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Serum Copper Homeostasis and Its Relationship with Iron Deficiency Anemia in Children

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Abstract

Copper-dependent ferroxidases are required for cellular iron export, but local pediatric data on copper status in iron deficiency anemia (IDA) are limited. This comparative cross-sectional study evaluated serum copper homeostasis in 300 children aged 1-15 years at Civil Hospital, Ghulam Muhammad Mahar Medical College, Sukkur, Pakistan. The study included 150 biochemically confirmed IDA cases and 150 age-matched healthy controls. Complete blood count, serum iron, ferritin, and copper were measured using validated hematological and biochemical procedures; serum copper was estimated by a bathocuproine-based method on the ADVIA 1800 Chemistry analyzer with trace-element-aware sample handling. Children with IDA showed significantly lower mean serum copper than controls (86.34 ± 0.83 vs 120.41 ± 1.02 µg/dL; $P < 0.001$). Serum iron (63.71 ± 1.54 vs 105.80 ± 1.52 µg/dL) and ferritin (8.57 ± 2.14 vs 35.68 ± 4.89 ng/mL) were also significantly reduced. Within the IDA group, serum copper correlated positively with serum iron ($r = 0.52$; $P < 0.001$) and ferritin ($r = 0.49$; $P < 0.001$). These findings indicate disturbed copper homeostasis accompanying pediatric IDA and support selective copper assessment in severe, recurrent, or treatment-resistant cases.

Keywords:Serum copper,Iron deficiency anemia,Ferritin,Bathocuproine,Children, Sindh

Introduction

Iron deficiency anemia (IDA) remains the most frequent nutritional anemia of childhood and continues to impair growth, immunity, neurodevelopment, and school performance, particularly in low- and middle-income settings where poor dietary diversity, infection, and undernutrition coexist [1-6]. Diagnostic interpretation is strengthened when hemoglobin is evaluated together with red-cell indices and biochemical indicators of circulating and stored iron, because hemoglobin alone may not fully explain the underlying micronutrient disturbance [3-6]. Anemia and iron deficiency are prevalent among children in Pakistan, and recent studies have shown that these conditions are affected by factors such as malnutrition, infections, and poverty [7-9]. Copper is involved in IDA due to the role of ferroxidases, which include ceruloplasmin and hephaestin, in oxidizing ferrous iron through ferroportin and transporting it as transferrin [10-12].

Copper deficiency may therefore disturb iron mobilization even when iron intake is being addressed. Clinical reports and reviews have also shown that hypocupremia may present with anemia, neutropenia, or cytopenia, whereas pediatric studies show that

copper status varies by age, nutritional status, and inflammation [13-16].

Published pediatric and adolescent studies from different regions have reported heterogeneous copper patterns in anemia, including higher, lower, or unchanged copper concentrations depending on nutritional status, inflammatory background, age group, and coexisting trace-element imbalance [17-24]. This variability supports the need for locally generated data rather than assuming a uniform copper response in all children with IDA. Recent pediatric trace-element reviews and biomarker studies further support a broader micronutrient approach in IDA, including interpretation of copper status together with iron and zinc rather than as an isolated marker [25, 26].

Sukkur and the adjoining districts of upper Sindh include many children from nutritionally vulnerable households who attend Civil Hospital, Ghulam Muhammad Mahar Medical College, Sukkur, for pediatric care. However, local data linking serum copper with biochemically confirmed pediatric IDA are scarce. Therefore, this study was designed to compare serum copper levels between children with IDA and healthy controls and to assess the relationship of serum copper with hemoglobin, serum iron, and ferritin.

Materials and Methods

Chemicals and Reagents

Analytical-grade nitric acid (HNO_3), perchloric acid ($HClO_4$), hydrochloric acid (HCl), acetate buffer reagents, hydroxylamine hydrochloride/ascorbic-acid reducing reagent, bathocuproine or bathocuproine-disulfonate chromogenic reagent, deionized water, reagent blanks, and aqueous copper standards were used for serum copper estimation. Ferrozine chromogenic reagent, acidic iron-release reagent, and reducing reagent were used for serum iron estimation, while commercial calibrators and kit reagents for ferritin were used. Chemicals were procured from certified suppliers, mainly Merck/Sigma-Aldrich (Darmstadt, Germany) grade reagents, and routine clinical chemistry reagents were supplied through Siemens-compatible laboratory channels (Siemens Healthcare Diagnostics, Tarrytown, NY, USA). Trace-element-free polypropylene tubes, acid-washed plasticware, and deionized water were used throughout to minimize external copper contamination.

