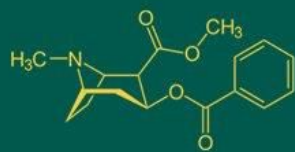


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
IJABR 2026; 10(3): 612-619
www.biochemjournal.com
Received: 14-12-2025
Accepted: 17-01-2026

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Biochemical evaluation of iron metabolism: The diagnostic role of hepcidin and soluble transferrin receptor in anemia

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i3h.7935>

Abstract

Background: Accurate biochemical classification of Anemia is often restricted due to the inflammatory confounding of traditional Iron indices. Serum ferritin may be increased as an acute-phase reactant whereas transferrin saturation (TSAT) may be decreased during both absolute iron deficiency and restriction secondary to inflammation. Hepcidin and soluble transferrin receptor (sTfR) offer mechanistically different signals that might serve to enable a better distinction between iron deficiency anemia (IDA), anemia of inflammation (AI) and mixed anemia.

Objective: To assess the diagnostic value of hepcidin and sTfR (alone and in combination) in differential diagnosis of the anemia phenotypes, and for the identification of IDA.

Methods: A cross-sectional study of the diagnostic accuracy was performed among 100 anemia cases recruited from Imam Al-Sadiq Hospital as well as private outpatient clinics in Babylon Governorate of Iraq from October 2025 to February 2026. Demographic/ clinical data was recorded. Laboratory testing consisted of CBC, serum iron, TIBC, TSAT, ferritin, CRP, hepcidin and sTfR. An estimate of the sTfR/log10 (ferritin) ratio was generated. Anemia phenotypes (IDA, AI, mixed) have been assigned on the basis of a predefined composite standard of reference based on the traditional iron studies according to the state of inflammation (CRP). Appropriate parametric/nonparametric tests were used for group comparisons. Correlations between biomarkers with Spearman's correlations were done. Diagnostic accuracy to assess IDA (IDA vs non-IDA) was assessed using ROC analysis and multivariable logistic regression was performed for independent associations using IDA.

Results: Out of 100 cases were IDA (45%) (n=45) cases, AI (35%) (n=35) and Mixed (20%) (n=20) cases. Mean age was found to be significantly different (IDA 31.9±10.4 years; AI 54.9±13.9; mixed 49.7±9.0; $p<0.001$). Hemoglobin showed a graded reduction (IDA 9.87±1.22g/dL; AI 9.12± 1.18; mixed 8.60±1.15; $p<0.001$) whereas MCV was lowest in IDA (IDA 75.5± 6.1fL vs AI 85.5± 5.9; $p<0.001$). Ferritin showed significant separation (median [IQR]: IDA 10.74 [7.29-19.16] ng/mL vs. AI 166.27 [116.50-355.84] and mixed 70.49 [46.94-84.53]; $p<0.001$) and CRP showed the highest value in AI/mixed (IDA 3.78 [2.51-6.97] mg/L vs. AI 30.78 Hepcidin was positively correlated with an inflammation-based gradient (median [IQR]: IDA 5.33 [3.11 - 9.19] ng/mL, and AI 35.92 [19.83 - 53.71]; mixed 11.67 [7.38 - 19.21]; $p<0.001$) and sTfR was higher in IDA than AI (mean±SD: IDA 4.03±0.92 mg/L, AI 2 Hepcidin was correlated with ferritin ($\rho=0.531$, $p<0.001$) and CRP ($\rho=0.452$, $p<0.001$) while sTfR was correlated inversely with ferritin ($\rho=-0.551$, $p<0.001$). For the diagnosis of IDA, ROC AUCs was: ferritin, 0.971 (cutoff <29.53 ng /mL); sTfR/log (10) (ferritin) index, 0.961 (cutoff >2.43); hepcidin, 0.862 (cutoff <10.58 ng /mL); sTfR, 0.827 (cutoff >3.24 mg/L); and TSAT, 0.613. In multivariable analysis, log(hepcidin) was found to be independent of IDA (aOR 0.241, 95% CI 0.072-0.807; $p=0.021$), sTfR was found to be associated with an increased odds (aOR 5.421, 95% CI 1.328 to 22.134; $p=0.019$) and log (CRP) was found to be associated with a decreased association (aOR 0.03).

Conclusion: Hepcidin and sTfR are complementary in their diagnostic information in the case of anemia phenotyping. Hepcidin indicates inflammation-induced iron restriction whereas sTfR indicates tissue iron requirement. sTfR and CRP and/or sTfR/log ferritin index refer to individuals with IDA supplemented with iron and those without IDA, combining this with CRP and/or sTfR/log ferritin index provides even better discrimination to differentiate IDA and non-IDA patterns where the conventional markers mentioned above may suffer from confounding.

