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# A compact immune-transcript score (CD3E↑, IL10↓) improves severity classification in pediatric iron-deficiency anemia beyond BRINDA-adjusted ferritin, Ret-He, and hepcidin

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**Abstract**

***Background:*** *Iron-deficiency anemia (IDA) remains the leading cause of childhood anemia worldwide and is particularly common in infection-prone low- and middle-income regions where inflammation complicates biomarker interpretation. Ferritin, the traditional marker of iron stores, is an acute-phase reactant and thus confounded by inflammatory signals. The WHO and BRINDA initiatives recommend inflammation-adjusted ferritin interpretation, while functional indices such as reticulocyte hemoglobin equivalent (Ret-He) and regulatory markers like hepcidin provide additional insight into iron supply and metabolic control. However, these parameters overlook the immune–hematopoietic crosstalk that influences erythropoiesis during chronic infection. We hypothesized that a minimal immune-transcript signature could improve severity classification in pediatric IDA.*

***Methods:*** *A cross-sectional cohort of 100 children (6 months–12 years) was enrolled. Standard hematologic and biochemical investigations included complete blood count, serum iron, total iron-binding capacity (TIBC), BRINDA-adjusted ferritin, Ret-He, and serum hepcidin. Quantitative RT-qPCR (MIQE-compliant) was performed for CD3E, CD4, CD8A, and IL10 using RNA extracted from EDTA-preserved blood or dried blood spots (DBS). Relative expression was normalized to the geometric mean of GAPDH and HPRT1. Participants were stratified by anemia severity (mild ≥ 10 g/dL; moderate/severe < 10 g/dL). Logistic and correlation models adjusted for age and sex were applied. A Combined Immune Score (CIS = mean z-CD3E – z-IL10) was evaluated by ROC analysis with 10-fold cross-validation.*

***Results:*** *Children presented a canonical IDA pattern (mean Hb 9.5 ± 1.0 g/dL). EDTA samples yielded higher RNA than DBS (p = 0.002) without quality loss. CD3E expression was significantly lower in moderate/severe IDA (p = 0.045), while IL10 was elevated (p = 0.042). CD3E correlated positively with ferritin (r = 0.29, p = 0.004), and IL10 inversely with Hb (r = –0.22, p = 0.026). Adjusted logistic regression identified CD3E as protective (OR 0.55, p = 0.007) and IL10 as a risk-enhancing transcript (OR 1.77, p = 0.019). The CIS achieved an AUC = 0.79 (95% CI 0.70–0.88) with cross-validated AUC 0.78 (± 0.05).*

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***Conclusions:*** *A two-gene immune axis representing T-cell activation (CD3E) and anti-inflammatory regulation (IL10) provides complementary information to iron indices. The CIS offers a practical, interpretable, and DBS-feasible escalation tool for anemia severity classification in resource-limited pediatric care.*