Study Design and Setting

This comparative cross-sectional study was conducted at Civil Hospital, Ghulam Muhammad Mahar Medical College, Sukkur, Sindh, Pakistan, from August 2024 to July 2025.

Study Population and Eligibility

A total of 300 children aged 1-15 years were enrolled, comprising 150 biochemically confirmed IDA cases and 150 apparently healthy age-matched controls. IDA was defined using age-appropriate hemoglobin cutoffs together with hematological evidence of microcytosis and hypochromia and biochemical evidence of low serum iron and low serum ferritin [3-6]. Children with known hemoglobinopathies, chronic liver disease, kidney disease, malignancy, acute infection at presentation, recent blood transfusion, or current micronutrient supplementation were excluded.

Ethical Considerations

The study protocol was reviewed within the relevant institutional ethical framework. Written informed consent was obtained from parents or legal guardians before sample collection, and assent was

obtained from older children where appropriate.

Instrumentation

Complete blood count was performed on an automated hematology analyzer (Nihon Kohden MEK-6420, Japan). Serum iron and ferritin were measured on the Siemens ADVIA 1800 Chemistry analyzer (Siemens Healthcare Diagnostics, USA) with routine calibration and internal quality-control procedures. Serum copper absorbance was read on the ADVIA 1800 colorimetric platform and cross-checked on a UV-Visible double-beam spectrophotometer (Lambda 365, Perkin-Elmer, USA).

Sample Collection and Handling

Before venipuncture, each participant underwent eligibility screening and clinical assessment. Approximately 3-5 mL of venous blood was collected aseptically during the morning hours. Blood for complete blood count was transferred into EDTA tubes, whereas blood for serum chemistry and trace-element analysis was transferred into plain/trace-element-aware tubes. Serum was separated after clotting and centrifugation and was handled in acid-washed polypropylene containers to avoid external metal contamination.

Hematological and Biochemical Analysis

Hemoglobin (Hb), hematocrit (HCT), red blood cell (RBC)count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were measured using the automated hematology analyzer. Serum ferritin was measured by routine immunoassay protocol. Serum iron was measured by the ferrozine colorimetric method, in which protein-bound iron is released under acidic conditions, ferric iron is reduced to ferrous iron, and ferrous iron forms a colored complex with ferrozine for absorbance-based quantification [28].

Calculation of Serum Iron

Serum iron was estimated by the ferrozine colorimetric method. In the automated ADVIA 1800 procedure, analyzer-controlled aliquots of serum, calibrator, reagent blank, and control were mixed with acidic releasing reagent to dissociate iron from transferrin. Ferric iron was reduced to ferrous iron by hydroxylamine/ascorbate-containing reducing reagent, and ferrous iron then reacted with ferrozine to form a violet Fe(II)-ferrozine complex. For a manual-equivalent description, 200 µL serum or standard may be mixed with 1.0 mL acidic buffer/reducing reagent, incubated for 5-10 min at room temperature, followed by 1.0 mL ferrozine-chromogen; absorbance was read at 562 nm against reagent blank. Aqueous iron standards were prepared in the working calibration range and processed similarly. Concentration was calculated from the calibration curve after blank subtraction and expressed as µg/dL; calibration solutions were maintained in µg/mL to avoid unit mixing.

Serum Copper Determination

Serum copper was quantified by a bathocuproine-based colorimetric method on the ADVIA 1800 Chemistry analyzer and cross-checked spectrophotometrically. For protein-bound copper release, 0.5 mL serum was digested with 2.0 mL freshly prepared nitric acid:perchloric acid mixture (4:1, v/v) in acid-washed glass tubes under controlled heating until a clear digest was obtained. After cooling, the digest was diluted to a defined volume with deionized water. A 1.0 mL aliquot of digested sample, standard or blank was treated with 2.0 mL acetate buffer, 0.5 mL reducing reagent to convert Cu(II) to Cu(I), and 1.0 mL bathocuproine/

bathocuproine - disulfonate chromogenic reagent. The final volume was made up with deionized water, mixed, and incubated for 10-15 min for color development. Absorbance of the Cu(I)-bathocuproine complex was measured at 580 nm against a reagent blank. Copper standards covering 0.25-4.0 µg/mL were processed in parallel, and serum copper concentration was obtained from the external calibration curve after blank correction and reported as µg/dL [27, 29].

