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Optimizing diagnostic biomarkers of iron deficiency anemia in community-dwelling Indian women and preschool children

Giridhar Kanuri,[1,2] Deepti Chichula,[1] Ritica Sawhney,[1] Kevin Kuriakose,[1] Sherwin De'Souza,[1] Faye Pais,[1] Karthika Arumugam[1] and Arun S. Shet[1,3]

[1]Wellcome Trust- DBT Hematology Research Unit, St. Johns Research Institute, Bangalore, Karnataka, India; [2]Department of Biotechnology, KLEF, Greenfields, Vaddeswaram, Andhra Pradesh, India and [3]National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA

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ABSTRACT

The detection of iron deficiency anemia is challenged by the paucity of diagnostic tests demonstrating high sensitivity and specificity. Using two biomarkers, zinc-protoporphyrin/heme and hepcidin, we established the diagnostic cut-off values for iron deficiency anemia in preschool children and women. We randomly selected non-anemic individuals (n=190; women=90, children=100) and individuals with iron deficiency anemia (n=200; women=100, children=100) from a preexisting cohort of healthy preschool children and their mothers. The diagnostic performance of these biomarkers was estimated by analyzing receiver operating characteristic curves. Diagnostic cut-offs with a high predictive value for iron deficiency anemia were selected. Median zinc-protoporphyrin/heme and hepcidin values in non-anemic children were 49 μmol/mol heme and 42 ng/mL, respectively, and in non-anemic women these values were 66 μmol/mol heme and 17.7ng/mL, respectively. Children and women with iron deficiency anemia had higher zinc-protoporphyrin/heme ratios (children=151 μmol/mol heme and women=155 μmol/mol heme) and lower hepcidin levels (children=1.2ng/mL and women=0.6ng/mL). A zinc-protoporphyrin/heme ratio cut-off >90 μmole/mole heme in children and >107 μmole/mole heme in women was associated with a high diagnostic likelihood for iron deficiency anemia (children, likelihood ratio=20.2: women, likelihood ratio=10.8). Hepcidin cut-off values of ≤6.8ng/mL in children and ≤4.5ng/mL in women were associated with a high diagnostic likelihood for iron deficiency anemia (children, likelihood ratio=14.3: women, likelihood ratio=16.2). The reference ranges and cut-off values identified in this study provide clinicians with guidance for applying these tests to detect iron deficiency anemia. Erythrocyte zinc-protoporphyrin/heme ratio is a valid point-of-care biomarker to diagnose iron deficiency anemia.

Introduction

Iron deficiency anemia (IDA) is the leading cause of anemia worldwide[1] with well established guidelines for diagnosis and treatment.[2,3] Typically, the diagnosis of IDA is made when the plasma hemoglobin (Hb) falls below normal (<11.0g/dL in children and <12g/dL in women) and the serum ferritin is <12 μg/L.[4] Unfortunately, the frequent coexistence of inflammation/infection confounds serum ferritin, which is an acute phase protein, mandating the performance of additional tests e.g., C-reactive protein (CRP) and serum transferrin receptor (sTfR). As a result, the diagnosis of IDA often requires a battery of diagnostic tests, trained technicians, and the use of expensive laboratory equipment, which increases costs and delays results. Clearly, developing biomarkers that quickly, easily and reliably detect IDA would be beneficial.

One such biomarker, zinc-protoporphyrin (ZPP), is formed in erythrocytes during iron-deficient erythropoiesis when the protoporphyrin ring incorporates an atom of zinc rather than iron. The ratio of zinc-protoporphyrin/heme (ZPP/H) can

Correspondence:
arun.shet@nih.gov

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Table 1. Demographic, hematological and biochemical parameters of study participants.