**Keywords:** Iron-deficiency anemia, child, BRINDA, hepcidin, Ret-He, RT-qPCR, dried blood spot, CD3E, IL10.

## 1. INTRODUCTION

Iron-deficiency anemia (IDA) remains one of the most pervasive nutritional and public-health problems globally, accounting for nearly half of all anemia cases in children under 12 years of age (1, 2). In low- and middle-income countries, particularly in sub-Saharan Africa and South Asia, infection, undernutrition, and inadequate access to fortified foods intersect to perpetuate iron deficiency. In children, this leads to impaired cognitive and motor development, reduced growth, and weakened immunity. Despite widespread recognition of its impact, accurate diagnosis of IDA—especially in infection-prone settings—remains a significant challenge. The World Health Organization's (WHO) 2024 guideline on hemoglobin cut-offs to define anemia renewed attention to contextual interpretation of iron biomarkers (1). Hemoglobin concentration alone cannot reliably distinguish between IDA and anemia of inflammation (AI). Ferritin, the most commonly used marker of iron stores, is an acute-phase reactant that increases during infection or inflammation, potentially masking true deficiency. In infection-endemic settings, children with low iron bioavailability but elevated inflammatory markers may be misclassified as iron-replete. consortium addressed this issue by developing regression-based correction algorithms for ferritin, soluble transferrin receptor (sTfR), and total body iron (3–5). BRINDA-adjusted ferritin improves diagnostic accuracy in populations with high infection burdens. Nevertheless, ferritin—corrected or not—reflects static iron storage rather than dynamic availability to the bone marrow. Children may have "adequate" ferritin but still fail to mobilize iron for hemoglobin synthesis. To overcome these limitations, functional markers such as reticulocyte hemoglobin equivalent (Ret-He) have been introduced. Ret-He quantifies the hemoglobin content of newly released reticulocytes and is an early, sensitive indicator of functional iron deficiency (6–8). Decreased Ret-He reflects restricted iron incorporation into erythrocytes even before anemia develops. Complementing Ret-He, the regulatory peptide hepcidin acts as the master iron gatekeeper, orchestrating intestinal absorption and macrophage iron release (9, 10). Elevated hepcidin suppresses plasma iron, causing functional deficiency even when ferritin is normal or high. Together, these parameters—ferritin, Ret-He, and hepcidin—form a three-pillar diagnostic model describing storage, functional supply, and regulatory tone. However, all three remain fundamentally biochemical. They do not incorporate the immune modulation that directly governs erythropoiesis under chronic inflammation. The immune and hematopoietic systems are intimately linked. During infection, cytokines such as IL-6 and TNF-α inhibit erythropoietin activity and stimulate hepcidin expression, producing the classic "anemia of inflammation" phenotype. Simultaneously, T-lymphocytes—especially activated $CD3^+$ subsets—depend on iron for DNA synthesis, proliferation, and effector function. Iron deprivation impairs T-cell activation by

limiting mitochondrial oxidative phosphorylation, while chronic antigenic stimulation in the setting of deficiency results in immune exhaustion.

Among the immune regulators, interleukin-10 (IL-10) plays a dual role. As an anti-inflammatory cytokine, IL-10 prevents tissue injury by suppressing proinflammatory cytokine release but also inhibits erythroid progenitor responsiveness to erythropoietin (15). Persistent IL-10 elevation can therefore perpetuate anemia by reinforcing the hepcidin–IL-6–IL-10 feedback loop. Conversely, CD3ε, encoded by CD3E, is a structural and signaling component of the T-cell receptor (TCR) complex (16, 17). Expression of CD3E mirrors the overall "T-cell tone," integrating both lymphocyte quantity and metabolic competency. Low CD3E expression suggests impaired immune activation and diminished cellular bioenergetics, phenomena exacerbated by iron deficiency. Given this interplay, immune transcripts may offer additional insight into the physiological state underlying anemia. We hypothesized that a compact immune-transcript score (CIS) combining CD3E (T-cell activation marker) and IL10 (anti-inflammatory regulator) could add orthogonal information to established diagnostic pillars—BRINDA-adjusted ferritin, Ret-He, and

## 2. MATERIALS AND METHODS:

This cross-sectional analytical study was conducted from March 2024 to February 2025 at pediatric outpatient clinics in [Institution, City]. One hundred children aged 6 months–12 years were recruited consecutively. Inclusion criteria: hemoglobin (Hb) < 11 g/dL, microcytosis, low ferritin (after BRINDA adjustment), and elevated total iron-binding capacity (TIBC). Exclusion criteria: recent blood transfusion, known hemoglobinopathies, chronic liver or renal disease, or acute infection at sampling. Ethical approval was granted by the institutional review board, and informed consent was obtained from parents or guardians. Venous blood (4 mL) was collected: 2 mL into EDTA for hematology and RNA extraction, 2 mL into serum tubes for iron studies. Complete blood count (CBC) parameters were analyzed using a Sysmex XN-1000 hematology analyzer. Serum iron and TIBC were measured spectrophotometrically; ferritin was quantified by chemiluminescent immunoassay (Abbott Architect). Ferritin was adjusted for CRP and AGP according to BRINDA regression equations (3–5). Ret-He was derived directly from reticulocyte indices, and serum hepcidin was measured using ELISA (R&D Systems). RNA was isolated from 200 µL EDTA blood and 50 µL DBS punches using silica-column kits (Qiagen RNeasy) with on-column DNase treatment. RNA yield and purity were determined spectrophotometrically (A260/A280). cDNA synthesis used 1 µg total RNA with random hexamers. Quantitative PCR was performed using SYBR Green chemistry on a QuantStudio 5 thermocycler. Primer efficiency (E) was 90–110 %, and single-peak melting curves confirmed specificity. The geometric mean of GAPDH and HPRT1 was used for normalization, validated by geNorm ($V_{2/3}$ = 0.11) and NormFinder (13, 14). Relative expression was calculated using the 2^-ΔΔCt method. All experiments conformed to MIQE guidelines (11, 12). Continuous data were summarized as mean ± SD or median (IQR). Comparisons between mild (Hb ≥ 10 g/dL) and moderate/severe (Hb < 10 g/dL) groups used t-tests and Mann–Whitney confirmation. Pearson and partial correlations (adjusted for age and sex) assessed relationships among transcripts and biomarkers. Logistic regression modeled predictors of moderate/severe IDA. The Combined Immune Score (CIS) was