Assay Calibration, Precision and Quality Control

The copper assay showed a linear response over 0.25-4.0 µg/mL with R^2 approximately 0.999. Reagent blank, aqueous standards, low and high quality-control sera, and duplicate samples were included in each analytical batch. Mean analytical recovery was 98.30%, RSD was 2.6%, and intra-assay and inter-assay coefficients of variation were below 5%, confirming acceptable precision for clinical trace-element comparison.

Statistical Analysis

Data were entered and analyzed using IBM SPSS Statistics for Windows, Version 26. Continuous variables were summarized as mean ± standard deviation. Between-group comparisons were performed using an independent-samples t-test. Severity-wise hematological comparisons were interpreted descriptively across mild, moderate, and severe IDA categories. Pearson correlation analysis was used to evaluate associations of serum copper with selected hematological and biochemical variables within the IDA group. Tests were two-tailed, and $P < 0.05$ was considered statistically significant.

Results and Discussion

A total of 300 children were included, with 150 IDA cases and 150 healthy controls. The case and control groups were age-matched, and the gender distribution was comparable, with boys forming approximately 47% and girls approximately 53% in each group (Table 1). This matched design minimized major demographic imbalance during the comparison of serum copper and iron-status indices.

Table 1. **Study design and participant distribution.**

Parameters	Description / Value
Study design	Comparative cross-sectional study
Study setting	Civil Hospital, Ghulam Muhammad Mahar Medical College, Sukkur, Sindh, Pakistan
Study period	August 2024 to July 2025
Age range	1-15 years
Total samples	300 children
IDA cases	150
Healthy controls	150
Sex distribution	Matched groups: boys about 47% and girls about 53% in each group
Primary outcome	Serum copper concentration in IDA versus healthy controls
Secondary analysis	Correlation of serum copper with Hb, serum iron, and ferritin in IDA

Anthropometric Z-scores were below zero in children with IDA, indicating that the mean nutritional profile was below the reference median for age and sex. Negative Z-scores do not indicate a mathematical error; they show that the child or group is below the WHO reference median. The more negative the value, the greater the deviation below expected growth. In the present cohort, both boys and girls showed evidence of underweight, stunting, and wasting, while girls showed slightly more negative values in weight-for-age and height-for-age indicators (Table 2). This finding suggests that IDA in the study population occurred within a broader

nutritional vulnerability rather than as an isolated biochemical abnormality.

Table 2. **Anthropometric measurements and classification of malnutrition among boys and girls with IDA.**

Indicator	IDA Boys	IDA Girls
Weight-for-age Z-score	-1.82 ± 0.61	-2.12 ± 0.57
Height-for-age Z-score	-1.56 ± 1.02	-1.61 ± 1.11
Weight-for-height Z-score	-1.32 ± 0.68	-1.17 ± 0.66
Underweight, n (%)	16 (46.14)	21 (51.23)
Stunting, n (%)	11 (42.51)	21 (46.16)
Wasting, n (%)	4 (13.51)	13 (22.57)

Hematological findings showed a severity-related microcytic-hypochromic pattern. Hemoglobin, HCT, RBC, MCV, MCH, and MCHC decreased from mild to moderate and severe IDA, while serum iron remained lower in all IDA categories than in healthy controls (Table 3). This pattern is consistent with impaired hemoglobin synthesis, reduced red-cell hemoglobinization, and progressive microcytosis due to iron depletion, as described in pediatric anemia guidelines and recent reviews [3-6, 26].

Serum copper, serum iron, and ferritin were significantly lower in IDA cases than in healthy controls (Table 4). Using the available mean ± SD values as the reported dispersion measure, the copper distribution was centered around 86.34 ± 0.83 µg/dL in IDA children compared with 120.41 ± 1.02 µg/dL in controls. Serum iron was also lower in IDA cases (63.71 ± 1.54 µg/dL) than in controls (105.80 ± 1.52 µg/dL), and ferritin showed marked depletion in IDA cases (8.57 ± 2.14 ng/mL) compared with controls (35.68 ± 4.89 ng/mL). The available grouped dataset did not provide individual minimum-maximum values; therefore, the low-to-high comparison is described using mean ± SD values. These findings support that reduced copper is associated with biochemical iron deficiency in this cohort. Fig. 1 illustrates the marked reduction of all three biochemical indices in IDA cases compared with healthy controls.