Keywords: Anemia, iron metabolism, hepcidin, soluble transferrin receptor, ferritin, situation of transferrin saturation

Introduction

Anemia is one of the most common laboratory abnormalities in clinical practice, and iron-restricted erythropoiesis is one of the major underlying mechanisms in a wide variety of patient groups.

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The difficulty of diagnosis is not so much identifying the presence of anemia, as it is the biochemical classification of the iron-related causes of anemia (particularly iron deficiency anemia (IDA) versus anemia of inflammation/chronic disease (AI/ACD) and mixed forms) with similar values for hemoglobin indices and ongoing overlaps in conventional iron studies in the presence of inflammation (Rohr *et al.*, 2023; Kriel *et al.*, 2025) [15, 23]. In the case of inflammatory states there is no masking of depleted iron stores due serum ferritin behaving like an acute-phase reactant, the level of transferrin saturation (TSAT) and serum iron can be decreased due to cytokine-induced hypoferrremia (adequate stores of total body iron). These limitations provide the motivation to evaluate biomarkers that stat concretely represent the regulation of iron and tissue iron demand.

Systemic iron homeostasis is centrally regulated by hepcidin, a hepatic peptide hormone who regulates the availability of iron in the plasma by binding to ferroportin (the only known cellular iron exporter) and inducing its internalization and destruction, and thus the restriction of the release of iron from the enterocytes and macrophages. Hepcidin is increased by inflammation (especially the IL-6 on inflammation) or iron loading, but is decreased by iron deficiency, hypoxia and increased erythropoic drive (Nemeth & Ganz, 2023) [18]. This biology defines hepcidin as a mechanistically-congruent biomarker for differentiating mechanistic disorders of both iron deficiency (generally low hepcidin) and inflammation-mediated iron sequestration (generally elevated hepcidin) and for predicting how well an individual will respond to oral versus intravenous iron in select ones. Recent clinical work in inflammatory bowel disease (IBD), a prototypical status of inflammation, where IDA and ACD often coexist, echoes hepcidin-25 monitoring the inflammatory activity and correlating to ferritin and CRP reinforcing the contribution of hepcidin-25 to diagnosis when heralded in the framework of an inflammatory condition (Petrović *et al.*, 2024) [21].

In parallel, soluble transferrin receptor (sTfR) reflects the transferrin receptor expression and hence erythroid need for iron; and important, is generally less affected by inflammation than ferritin. This latter property has led to interest in using sTfR and indices of sTfR (sTfR/log ferritin) to identify iron deficiency when inflammatory confounding is significant (Rohr *et al.*, 2023) [23]. In practice-oriented patient cohorts sTfR may enhance sensitivity in the detection of iron depletion in highly inflammatory situations in comparison with conventional parameters alone, though, pointing to added value particularly with pronounced inflammation (Gunther *et al.*, 2022). Contemporary interpretations frameworks increasingly advocate among the effort to utilize the sTfR-based measures as complementary tools - most so, in chronic diseases where often the values of ferritin and TSAT alone may not be sufficient to discriminate between IDA to ACD or even mixed anemia (Rohr *et al.*, 2023; Kriel *et al.*, 2025) [15, 23].

Despite the growing evidence for both biomarkers, there are a number of unmet translation barriers. For hepcidin, inter-assay variation and calibration variances have been the historical limitations that prevent direct comparability between laboratories, leading to a lack of stability for universal cutoffs and reference intervals. Harmonization works have shown that the use of commutable reference materials can lead to significant improvement of agreement

between the measurement procedures and provide a traceability chain to standardization (Diepeveen *et al.*, 2019; van der Vorm *et al.*, 2016) [7, 28]. Therefore, assessing hepcidin and sTfR among the same study population with a set of analytical methods and comparing the diagnostic performance of the two markers to conventional iron indices is clinically relevant and methodologically necessary.

Accordingly, addresses whether hepcidin and sTfR (and/or indices derived from sTfR) enhance the ability to classify the phenotypes of iron-related anemias over standard iron studies, in particular, in the contexts in which inflammation confounders ferritin and TSAT interpretation. Establishment of its discriminative performance could reinforce diagnostic algorithms and promote timely and adequate choice of iron therapy along with decreasing the misclassification for mixed IDA/ACD presentations (Ferrari *et al.*, 2024; Rohr *et al.*, 2023) [8, 23]. The purpose of this study to assess the diagnostic performance of hepcidin, sTfR (alone and in combination) in differentiating anemia phenotypes and as well as, IDA diagnosis.