	Non-anemic		Iron deficiency anemia (IDA)	
	Women (n=90)	Children (n=100)	Women (n=100)	Children n=100)
Age ± SD	26±3.7	3.7±0.9	25±3.8	2.4±0.8[a]
Sex M:F	na	49:51	na	56:44
Hemoglobin ± SD(g/dl)	13.2±0.7	11.9±0.6	10±1.2[a]	9.6±0.8[a]
MCV ± SD (fL)	86±4.5	77±3.7	74±7.6[a]	65±5.9[a]
WBC ± SD x 10^3/μL	8.7±2.0	9.8±2.4	7.8±2.0	10.3±2.8
Platelet count ± SD x 10^5/μL	3±0.7	3.9±1	3.4±0.7	4.6±1.2
Biochemical parameters				
Serum Ferritin*(ng/mL)	39 (33, 48)	35 (32, 45)	3.7[a] (2.9, 5.3)	3.8[a] (2.6, 5.1)
Serum sTfR* (mg/L)	1.3(1.1, 1.5)	1.7 (1.5, 2.0)	3[a] (2.2, 4.0)	3.9[a] (3, 5.1)
sTfR/logferritin index	0.8 (0.7, 0.9)	1.1 (0.9, 1.2)	5.1[a] (3.5, 8)	6.9[a] (4.5, 11.2)
CRP* (mg/L)	1.4 (0.4, 3.4)	1.1 (0.4, 2.2)	0.6[a] (0.1, 2)	0.5[a] (0.06, 1.6)
Biomarkers				
ZPP/H* (mol/mol heme)	66 (53, 83)	49 (39, 60)	155[a] (101, 243)	151[a] (104, 263)
Hepcidin* (ng/mL)	17.7 (9, 38)	42.6 (25, 62)	0.6[a] (0.2, 1.3)	1.2[a] (0.5, 3.6)

*Data represented as median (inter quartile range). [a]P<0.05 compared with the appropriate non-anemic control group. SD: standard deviation; MCV: mean corpuscular volume; WBC: white blood cells; Stfr: serum transferrin receptor; CRP: C-reactive protein; ZPP/H: zinc protoporphyrin/heme.

be determined rapidly at the point-of-care (POC) by a hematofluorometer.[5,6] Serum hepcidin, a key regulator of iron homeostasis, is an important biomarker because its levels determine how well oral iron is absorbed, with low hepcidin levels indicating both a requirement for iron and an ability to utilize it if provided.[7,8]

Although ZPP/H reference values are available for iron deficient pregnant women[5] and children,[6] cut-off values that establish a diagnosis of IDA with acceptable sensitivity and specificity are lacking. Moreover, reference ranges for hepcidin in healthy rural Indian women and children are not defined. Indian women and preschool age children account for one-third of the global burden of anemia.[3,9] Establishing reference ranges for these two biomarkers among healthy individuals and determining cut-off values for the diagnosis of IDA could facilitate their use and the development of novel POC assays. Therefore, we sought to define the median erythrocyte ZPP/H and serum hepcidin levels and select optimal cut-off values for the diagnosis of IDA in healthy rural preschool children and their mothers.

Methods

Definition of study groups and sample selection

In total, there were 2227 samples that were divided into three groups (non-anemic individuals, and those with IDA or iron deficiency without anemia) (Figure 1). Women and children with anemia (WHO recommended Hb concentrations anemia<11g/dL for children and <12g/dL for women[2]) with absent body iron stores (serum ferritin <12ng/mL[4]), were categorized into the IDA group (women n=334 and children n=560). Women and children without anemia (Hb≥11g/dL for children and ≥12g/dL for women) having normal iron stores (serum ferritin≥30ng/mL) were categorized into the non-anemic group (women n=99 and children n=173). Women and children with normal Hb but low body iron stores (ferritin<30ng/mL) were categorized as having iron deficiency without anemia and excluded from the study (n=1061). Subsequently, using a computerized random number generator we selected 200 samples from the IDA group (100 each from both women and children) and 190 samples from the non-anemic group (Children=100; Women=90) and performed biomarker measurements.[10] Using a sTfR/log ferritin index >2, we diagnosed nutritional IDA without coexisting anemia of inflammation.[11,12] Study location, sample size, and processing are detailed in *Online Supplementary Methods*.

