron, N=600	Total, N=2100
Age, y	23.7 ±4.1	24.1 ±4.2	24.2 ±4.0	24.0 ±4.1
18 – 20 y, %	24%	21%	20%	22%
>20 – 25 y, %	46%	47%	47%	47%
>25 y, %	30%	32%	33%	32%
Gestational age at enrolment, weeks	17.9 ±4.3	17.8 ±4.4	20.1 ±3.8	18.5 ±4.3
4 – 13 weeks, %	16%	18%	6%	14%
>13 – 20 weeks, %	55%	53%	46%	51%
>20 – 27 weeks, %	29%	30%	49%	35%
Gravidity, *n* (%)				
Primigravida	61%	55%	53%	57%
Secundigravida	39%	45%	47%	43%
Body mass index (BMI), kg/m^2	24.5 ±4.4	25.4 ±4.7	24.2 ±3.9	24.4 ±4.4
<18.5 kg/m^2, %	4%	5%	3%	4%
18.5 to <25 kg/m^2, %	58%	55%	62%	58%
25 to <30 kg/m^2, %	26%	29%	26%	27%
≥30 kg/m^2, %	12%	11%	9%	11%
Education, y, *n* (%)				
0 – 7 y	58%	56%	56%	57%
>7 – 11 y	29%	28%	27%	28%
>11 y	13%	16%	17%	15%
Marital status, *n* (%)				
Married or cohabiting	80%	81%	81%	80%
Never married, widowed, or divorced	20%	19%	19%	20%
Occupation, *n* (%)				
Unemployed	50%	48%	48%	49%
Informal – skilled/unskilled	31%	32%	33%	32
Skilled formal	6%	8%	9%	7%
Business/professional	13%	12%	10%	12%
Meat consumption, grams per week, *n* (%)				
≥ 75g	71%	72%	81%	74%
< 75g	29%	28%	19%	26%
Number of household assets, *n* (%)[2]				
0 – 1	14%	13%	15%	14%
2 – 3	37%	36%	35%	36%
4 – 5	49%	51%	50%	50%
Hemoglobinopathy, *n* (%)[3]				

Characteristics	Iron replete, received iron, N=750	Iron replete, received placebo, N=750	Iron deficient, received iron, N=600	Total, N=2100
Not suggestive	85%	82%	85%	84%
Suggestive of Thalassemia	15%	18%	15%	16%
Duration of use of iron supplements, days				
<90	56%	56%	47%	53%
≥90	44%	44%	54%	47%
Study site, *n* (%)				
AmtullabaiKarimjee	63%	64%	55%	61%
Magomeni	11%	11%	0%	8%
Sinza	26%	25%	45%	31%
Season of enrolment				
Dry (Nov – Mar)	33%	31%	46%	36%
Long rains (Apr – May)	20%	20%	17%	19%
Harvest (Jun – Sep)	30%	31%	20%	28%
Short rain (Oct – Nov)	17%	18%	17%	18%
Multiple gestation, *n* (%)				
Singleton pregnancy	98%	98%	99%	99%
Twin gestation	2%	2%	1%	1%

[1] Values in the table are means (±SD) and n (%). Percentages may not add up to 100% due to rounding.

[2] Number of household assets was computed from a simple list of assets owned by participant –car, generator, bike, sofa, television, radio, refrigerator, fan, electricity and potable water.

[3] Hemoglobin electrophoresis was conducted in a randomly selected subsample of the iron-replete (n=484) and iron deficient (n=311) participants

Table 2.