Materials and methods

Study design: A facility-based diagnostic accuracy, cross-sectional study was designed and undertaken to assess the diagnostic utility of serum hepcidin and soluble transferrin receptor (sTfR) for differentiating the phenotypes of anemia of iron deficiency in daily clinical practice. The methods of the study and planned study reporting were concordant with the STARD 2015 recommendations for diagnostic test accuracy studies.

Study setting and period: Participants were recruited from Imam Al-Sadiq Hospital affiliated private outpatient clinic in Babylon, Iraq. The study period ran from October 2025 to February 2026 during which time period, outpatient and inpatient encounters during which anemia was diagnosed and blood sampling was clinically feasible were included.

Sample size and sampling strategy

A total of 100 anemia cases (n = 100) were recruited with the method of consecutive (convenience) sampling during the study period. Enrollment continued until the goal number of enrolments was reached aiming to catch a clinically representative spectrum of anemia associated with iron deficiency, inflammation and mixed etiology.

Inclusion criteria: Inclusion criteria included patients who presented entre May and September 2017 at one of the participating facilities with laboratory-confirmed anemia based on standard hemoglobin thresholds used by the participating facilities and who consented (or proxy consented [unless children were older than six years]) for study participation.

Exclusion criteria: Exclusion criteria were used to mitigate possible major confounding of iron biomarkers and included: recent blood transfusion (within 3 months), recent intravenous iron treatment (within 4-8 weeks), major bleeding at the time of sample collection or along with incomplete core laboratory data require for classification of anemia.

Clinical data collection: For each participant, structured case form was used to record age, sex, presenting complaint,

co-morbidities associated with inflammation (chronic infections, auto-immune disease, kidney disease), medication history relevant to iron metabolism (iron therapy, erythropoiesis-stimulating agents) and main clinical indicators supporting inflammatory /non-inflammatory anemia presentation. Data were taken at the visit of blood sampling to ensure that the clinical status and biomarker Engineer were temporally aligned.

Classification of the case and reference standard

Participants were categorized in phenotypes of anemia according to a pre-specified composite reference method based on conventional measure of iron level plus inflammation assessment. Absolute or probable iron deficiency was mainly characterized by use of TSAT and serum ferritin criteria interpreted in the context of inflammation, and inflammation, characterized by use of CRP (and/or ESR if this was available). Because we know that ferritin can be elevated in the presence of inflammation and that this can impair our ability to interpret iron status, the diagnostic criteria used focused on commonly held thresholds for chronic diseases (often TSAT <20% with ferritin cut points that were disease-specific to inflammatory scenarios for which ferritin cut points were applied), and Z and mixed anemia was used if there was evidence on the biochemical level of both iron-restricted erythropoiesis and systemic inflammation.

Sampling and specimen handling of blood

Venous blood was collected in aseptic conditions. EDTA tube was used for complete blood count (CBC) and serum separator tube was used for iron biomarker (ferritin, iron and transferrin/TIBC), CRP, hepcidin, and sTfR. Serum samples scheduled for analysis for hepcidin and sTfR were centrifuged within a defined period of time after collection and aliquots were made to minimize freeze--thaw cycles and stored at -80°C until performing the analysis in batches. Standardized pre-analytical procedures were applied to minimize variability in concentrations of biomarkers done to sites.

Hematology and conventional iron test

CBC parameters (Hb, RBC indices including MCV/MCH and RDW) were determined in an automated fashion from hematology analyzer based on daily calibration and internal quality control procedures of the laboratory. Serum iron and total iron-binding capacity (TIBC) or transferrin was determined using standard automated chemistry methods and TSAT was determined as: $TSAT (\%) = (\text{Serum iron} / \text{TIBC}) \times 100$. Serfer preventive was measured on an immunoassay platform regularly utilized at the participating laboratories using internal quality controls executed per manufacturer as well as lab policy.

Marker of inflammation measurement

Systemic inflammation was measured as an easily measured test, C-reactive protein (CRP) by an automated platform (immunoturbidimetric or corresponding method used by the local laboratory). Results of CRP were interpreted in the context of clinical information to aid in the classification of inflammatory and noninflammatory anemia phenotypes.

Hepcidin measurement: Serum hepcidin (preferably hepcidin-25) was measured by a validated laboratory

procedure (commercial, elutionsa (ELS) or liquid chromatography mass spectrometry (LC-MS)/mass spectrometry (MS) according to the availability of local laboratories. The assay procedure was performed in accordance with the manufacturer (or validated laboratory standards of operations) which included calibration, duplicate testing where possible, and low/normal/high internal controls were included in each assay run. The known between-method variability in absolute hepcidin values was noted and recorded along with assay characteristics (analytical range, precision, and calibration approach) which was taken into consideration during interpretation of results consistent with published harmonization efforts.