Ethics

The study was approved by the St. Johns National Academy of Health Sciences Institutional Ethical Committee (IEC115/2012, IEC119/2013, and IEC121/2015).

Biomarkers, ferritin, sTfR and Inflammation assays

Serum hepcidin was quantified using an enzyme-linked immunosorbent assay (Peninsula Labs, San Carlos, CA, USA). The hepcidin concentration was extrapolated from a standard curve generated by four parametric logistic regression in accordance with manufacturer instructions. ZPP/H was measured using a hematofluorometer (Aviv Biomedical, Lakewood, NJ, USA) calibrated with commercially available standards. Samples were measured in triplicate and the average value obtained was expressed in μmole/mole heme. Due to a high baseline prevalence of inflammation in this population, we measured sTfR, an indicator of iron status that is not affected by the acute phase response. We calculated the sTfR/log ferritin index to accurately distinguish IDA from anemia of inflammation.[13] Serum ferritin and sTfR levels were both measured by paramagnetic particle chemiluminescent immunoassay

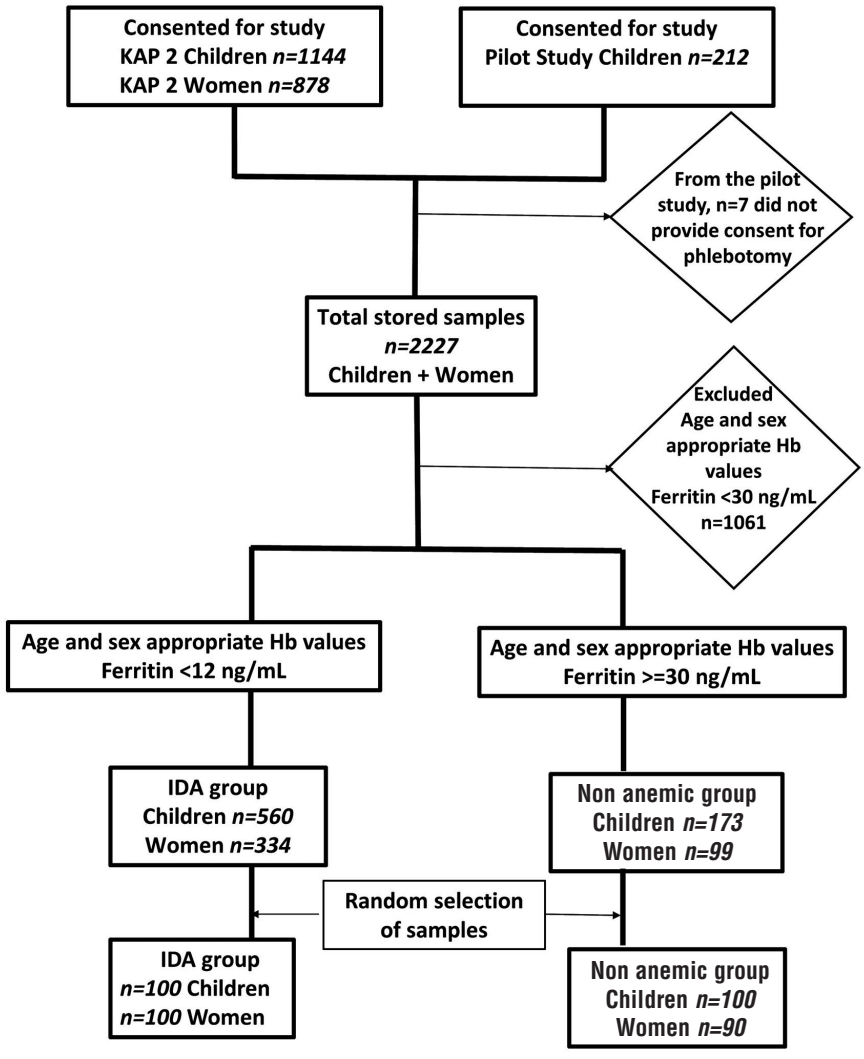

Figure 1. Flow diagram of participants and categorization of study groups. Schematic representation of the study design and sample selection. Using age and gender-adjusted WHO definitions for Hb and serum ferritin, samples were divided into a non-anemic group and a group with iron deficiency anemia. Those individuals with normal Hb values but low serum ferritin were defined as having iron deficiency and excluded. Using a computer random number generator, 200 samples from the iron deficiency anemia group (children = 100; women = 100) and 190 samples from the non-anemic group (Children = 100; Women = 90) were randomly selected for biomarker measurements. IDA: iron deficiency anemia; Hb: hemoglobin.

(Access 2, Beckman Coulter). Serum high sensitivity (hs)-CRP, a biochemical measure of inflammation, was determined by quantitative sandwich enzyme-linked immunosorbent assay ([ELISA] R&D systems, Minneapolis, MN, USA).

Statistical analysis

Variables were aggregated into mean±SD (continuous variables with a Gaussian distribution, t-test for departure from no difference) and median with the interquartile range ([IQR] continuous variables with non-Gaussian distribution, Mann-Whitney test for departure from no difference).