Baseline hematologic and inflammatory biomarkers among Tanzanian pregnant women using conventional cutoffs

Biomarker[1]	Iron replete, received iron, N=750	Iron replete, received placebo, N=750	Iron deficient, received iron, N=600	Total, N=2100
Hemoglobin, *n*=2078				
Median (IQR)	116 (108, 125)	117 (109, 124)	102 (92, 111)	113 (103, 121)
%Deficient, <110g/L	28%	26%	70%	39%
Ferritin, *n*=2077				
Median (IQR)	30.7 (18.9, 47.9)	30.0 (19.3, 51.0)	5.8 (2.9, 9.2)	21.6 (10.9, 41)
%Deficient, ≤15 µg/L	13%	13%	93%	36%
%hID	29%	32%	98%	49%
%rID	29%	30%	96%	49%
ZPP, *n*=618				
Median (IQR)	50 (41, 68)	52 (41, 68)	73 (54, 101)	57 (44, 83)
%Deficient, >70 mmol/L	21%	22%	59%	37%
Hepcidin, *n*=771				
Median (IQR)	2.2 (1.4, 4.3)	2.0 (1.3, 4.9)	1.0 (0.9, 1.5)	1.4 (1.0, 2.6)
%Deficient, ≤13.3µg/L	96%	94%	100%	97%
sTfR, *n*=746				
Median (IQR)	2.0 (1.1, 2.8)	1.9 (1.1, 2.9)	3.4 (2.1, 4.8)	2.5 (1.5, 3.9)
% Deficient, >4.4mg/L	7%	8%	32%	20%
CRP, *n*=2064				
Median (IQR)	4.7 (2.1, 8.3)	4.2 (2.2, 7.8)	3.8 (2.0, 7.0)	4.3 (2.1, 7.7)
% Elevated, >8.2mg/L	25%	23%	19%	23%
AGP, *n*=975				
Median (IQR)	59.8 (47.1, 71.5)	60.4 (49.2, 72.4)	51.6 (41.4, 64.3)	55.3 (44.5, 68.9)
% Elevated, >1mg/L	100%	100%	94%	97%

[1] Hemoglobin and ZPP were measured in whole blood. Ferritin, hepcidin and sTfR were measured in serum.

[2] hID refers to iron deficiency determined based on the higher ferritin cutoff approach. *rID* refers to iron deficiency determined based on regression-correction approach.

Values are median (IQRs) or percentages.

Table 3:

Analytic Validity of Baseline Hematologic Biomarkers among Tanzanian pregnant women[1,2]

Biomarker		Iron Deficiency (ID)[3]	Higher cutoff iron deficiency (hID)	Regression-corrected iron deficiency (rID)	Iron deficiency anemia (IDA)	Higher cutoff iron deficiency anemia (hIDA)	Regression-corrected iron deficiency anemia (rIDA)
Hemoglobin, g/L							
>110[c]	Sensitivity	100%	57%	55%			
	Specificity	85%	70%	74%			
ZPP							
>70[c]	Sensitivity	60%	89%	45%	70%	66%	62%
	Specificity	79%	50%	88%	78%	80%	79%
sTfR, mg/L							
>4.4[c]	Sensitivity	31%	25%	26%	37%	35%	35%
	Specificity	91%	93%	93%	91%	92%	91%
Hepcidin, µg/L							
≤13.3[c]	Sensitivity	99%	100%	100%	100%	100%	100
	Specificity	4%	7%	6%	4%	4%	4%
≤1.6[j]	Sensitivity	82%	76%	75%	86%	84%	83%
	Specificity	61%	79%	74%	58%	61%	60%

[c] Cut-off is conventional;

[j] Cut-off identified using Youden Index;

* P-value <0.05;

** P-value<0.01;

*** P-value <0.001

[1] All biomarkers tested at baseline.

[2] Sensitivity was calculated as a/a+c, where a=number of patients meeting both the test biomarker threshold (ZPP or sTfR or Hepcidin) and the comparator threshold (hemoglobin or ferritin or both), and c=number of patients meeting comparator threshold but not the test biomarker threshold. Specificity was calculated as d/b+d, where d=number of patients who did not meet both the test biomarker threshold and the comparator threshold, and b=number of patients meeting the test biomarker threshold but not the comparator threshold. In some cases, a, b, c or d were equal to zero, and these were replaced with 0.5. The Youden index is a summary statistic of the ROC curve and maximizes the sum of the sensitivity and specificity

[3] Anemia was defined as hemoglobin <110g/L. Iron deficiency was defined as ferritin ≤15 μg/L regardless of inflammation. Higher cutoff iron deficiency was defined depending on the presence (ferritin, <30 μg/L; CRP, >8.2 μg/mL) or absence (ferritin, <15 μg/L; CRP, ≤8.2 μg/mL) of inflammation

Values are percentages.