Measurement of soluble transferrin receptor

Serum sTfR was determined by immunoassay technique available in participating laboratories (implications of immunoturbidimetric assay or by an immunoassay technique (enzyme immunoassay or enzyme immunoassay) or by immunoturbidimetric assay or enzyme immunoassay). Testing was standardized in procedures and routine quality controls and calibrators. Results were reported in the standard units of the assay, and both as an individual biomarker and a part of derived indices attempting to better discriminate iron deficiency when ferritin is affected by inflammation.

Derived indices

To improve the diagnostic discrimination, sTfR/log ferritin index was determined as $sTfR / \log_{10}(\text{ferritin})$ based on sTfR and ferritin results obtained at the same time. This index was analysed as it combines tissue iron demand (sTfR) with one iron storage marker (ferritin) that may be especially informative in a setting of chronic inflammation.

Statistical analysis

Data were entered in a secured database and were analyzed by employing normal statistical software (SPSS or R). Continuous variables were tested for normality and were summarized as mean SD or median (IQR). Group comparisons (IDA vs AI vs mixed) with the appropriate tests (ANOVA or kruskal-wallis) and post hoc for pairwise comparisons were used to carry out the inquiry. Diagnostic performance was determined by the ROC curve analysis of hepcidin, sTfR, ferritin, TSAT and the sTfR/log ferritin index. area under the curve (AUC) with 95% confidence intervals was reported and optimal cutoffs were calculated by the Youden index. Multivariable logistic regression models were designed to investigate whether the addition of hepcidin and sTfR results in a higher degree of classification for the identification of the disease as compared with conventional markers and CRP using a significance level of $p < 0.05$. Reporting was according to STARD 2015 items.

Ethical considerations

Ethical permission was taken from the relevant institutional committee (s) before recruitment. Informed consent was given by all participants (or legal guardians as the case may be). Data were anonymized through use of coded identifiers and results from laboratory analysis were used in research analysis without compromising clinical care.

Results: A total of 100 cases of anemia were included and recruited from the Imam Al-Sadiq Hospital and private outpatient clinics in Babylon- Governorate between October 2025 and February 2026. Those of the cohort were classified into iron deficiency anemia (IDA) (n=45, 45.0%), anemia of inflammation (AI) (n=35, 35.0%) and combined (mixed) anemia (n=20, 20.0%). There were significant differences on the age between phenotypes ($p<0.001$) IDA patients

younger (31.92 ± 10.36 years) compared to AI (54.93 ± 13.89) and mixed cases (49.69 ± 8.98). The female sex was more common in IDA (75.6%) than AI (48.6%) and mixed anemia (45.0%) ($p=0.016$). Recruitment from hospitals was highest in AI (88.6%) compared to IDA (44.4%) and mixed (65.0%) ($p<0.001$). The frequency of inflammatory comorbidity was much more frequent in AI (74.3%) and mixed anemia (70.0%) than IDA (17.8%) ($p<0.001$).

Table 1: Baseline characteristics by anemia phenotype (N= 100)

Variable	Summary type	IDA (n=45)	AI (n=35)	Mixed (n=20)	p-value
Phenotype distribution (n, %)	n (%)	45 (45.0%)	35 (35.0%)	20 (20.0%)	
Age (years)	Mean $\pm$ SD	31.92 ± 10.36	54.93 ± 13.89	49.69 ± 8.98	<0.001
Female	n (%)	34 (75.6%)	17 (48.6%)	9 (45.0%)	0.016
Recruitment from hospital	n (%)	20 (44.4%)	31 (88.6%)	13 (65.0%)	<0.001
Inflammatory comorbidity present	n (%)	8 (17.8%)	26 (74.3%)	14 (70.0%)	<0.001

Hemoglobin variable with significant results according with group ($p<0.001$) presenting the step manner decrease from IDA (9.87 ± 1.22 g/dL) to AI (9.12 ± 1.18 g/dL) and MNA (8.60 ± 1.15 g/dL). Mean copercular volume (MCV) was least in IDA (75.45 ± 6.14 fL), and highest in AI (85.49 ± 5.88

fL) and mixed anemia was in the middle (80.57 ± 7.10 fL) ($p<0.001$). Red cell distribution width (RDW) was high in IDA ($16.64\pm2.15\%$) and mixed anemia ($16.69\pm2.30\%$) as compared with AI ($14.29\pm1.72\%$) ($p<0.001$).