To determine ZPP/H and serum hepcidin cut-off values for IDA diagnosis, we used receiver operating characteristic (ROC) curves with IDA defined as Hb below the normal range combined with serum ferritin<12ng/mL. Youden index (J=sensitivity+specificity-1) and likelihood ratios [LR+=sensitivity/(1-specificity)] [LR-=(1-sensitivity)/specificity] were calculated for each individual cut-off value of ZPP/H and hepcidin. This iterative process began with the selection of the highest Youden indices corresponding with the highest positive likelihood ratio for IDA.[14] The conventional threshold of <0.05 was used for statements about statistical significance. All statistical analyses were done using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) and ROC curves were performed using MedCalc software (MedCalc, Ostend, Belgium).

Results

Comparison of baseline characteristics between the two groups (non-anemic and IDA) for children and women are presented as mean±SD for variables with a normal distribution and as the median ± interquartile range for variables that are not normally distributed. Age, gender, hematological and biochemical parameters are presented in Table 1 and ROC estimates, Youden indices, and likelihood ratios are presented in Table 2.

Baseline characteristics of the anemic and non-anemic groups

Non-anemic children were slightly older than children with IDA (3.7±0.9 *vs.* 2.4±0.8 years) (Table 1) while the ages of non-anemic women and women with IDA did not differ (26±3.7 years *vs.* 25±3.8 years) (Table 1). Women and children with IDA had significantly lower mean Hb

Table 2. Properties of selected hepcidin and ZPP/H cut-off values for iron deficiency anemia diagnosis.

Study group	Biomarker Cut-off Value	Sensitivity	95% CI	Specificity	95% CI	LR+	LR-	Youden Index	Positive Predictive	Negative Predictive
	ZPP/H µmol/mol heme									
Children	>90	81	71.9 - 88.2	96	90.1 - 98.9	20.25	0.2	0.77	95.29	83.48
Women	>107	73	63.2 - 81.4	93	85.9 - 97.5	10.83	0.29	0.66	91.25	77.5
	Hepcidin ng/mL									
Children	≤6.85	86	77.6 - 92.1	94	87.4 - 97.8	14.33	0.15	0.80	93.48	85.45
Women	≤4.52	90	82.4 - 95.1	94	87.5 - 98.2	16.2	0.11	0.84	93.75	90.38

CI: confidence interval; +LR: positive likelihood ratio; -LR: negative likelihood ratio; ZPP/H: zinc protoporphyrin/heme.