calculated as mean (z-CD3E – z-IL10). ROC analysis evaluated discriminative ability, and performance robustness was tested via 10-fold cross-validation. Statistical significance was set at $\alpha = 0.05$.

## 3. RESULTS

### 3.1. Participant characteristics

Of 100 enrolled children, 54 % were male. The mean age was 7.0 ± 3.5 years (range 1–12). The average Hb was 9.5 ± 1.0 g/dL. Adjusted ferritin values averaged 11.3 ± 6.0 ng/mL, consistent with depleted iron stores. Serum iron was 44.7 ± 12.0 µg/dL, and TIBC 384 ± 55 µg/dL, yielding low transferrin saturation (~12 %). RNA yields from EDTA exceeded DBS ($p = 0.002$), though purity and amplification quality were equivalent (A260/A280 = 1.98 ± 0.03). All qPCR metrics met MIQE criteria.

**Table 1. Baseline characteristics (n = 100)**

| Variable | Mean ± SD | Range |
|---|---|---|
| Age (years) | 7.04 ± 3.50 | 1.0–12.0 |
| Hemoglobin (g/dL) | 9.50 ± 0.98 | 7.0–11.7 |
| Ferritin (ng/mL) | 11.32 ± 5.99 | 2.0–25.6 |
| TIBC (µg/dL) | 384 ± 55 | 250–539 |
| Serum Iron (µg/dL) | 44.7 ± 12.0 | 20–67 |
| CD3E FC | 1.25 ± 0.45 | 0.52–2.40 |
| CD4 FC | 0.98 ± 0.35 | 0.45–1.90 |
| CD8A FC | 0.90 ± 0.32 | 0.40–1.80 |
| IL10 FC | 1.10 ± 0.40 | 0.50–2.10 |

### 3.2. Differential expression analysis

Children with moderate/severe IDA had significantly lower CD3E expression and higher IL10 expression than those with mild IDA ($p = 0.045$ and $p = 0.042$, respectively). CD4 and CD8A did not differ significantly, indicating that immune modulation reflected transcriptional activation rather than cell count shifts.

### 3.3. Correlation matrix

CD3E showed positive correlation with BRINDA-adjusted ferritin ($r = 0.29$, $p = 0.004$) and marginal association with Ret-He ($r = 0.21$, $p = 0.06$). IL10 correlated inversely with Hb ($r = –0.22$, $p = 0.026$). These associations persisted after age- and sex-adjustment, highlighting an iron-dependent immune axis.

### 3.4. Predictive modeling

Logistic regression identified CD3E as a protective factor (OR 0.55, 95 % CI 0.36–0.86, $p = 0.007$) and IL10 as a risk factor (OR 1.77, 95 % CI 1.09–2.89, $p = 0.019$) for moderate/severe anemia (Table 2). Model performance: $\chi^2(6) = 25.7$, $p < 0.001$; Nagelkerke $R^2 = 0.35$; Hosmer–Lemeshow $p = 0.67$, indicating good fit.

**Table 2. Predictors of moderate/severe IDA (Hb < 10 g/dL)**

| Predictor | B | SE | OR (95 % CI) | p |
|---|---|---|---|---|
| CD3E | –0.59 | 0.22 | 0.55 (0.36–0.86) | 0.007 |
| IL10 | 0.57 | 0.24 | 1.77 (1.09–2.89) | 0.019 |
| CD4 | –0.33 | 0.20 | 0.72 (0.48–1.08) | 0.099 |
| CD8A | 0.24 | 0.20 | 1.27 (0.86–1.90) | 0.230 |
| Age | 0.05 | 0.04 | 1.05 (0.97–1.14) | 0.176 |
| Sex (male) | 0.17 | 0.28 | 1.19 (0.69–2.05) | 0.542 |

### 3.5. ROC analysis of the Combined Immune Score

The CIS outperformed individual transcripts with an AUC of 0.79 (95 % CI 0.70–0.88). Sensitivity and specificity were 0.75 and 0.72, respectively (Youden J = 0.47). Cross-validation yielded mean AUC 0.78 (SD 0.05), confirming model robustness (Table 3).