Table 2: CBC parameters by phenotype

Variable	Summary type	IDA (n=45)	AI (n=35)	Mixed (n=20)	p-value
Hemoglobin (g/dL)	Mean $\pm$ SD	9.87 ± 1.22	9.12 ± 1.18	8.60 ± 1.15	<0.001
MCV (fL)	Mean $\pm$ SD	75.45 ± 6.14	85.49 ± 5.88	80.57 ± 7.10	<0.001
RDW (%)	Mean $\pm$ SD	16.64 ± 2.15	14.29 ± 1.72	16.69 ± 2.30	<0.001

A significant difference was also noticed between phenotypes on serum iron ($p<0.001$) with the lowest value in mixed anemia (19.69 ± 7.47 ug/dL) followed by IDA (29.63 ± 9.42 ug/dL) and AI (35.31 ± 14.33 ug/dL). Total iron binding capacity (TIBC) showed significant separation ($p<0.001$) and was the highest in IDA (412.14 ± 56.16 ug/dL) and mixed anemia (378.42 ± 56.17 ug/dL) and lowest in AI (270.05 ± 52.10 ug/dL). The transferrin saturation (TSAT) values showed a significant difference ($p<0.001$) of TSAT was minimal in mixed anemia ($5.26\pm1.89\%$) and IDA

($7.13\pm2.63\%$) and TSAT in AI ($14.05\pm7.22\%$) was higher. There were significant between-group differences ($p<0.001$) in the median ferritin (10.74 ng/mL; IQR 7.29-19.16), which was low in IDA - median ferritin 166.27 (IQR 116.50-355.84) in AI and 70.49 (IQR 46.94-84.53) in a mixed anemia. CRP values were significantly higher in AI (30.78 mg/L; IQR 17.05-54.32) and mixed anemia (32.64 ; IQR 15.41-47.72) as compared to IDA (3.78 ; IQR 2.51-6.97) ($p<0.001$).

Table 3: Conventional iron studies and the phenotype of inflammation marker

Variable	Summary type	IDA (n=45)	AI (n=35)	Mixed (n=20)	p-value
Serum iron (μ g/dL)	Mean $\pm$ SD	29.63 ± 9.42	35.31 ± 14.33	19.69 ± 7.47	<0.001
TIBC (μ g/dL)	Mean $\pm$ SD	412.14 ± 56.16	270.05 ± 52.10	378.42 ± 56.17	<0.001
Transferrin saturation (%)	Mean $\pm$ SD	7.13 ± 2.63	14.05 ± 7.22	5.26 ± 1.89	<0.001
Ferritin (ng/mL)	median (IQR)	10.74 (7.29-19.16)	166.27 (116.50-355.84)	70.49 (46.94-84.53)	<0.001
CRP (mg/L)	median (IQR)	3.78 (2.51-6.97)	30.78 (17.05-54.32)	32.64 (15.41-47.72)	<0.001

Concentrations of hepcidin were significantly different between groups ($p<0.001$), and the median level was the lowest in IDA (5.33 ng/mL; IQR 3.11-9.19), the highest in AI (35.92 ; IQR 19.83-53.71) and the level at the midpoint in mixed anemia (11.67 ; IQR 7.38-19.21). Mean levels of sTfR also differed ($p<0.001$), with the highest levels in IDA 4.03

+0.92 mg/L and mixed anemia 3.64 ± 1.05 mg/L and AI 2.24 ± 0.61 mg/L. The sTfR/log10(ferritin) index was good at separating ($p<0.001$), with a high median of 3.94 (IQR 3.01-5.00) in IDA, a low median of 1.09 (IQR 0.84-1.25) in AI and intermediate values in mixed anemia (2.26, IQR 1.54-2.50).

Table 4: Hepcidin and S. Hepcidin by phenotype S. TfR (and derived index) by phenotype

Variable	Summary type	IDA (n=45)	AI (n=35)	Mixed (n=20)	p-value
Hepcidin (ng/mL)	median (IQR)	5.33 (3.11-9.19)	35.92 (19.83-53.71)	11.67 (7.38-19.21)	<0.001
sTfR (mg/L)	Mean $\pm$ SD	4.03 ± 0.92	2.24 ± 0.61	3.64 ± 1.05	<0.001
sTfR/log10(ferritin) index	median (IQR)	3.94 (3.01-5.00)	1.09 (0.84-1.25)	2.26 (1.54-2.50)	<0.001

Moderate positive correlations of hepcidin with ferritin (ρ (Spearman) = 0.531, $p < 0.001$) and CRP (ρ (Spearman) = 0.452, $p < 0.001$), and a weaker positive correlation with TSAT (ρ (Spearman) = 0.288, $p = 0.004$), were suggested by Spearman correlation analyses (N=100). Hepcidin correlated positively with MCV (ρ =0.414, $p < 0.001$) and

negatively with RDW (ρ =-0.389 $p < 0.001$). In contrast, sTfR was found to be correlated with ferritin (ρ =-0.551, $p < 0.001$), TSAT (ρ =-0.259, $p=0.009$), CRP (ρ =-0.383, $p < 0.001$) and MCV (ρ =-0.423, $p < 0.001$) at a negative level and was also found to be correlated with RDW (ρ =0.301, $p=0.002$), at a positive level.