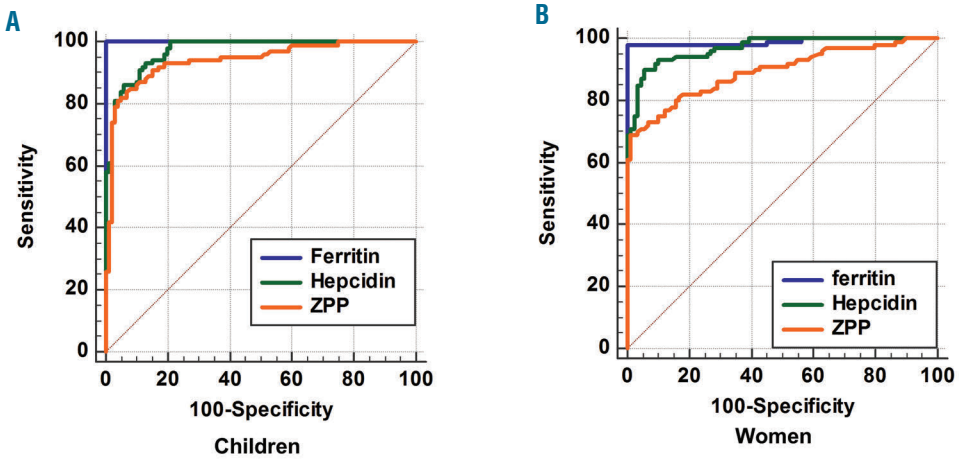

Figure 2. Receiver operating characteristic curves for ferritin, hepcidin and ZPP/H. Pairwise comparison of area under ROC curves of hepcidin and ZPP/H with ferritin as a gold standard for IDA diagnosis in (A) children and in (B) women. As noted, the AUC is similar between the gold standard (ferritin) and either hepcidin or ZPP/H indicating the inherent ability of these two biomarker tests to discriminate between the non-anemic and IDA groups. ZPP: zinc protoporphyrin.

(Children: 9.6 ± 0.8g/dL *vs.* 11.9 ± 0.6g/dL; Women: 10 ± 1.2g/dL *vs.* 13.2 ± 0.7 g/dL), mean corpuscular volume (Children: 65 *vs.* 77fl, Women: 74 *vs.* 86fl) and median ferritin (Children: 3.8 *vs.* 35ng/mL, Women: 3.7 *vs.* 39ng/mL) compared with non-anemic women and children, respectively (Table 1).

There were no differences in white blood cell and platelet counts between the two study groups (Table 1). Serum CRP values were low in both study groups (Table 1). As expected, sTfR was significantly higher in children and women with IDA compared with their non-anemic counterparts (Children: 3.9 *vs.* 1.7mg/L; Mothers: 3 *vs.* 1.3mg/L) (Table 1). Moreover, sTfR/log ferritin index values >2 in women and children with IDA confirmed a diagnosis of IDA and ruled out coexisting anemia of inflammation (Table 1).

Erythrocyte ZPP/H and serum hepcidin values in anemic and non-anemic groups

The median ZPP/H in children and women with IDA was higher when compared with their non-anemic counterparts (children: 151 µmol/mol heme [IQR: 104, 263] *vs.* 49 µmol/mol heme [IQR: 39, 60] and women: 155 µmol/mol heme [IQR: 101, 243] *vs.* 66 µmol/mol heme [IQR: 53, 83], $P<0.001$ for both) (Table 1). Median hepcidin concentration was markedly higher in non-anemic children and women when compared with their counterparts who had IDA (children: 42ng/mL [IQR: 25, 62] *vs.* 1.2ng/mL [IQR: 0.5, 3.6] and women: 17.7ng/mL [IQR: 9, 38] *vs.* 0.6ng/mL [IQR: 0.2, 1.3], $P<0.05$ for both) (Table 1).