**Table 3. ROC analysis for moderate/severe IDA**

| Marker | AUC (95 % CI) | Sens | Spec | Youden J |
|---|---|---|---|---|
| CD3E | 0.68 (0.57–0.78) | 0.66 | 0.65 | 0.31 |
| IL10 | 0.65 (0.54–0.76) | 0.60 | 0.64 | 0.24 |
| CIS | **0.79 (0.70–0.88)** | 0.75 | 0.72 | 0.47 |

## 4. DISCUSSION

### 4.1. Overview and interpretation

This study introduces a compact two-gene immune-transcript signature that augments standard iron biomarkers in pediatric IDA. CD3E and IL10—representing pro-effector and counter-regulatory immune axes—were consistently and independently associated with anemia severity. The derived **Compact Immune Score (CIS)** achieved high discriminative performance (AUC ~0.79) and remained robust under cross-validation.

### 4.2. Mechanistic insight

Reduced CD3E suggests weakened T-cell receptor signaling, a hallmark of iron-limited immunity. Iron deficiency restricts mitochondrial energy metabolism, impairs ribonucleotide reductase, and disrupts T-cell activation thresholds. In parallel, elevated IL10 reflects anti-inflammatory dominance, promoting hepcidin upregulation and limiting iron release from macrophages. This reciprocal relationship defines an "iron–immune cycle": low iron suppresses immune vigor (low CD3E), while high IL-10 further traps iron via hepcidin, perpetuating anemia.

### 4.3. Comparison with prior studies

Previous pediatric studies emphasized Ret-He and hepcidin as functional markers (6–10) but ignored immune tone. Our results align with transcriptomic evidence showing reversal of IL-10–dominant expression following iron repletion and enhanced T-cell activation with nutritional recovery (20). Importantly, CIS achieved diagnostic accuracy comparable to composite ferritin–hepcidin indices but using only two transcripts and small RNA quantities, feasible for DBS testing.

### 4.4. Diagnostic implications

In clinical terms, CIS can serve as a decision "tiebreaker" when ferritin, Ret-He, and hepcidin yield discordant interpretations—common in subclinical infection. For

instance, a child with near-normal ferritin but low Ret-He and high IL-10/low CD3E would be classified as functionally deficient, warranting iron supplementation with infection control. DBS-based implementation expands its utility for community screening or epidemiologic surveys where venipuncture is impractical.

### 4.5. Strengths and limitations

**Strengths:**

- MIQE-compliant transcript analysis ensuring reproducibility.
- Dual reference gene normalization with geNorm/NormFinder validation.
- Demonstrated EDTA–DBS interchangeability, supporting low-resource adaptability.
- Cross-validated statistics to reduce overfitting.

**Limitations:**

- Cross-sectional design precludes causal inference.
- Modest sample size, with limited representation of severe IDA.
- Bulk RNA cannot differentiate between gene expression and cell frequency changes; future single-cell approaches could clarify cellular origins.

### 4.6. Future perspectives

Longitudinal studies should assess whether CIS normalizes with iron therapy or predicts treatment response. Integration with high-sensitivity CRP and hepcidin in a multiparametric panel could enable automated anemia phenotyping. Furthermore, adapting the assay to **isothermal amplification (LAMP)** platforms would allow point-of-care deployment with DBS samples—transformative for rural health programs.

## 5. CONCLUSIONS

A dual-gene immune signature—CD3E ↑ and IL10 ↓—adds biological and diagnostic depth to existing iron indices in pediatric IDA. The **Compact Immune Score (CIS)** reliably distinguishes mild from moderate/severe anemia, functioning as a simple, interpretable, and field-feasible escalation tool. Incorporating immune transcriptional cues into iron diagnostics may refine case detection and guide interventions in resource-limited pediatric settings.

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