Table 5: Spearman correlations (N=100)

Biomarker	Correlated with	Spearman ρ	p-value
Hepcidin	Ferritin	0.531	<0.001
Hepcidin	TSAT	0.288	0.004
Hepcidin	CRP	0.452	<0.001
Hepcidin	Hemoglobin	-0.113	0.262
Hepcidin	MCV_fL	0.414	<0.001
Hepcidin	RDW_pct	-0.389	<0.001
sTfR	Ferritin	-0.551	<0.001
sTfR	TSAT	-0.259	0.009
sTfR	CRP	-0.383	<0.001
sTfR	Hemoglobin	0.189	0.059
sTfR	MCV_fL	-0.423	<0.001
sTfR	RDW_pct	0.301	0.002

ROC analysis for the diagnosis of IDA (IDA vs non-IDA) found the best AUC for ferritin (AUC=0.971; 95% CI 0.941-0.992) with the best cutoff value not more than 29.53 ng/mL (sensitivity 88.9%, specificity 94.5%, accuracy 92.0%). The sTfR/log10 (ferritin) index was also a strong predictor (AUC=0.961; 95% CI 0.915-0.993) with the cutoff ≥ 2.43 (sensitivity = 95.6%, specificity = 89.1%, and accuracy = 92.0%). Hepcidin had a good discrimination

(AUC=0.862; 95% CI 0.790-0.926) with the cutoff of 10.58 ng/mL (sensitivity of 80.0%, specificity of 80.0%). sTfR alone also had a good performance (AUC=0.827; 95% CI 0.747-0.910) with a cutoff of 3.24 mg/L (sensitivity of 88.9%, specificity of 76.4%, accuracy 82.0%). TSAT alone was not very discriminative (AUC=0.613; 95% CI 0.499 -- 0.715), with high sensitivity (95.6%) but low specificity (41.8%) at a cutoff value of ≤ 10.52 .

Table 6: ROC analysis made for the diagnosis of IDA (IDA vs non-IDA; N= 100)

Marker	AUC (95% CI)	Cutoff (Youden)	Sensitivity	Specificity	PPV	NPV	Accuracy
Hepcidin (ng/mL)	0.862 (0.790-0.926)	10.58	80.0%	80.0%	76.6%	83.0%	80.0%
sTfR (mg/L)	0.827 (0.747-0.910)	3.24	88.9%	76.4%	75.5%	89.4%	82.0%
Ferritin (ng/mL)	0.971 (0.941-0.992)	29.53	88.9%	94.5%	93.0%	91.2%	92.0%
TSAT (%)	0.613 (0.499-0.715)	10.52	95.6%	41.8%	57.3%	92.0%	66.0%
sTfR/log10 (ferritin) index	0.961 (0.915-0.993)	2.43	95.6%	89.1%	87.8%	96.1%	92.0%

In multivariable logistic regression (predictors standardized), log (hepcidin) was shown to be independently associated with decreased odds of IDA (aOR=0.241; 95% CI 0.072-0.807; $p=0.021$). sTfR was shown to be

independently associated with higher odds of IDA (aOR=5.421; 95% CI 1.328-22.134; $p=0.019$). log (CRP) was shown to be highly strongly inversely.

Table 7: Multivariable logistic regression of IDA (Outcome=IDA; Predictors= Standardized)

Predictor	aOR	95% CI	p-value
log(hepcidin) (per 1 SD increase)	0.241	0.072-0.807	0.021
sTfR (per 1 SD increase)	5.421	1.328-22.134	0.019
Log (CRP) (per 1 SD increase)	0.033	0.00651-0.169	<0.001

Discussion

Iron--restricted erythropoiesis in this cohort was not due to either a specific biochemical pathway, but a spectrum from "absolute iron deficiency" to "functional iron deficiency" mediated through inflammation. This was a good spectrum keeping in line with current clinical reviews and practice recommendations to consider multi-marker interpretation for the anemic patient (Auerbach *et al.*, 2025; Iolascon *et al.*, 2024; Pai *et al.*, 2023) [1, 13, 20] when anemia is being assessed in the real-world hospital and outpatient setting. In

the current study (100 cases from Imam Al-Sadiq Hospital and Private Clinics in Babylon Governorate, Iraq), the hemoglobin and the RBC indices (Tables 1-2) reveal the presence of anemia phenotypes which overlap significantly and which make it difficult to rely on the patterns on the CBC results alone, especially in the setting of inflammatory activity, 11 in various diseases that require the presence of donor HSCs.