Diagnostic cut-off for erythrocyte ZPP/H

ZPP/H >90 µmole/mole heme resulted in IDA diagnosis in children with 81% sensitivity and 96% specificity (Table 2). This cut-off had a positive likelihood ratio of 20.25 and a positive predictive value of 95.2% (Table 2). In women, a higher ZPP/H >107 µmole/mole heme resulted in IDA diagnosis with 73% sensitivity and 93% specificity. This cut-off in women yielded a positive likelihood ratio of 10.8 and a positive predictive value of 91.2% (Table 2). ROC curves for ZPP/H to diagnose IDA revealed an area under the curve $(AUC)^{ROC}$ of 0.94 in children (Figure 2A) and 0.89 ($P<0.0001$) in women (Figure 2B).

Diagnostic cut-off for serum hepcidin

Serum hepcidin value ≤6.85ng/mL yielded an IDA diagnosis in children with 86% sensitivity and 94% specificity (Table 2). This cut-off had a likelihood ratio of 14.3 with a positive predictive value of 93.4% (Table 2). In women, a hepcidin value of ≤4.5ng/mL resulted in an IDA diagnosis with 90% sensitivity and 94% specificity (Table 2). This cut-off corresponded with a likelihood ratio of 16.2 and a positive predictive value of 93.7%. ROC curves for hepcidin to diagnose IDA revealed an AUC^{ROC} of 0.97 in children (Figure 2A) and 0.96 ($P<0.0001$) in women (Figure 2B).

Discussion

In this study of healthy rural community-dwelling non-anemic Indian women and children and their counterparts with biochemically defined IDA, we 1) report the median values for the iron biomarkers erythrocyte ZPP/H and serum hepcidin, 2) analyze ROC curves for erythrocyte ZPP/H and serum hepcidin, and 3) define the ZPP/H ratio and serum hepcidin cut-off values for IDA diagnosis and estimate the post-test probability of IDA for these cut-off values. Overall, these findings demonstrate the utility of erythrocyte ZPP/H as a POC biomarker for IDA diagnosis, particularly in women and children from low-middle income settings.

We found similar median ZPP/H levels in non-anemic children to those reported previously (47.5 and 58 μmol/mol heme).[15,16] Although evaluated systematically in children and non-anemic pregnant women with iron deficiency,[5,17] ZPP/H levels have not been studied either in women or preschool children using rigorous criteria for nutritional IDA. Only one large Indian study of tribal adults and children (<18 years) previously used ZPP/H to detect IDA in a subset (n=100) of anemic individuals (mean Hb 8.4) with normal Hb phenotype.[18] The authors reported a higher mean ZPP/H value (214.9 ± 120.1) than in our study. This discrepancy may be explained by either the difference in the two study populations (severity of anemia or undetected Hb disorders) or methodological differences (i.e., whole blood *vs.* washed erythrocytes).[19] The median ZPP/H ratios reported in our study probably reflect values encountered in healthy women and children residing in rural Indian communities.

Serum hepcidin values in non-anemic children in our study are concordant with reports in European children,[20,21] but higher than values reported in Asian[22] and African children.[23,24] The inclusion of <12-month-old non-anemic children in the latter studies explain these differences, since hepcidin concentrations are decreased between three and six months of age.[24] Children with IDA in our study had serum hepcidin levels comparable with those reported in anemic children from Asia[25] and Africa.[23] Non-anemic women in our study had variable levels compared with those previously reported in European studies,[26,27] discrepancies that are possibly explained by socioeconomic and dietary differences between these populations. Some of the inter-study variability is also possibly attributable to differences in the methodological assays used to estimate hepcidin.[28] Women with IDA in our study had very low median hepcidin levels concordant with previously published studies.[29]