Table 4.

Relationship of baseline biomarker categories and the occurrence of clinical outcomes at delivery among Tanzanian pregnant women who did not receive iron supplements

	Anemia at delivery		Higher cutoff iron deficiency (hID) at delivery		Regression-corrected iron deficiency (rID) at delivery		Higher cutoff iron deficiency anemia (hIDA) at delivery		Regression-corrected iron deficiency anemia (rIDA) at delivery	
Biomarker	***n/N*[1]**	**Multivariate RR (95% CI)[2]**	***n/N***	**Multivariate RR (95% CI)**	***n/N***	**Multivariate RR (95% CI)**	***n/N***	**Multivariate RR (95% CI)**	***n/N***	**Multivariate RR (95% CI)**
Hemoglobin										
≥110g/L[c]	245/495	0.59 (0.49, 0.70)	224/482	0.82 (0.61, 1.09)	180/488	0.94 (0.67, 1.32)	119/464	0.56 (0.38, 0.81)	99/470	0.73 (0.47, 1.13)
Zinc protoporphyrin (ZPP)										
<70 mmol/L[c]	72/120	0.93 (0.72, 1.20)	57/127	0.95 (0.50, 1.79)	45/127	1.16 (0.50, 2.72)	38/117	0.75 (0.36, 1.53)	31/117	0.97 (0.37, 2.52)
Hepcidin										
>13.3μg/L[c]	87/182	0.96 (0.43, 2.15)	78/178	0.59 (0.18, 2.01)	60/179	0.59 (0.13, 2.60)	41/174	0.99 (0.21, 4.68)	33/175	0.45 (0.05, 3.74)
>4.3μg/L[s]	87/182	0.61 (0.39, 0.95)	78/178	0.78 (0.44, 1.38)	60/179	0.73 (0.37, 1.44)	41/174	0.79 (0.35, 1.81)	33/175	0.50 (0.19, 1.36)
>1.8μg/L[j]	87/182	0.58 (0.41, 0.81)	78/178	0.99 (0.62, 1.58)	60/179	1.06 (0.62, 1.81)	41/174	0.51 (0.26, 0.99)	33/175	0.54 (0.26, 1.13)
>1.6μg/L[k]	87/182	0.51 (0.35, 0.73)	78/178	1.00 (0.62, 1.64)	60/179	1.06 (0.60, 1.88)	41/174	0.50 (0.26, 0.97)	33/175	0.55 (0.26, 1.17)
Transferrin receptor (sTfR)										
<4.4mg/L[c]	88/186	0.97 (0.51, 1.87)	81/183	1.01 (0.35, 2.86)	62/184	0.83 (0.29, 2.40)	40/179	0.98 (0.22, 4.34)	33/180	0.80 (0.18, 3.59)

[c] Cut-off is conventional;

[s] Cut-off at 95% sensitivity for regression-corrected ID;

[j] Cut-off identified using Youden Index for regression corrected ID;

[k] Cut-off identified using Youden Index for regression-corrected IDA;

* P-value <0.05;

** P-value<0.01;

*** P-value <0.001

[1] Values in column are number of events/number of observations.

[2] Log-binomial regression models were estimated to obtain the RR of clinical outcomes. RR > 1 implies the outcome is more likely to occur as the biomarker level increases. RR < 1 implies the outcome is less likely as the biomarker level increases. Multivariate models were adjusted for age (18 – 25, 26 – 35 and >36 years), gestational age at enrollment (weeks), years of formal education (0 – 7 years, 8 – 11 years and ≥12 years), number of household assets(0 – 1, 2 – 3 and 4 – 5), consumption of meat (<75g, ≥75g per week), season of enrolment [December – March (long rains), April – May (harvest), June – September (post-harvest) and October – November (short rains)], multiple gestation (yes, no), and clinic attended.