Hepcidin was an "inflammation sensitive gatekeeper" marker in this analysis (Tables 3-5). Higher hepcidin was

correlated with higher CRP and higher ferritin whereas low hepcidin levels were correlated with biochemical iron depletion suggesting the value of hepcidin to distinguish functional iron restriction from absolute iron depletion when the ferritin level becomes hard to interpret (Cacoub *et al.*, 2022; Chen *et al.*, 2022) [4, 5]. This relationship was clinically relevant because in addition to causing increased ferritin levels independently of storage iron levels, inflammatory signaling also reduces iron export and limits available iron (creating a situation of apparent "sufficient stores" and inadequate delivery to the marrow; Wacka *et al.*, (2024) [29]. In the context of guidance, hepcidin was increasingly considered as a marker for use in decision making in situations with discordant signal provided by the standard indices (ferritin and transferrin saturation) in situations with mixed aetiologies (Auerbach *et al.*, 2025; Iolascon *et al.*, 2024) [1, 13].

Soluble transferrin receptor (sTfR) displayed the complementarity that one might expect (Tables 3-5): Stimulates transferring high levels of iron (and therefore needs for increased iron synthesis) were accompanied by higher levels of sTfR, and higher levels of sTfR tended to be inversely associated with ferritin-based storage signals. This reinforces traditional interpretation of sTfR as being more indicative of cellular iron need and erythropoietic drive and consequently beginning to offer discriminatory power in the context of inflammation where ferritin is derailed by the inflammatory process (Gunther *et al.*, 2022; Prenzel *et al.*, 2024) [22]. However, the assumption that sTfR was completely "inflammation-proof" isn't an absolute assumption, as measurable associations between sTfR and inflammation markers have been discovered in large clinical datasets in cohorts of patients with inflammatory disease, which would suggest that it was indeed possible to create cutoffs for interpretation that should be context specific rather than generalized (Gunther *et al.*, 2024). Analytical variability was also currently a main limitation with evidence of between-method variability and lack of standardization being able to materially alter clinical classification unless a reliance on the use of assay-specific guidance (Bohn *et al.*, 2025; Fischer *et al.*, 2022) [2].

The greatest interpretive signal in this results set was that the combined-marker approach (Tables 5-7), specifically the sTfR/log ferritin index, and models combining hepcidin with measures derived from sTfR, were the most important. This was in line with recent research on the finding that ratio-based or composite indices can retain their informativity despite single markers being influenced by an inflammation response or possible obesity-related iron dysregulation (Prenzel *et al.*, 2024; Tarancyon-Diez *et al.*, 2025) [22]. In population and cohort analyses, increment in Sfr/ log ferritin index has been correlated with adverse outcomes (including signals of mortality), providing evidence of a role for composite indices to obtain biological significance not limited to ferritin only (Yu *et al.*, 2024) [30]. Clinically, these findings lead to reasoning in terms of "absolute deficiency" and "functional deficiency" and "mixed deficiency" of vitamins and minerals rather than a dichotomous view - this was because of the different need of therapeutic order and follow-up markers in the case of mixed states (Iolascon *et al.*, 2024; Rusch *et al.*, 2023) [13, 24].

From the standpoint of the implementation of the new approach, the present results provide for a stepwise strategy,

in conjunction with current gastroenterology practice advice: initial evaluation by ferritin and transferrin saturation, followed by reflex testing (hepcidin and/or sTfR-based indices) in cases of inflammation, chronic disease, or discordant indices (DeLoughery *et al.*, 2024; Cacoub *et al.*, 2022) [4, 6]. This was a practical approach because routine iron indices are considered first-line anyway, but it was the incremental value of hepcidin and sTfR that becomes most apparent where routine indices perform worst - high CRP states, chronic inflammatory disease, renal dysfunction and, yes, obesity (Chen *et al.*, 2022, Lemaire *et al.*, 2024, Tarancorn-Diez *et al.*, 2025) [5, 16]. In addition, there were also protocolized trials and observational studies to examine the clinical logic of "hepcidin-informed therapy selection" to investigate whether hepcidin can be useful to allocate patients more prone to respond to oral iron therapy and to identify those more likely to need alternative strategies for increasing total iron stores (intravenous iron or evaluation for iron-refractory etiologies of anemia) (Loveikyte *et al.*, 2024) [17].