Using ROC analysis, we selected cut-off values for ZPP/H that detected IDA in both women and children with >90% specificity. Cut-off values selected in recent studies were lower (>40 μmole/mole[30,31] heme and >70 μmol/mole heme[6]) and lacked specificity (56% and 60%, respectively), perhaps because they were selected to detect iron deficiency, not IDA. Another recent study utilized a ZPP/H cut-off value of 70 μmole/mole to diagnose IDA in a pediatric population, but this value had a low specificity (75%).[32] The large Indian study referenced previously used a cut-off value of >80 μmole/mole heme to define IDA in a mixed population of healthy individuals and those with sickle cell trait or sickle cell anemia.[18] The scientific rationale for this cut-off value and the validity of ZPP/H as a stand-alone diagnostic assay for IDA in individuals with sickle cell anemia, and possibly α-thalassaemia,[33] is uncertain. In contrast, our study used rigorous biochemical criteria to define IDA in a representative sample of healthy community-dwelling women and children. Consequently, this is the first study to demonstrate the utility of ZPP/H as a biomarker of IDA and define cut-off values with which to establish an IDA diagnosis in healthy rural women and children.

The hepcidin cut-off values selected to diagnose IDA in children in our study were higher than those selected previously in Korean children (≤6.85ng/mL *vs.* ≤2.735ng/mL) and yielded higher AUCs (0.97 *vs.* 0.90).[25] The higher AUC value indicates a better discriminative power of hepcidin in detecting IDA. However, these cut-off values were similar to values that detect IDA in six to 60-month-old Gambian and Tanzanian children (5 and 8 ng/ml, respectively).[7]

The proposed cut-off values, with their high sensitivity and specificity, increase the probability of a diagnosis of IDA. However, we also determined the predictive values of these tests by estimating their likelihood ratios. The likelihood ratio indicates how many times more likely a particular test result is, in a patient with that particular condition, with a likelihood (LR) ratio value >10 providing robust diagnostic evidence.[34] Assuming a pre-test probability of IDA of 50% in any given population, the selected ZPP/H and hepcidin cut-off values had likelihood ratios of ~10, which according to the Fagan nomogram corresponded with a 90% post-test probability of having an IDA diagnosis.[35] Thus, even in populations with lower pre-test probabilities of having IDA, these diagnostic cut-offs are valid.

Measuring erythrocyte ZPP/H is procedurally simple, technically feasible by field health workers possessing <12th-grade education, and could provide rapid results at the primary health center. Rapid diagnosis would facilitate therapeutic decision-making in a single visit and favor patient convenience, an important consideration in low-middle income settings. Although not a prior study objective, informal assay cost estimates in our laboratory indicate that ZPP/H measurement is cheaper than hepcidin. Thus, our findings suggest that ZPP/H has greater utility as a POC diagnostic test to detect IDA in women and children. Although hepcidin is extremely useful in predicting iron absorption and incorporation into erythrocytes,[8] potential limitations of its use in this setting include higher costs, lack of hepcidin standardization and the requirement to convert the immunological assay into a POC assay.[36]

The strengths of this study are its inclusion of a large representative sample of healthy community dwelling women and children, a random selection of blood samples for biomarker measurements, and rigorous definition of IDA using multiple biomarkers.[4,37] Using a combination of low Hb with ferritin as a gold standard for IDA instead of bone marrow aspiration with perls staining raises potential concerns regarding diagnostic accuracy. Reassurance against this concern is provided by the sTfR/log ferritin index >2 and near normalization of Hb in response to iron therapy in children with IDA after six months treatment (baseline Hb for children in the IDA group = 9.6±0.8g/dL; six-month post-treatment Hb = 10.5±1.3g/dL). Finally, estimates of the diagnostic accuracy for the proposed cut-off values highlight the clinical applicability of these find-

ings for IDA diagnosis.[38] The results of this study are generalizable to women and children from similar agrarian parts of India and possibly to other similar low-middle income settings around the world.

In conclusion, the findings of this study provide a scientific rationale for the use of ZPP/H as a POC biomarker to establish the diagnosis of IDA in women and children from low-middle income settings. The diagnostic cut-off values and their accompanying likelihood ratio's provide clinicians with guidance for using these biomarkers to diagnose IDA.

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