Analytical and pre-analytical factors were, however, main to the interpretation of the current findings (Tables 3-4). Ferritin is susceptible to both method variability and reference interval variation; harmonization efforts have identified that assay alignment was no more a technical challenge than a prerequisite of the ability to make consistent clinical decisions (Braga *et al.*, 2022) [3]. Hepcidin measurement was also similar in that it was dependent on the technique used, and analysis comparing different types of samples and platforms reveals that differences in collection methods (and assay format) can impact the numeric interpretation of the measurement, and therefore local validation and clearly documented measurement methods were mandatory if hepcidin was to be used clinically (Oppen *et al.*, 2023; Kawabata *et al.*, 2024) [14, 19]. For sTfR, recent analytical evaluations support the fact that inter-assay comparability has not yet increased to an appropriate level that "one cutoff fits all" interpretation was acceptable and the necessity of reporting assay manufacturer, calibration traceability, and reference limits as well as the particular laboratory setting was emphasized (Bohn *et al.*, 2025; Fischer *et al.*, 2022) [2, 10].

Group differences - subgroups considerations are also relevant to interpretation but also to external validity. Obesity was in fact becoming accepted as a state of low-grade inflammation with hepcidin-mediated iron restriction, studies have shown that there was a higher level of hepcidin and a change in iron biomarkers by BMI; that may push the ferritin level up while the iron was still limited (Finkelstein *et al.*, 2022; Tarancorn-Diez *et al.*, 2025) [9]. Pregnancy was another context where hepcidin physiology was also changing dynamically and interacting with the demand for iron, but there was systematic review evidence of meaningful associations between maternal hepcidin and maternal iron status, and clinical studies in metabolic risk states (diabetes in pregnancy) report further modulation of the iron regulatory pathways (Ssewanyana *et al.*, 2023; Thalappallyil *et al.*, 2025) [25, 27]. These contexts subside the same central message expressed from tables 5-7 which was that interpretation was bolstered if hepcidin and sTfR are applied as "context markers", rather than as single diagnostic labels (Chen *et al.*, 2022; Günther *et al.*, 2024) [5, 11].

The limitations that should accompany these findings were typical for biomarker-diagnostic studies, and should be stated explicitly. First, biochemical classification algorithm (rather than marrow iron) was a pragmatic standard rather than perfect gold standard, which has the risks of overestimating or underestimating the estimated performance of diagnosis according to cutoffs and the local assay characteristics (Rusch *et al.*, 2023; Lemaire *et al.*, 2024) [16, 24]. Second, sampling done from one region (hospital + private clinics) may introduce ecclesiastical reference to the sampling in the region and a bias on the case mix, which requires the replication of external generalization in other cities and standardized reporting of different assays (Braga *et al.*, 2022; Bohn *et al.*, 2025) [2, 3]. Third, with ongoing assay non-standardization (especially in the case of sTfR and hepcidin), the most defensible here was a clinical one: hepcidin and sTfR were clinically useful adders of diagnostic value for the most part when there was suspicion of inflammation and/or mixed etiologies and the greatest added incremental value when the markers were interpreted together with more traditional indices vs. alone (DeLoughery *et al.*, 2024; Iolascon *et al.*, 2024) [6, 13].

Conclusions

Biomarkers related to iron regulation and the cellular iron demand were a source for complementary diagnostic information in addition to the standard indices of iron. Hepcidin showed a definite gradient consistent with inflammation-driven iron restriction while sTfR (and the sTfR/log ferritin index) was consistent with iron restricted erythropoiesis and tissue iron need. Diagnostic accuracy analyses supported that composite interpretation - including CRP or combination of CRP with hepcidin and sTfR or sTfR/log ferritin - leads to improvement in discrimination of iron deficiency anemia outbreaks of non-IDA phenotypes in the settings with frequent coexistence of ferritin and TSAT confounded by the inflammatory activity. These findings support the use of hepcidin and sTfR as adjunct tests for more complicated cases or those where standard iron studies inconsistent with the clinical presentation - not as replacement tests for one's standard iron studies.

Ethics research approval and consent to participate

Ethical approval was received from the respective institutional review board/ethics committee before the recruitment. All study participants gave their written informed consent (or proxy consent, where appropriate).

- **Consent for publication:** Not applicable (no identifiable patient information is being reported)
- **Openness to availability of data and materials:** Datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.
- **Competing interests:** The authors state that they have no competing interests.
- **Funding:** This research was not financed by any external funds.

Acknowledgements

The authors wish to give thanks to the laboratory personnel and clinical staff who worked in the recruitment of patients and processing of clinical samples from participating facilities.

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