Table 3. **Hematological parameters across IDA severity categories and healthy controls.**

Parameters	Mild IDA	Moderate IDA	Severe IDA	Healthy control
Hb (g/dL)	11.25 ± 0.67	9.12 ± 0.47	7.86 ± 0.73	12.11 ± 0.75
HCT (%)	35.51 ± 1.43	31.26 ± 2.67	26.21 ± 2.23	36.21 ± 3.71
RBC (×10⁶/µL)	4.11 ± 0.37	3.71 ± 0.765	3.57 ± 0.62	4.20 ± 0.32
MCV (fL)	74.43 ± 5.16	71.18 ± 11.16	65.52 ± 8.18	85.71 ± 3.41
MCH (pg)	24.98 ± 1.84	21.54 ± 1.23	20.43 ± 2.23	28.51 ± 1.41
MCHC (g/dL)	31.41 ± 1.35	28.50 ± 1.72	27.37 ± 1.84	33.80 ± 2.51
Serum iron (µg/dL)	63.71 ± 1.54	32.43 ± 5.43	27.23 ± 20.56	105.80 ± 1.52

Table 4. **Serum copper and iron-status indices in children with IDA and healthy controls.**

Variables	IDA mean ± SD	Healthy mean ± SD	P-value
Copper (µg/dL)	86.34 ± 0.83	120.41 ± 1.02	< 0.001
Serum iron (µg/dL)	63.71 ± 1.54	105.80 ± 1.52	< 0.001
Ferritin (ng/mL)	8.57 ± 2.14	35.68 ± 4.89	< 0.001

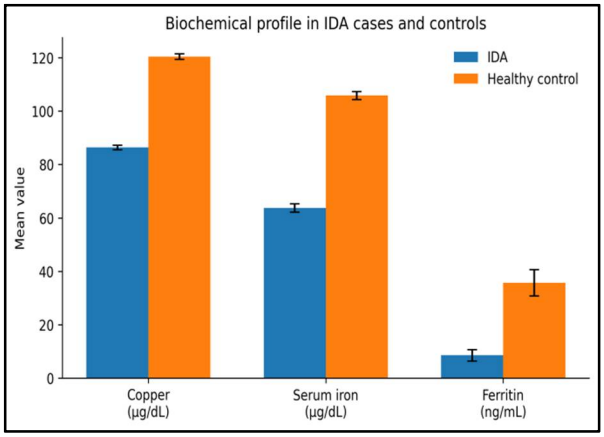

Figure 1. **Comparison of serum copper, serum iron, and ferritin between children with IDA and healthy controls**

Pearson correlation analysis within the IDA group showed that serum copper had a positive association with serum iron ($r = 0.52$; $P < 0.001$) and ferritin ($r = 0.49$; $P < 0.001$), indicating that lower copper tended to accompany lower circulating iron and depleted iron stores (Table 5). This association is biologically supported by the copper-dependent ferroxidaseceruloplasmin and hephaestin, which facilitate iron oxidation, export, and transferrin loading [10-12]. Similar clinical and pediatric reports have emphasized that copper disturbance may coexist with anemia and other cytopenias, particularly in malnutrition, chronic disease, or altered trace-element status [13-16]. The inverse relationship with hemoglobin ($r = -0.44$; $P < 0.01$) was interpreted cautiously because Hb is influenced by severity category, erythropoietic response, infection history, growth status, and nutritional background.

The biological explanation for reduced copper in this cohort is that iron deficiency in these children was likely part of a broader micronutrient imbalance. Copper participates in iron export and mobilization through ceruloplasmin and hephaestin; therefore, inadequate copper status may interfere with the oxidation of Fe^{2+} to Fe^{3+} and reduce efficient binding of iron to transferrin [10-12]. In practical terms, copper depletion may contribute to poor iron transport from intestinal cells, macrophages, and hepatic stores to sites of erythropoiesis. This mechanism supports the observed positive correlations of serum copper with serum iron and ferritin. Comparison with published literature demonstrates that copper findings in anemia are not uniform. Studies from Turkey reported higher serum copper in pediatric IDA, while Bangladeshi adolescent data also showed hypercupremia with hypozincemia; these patterns may reflect inflammatory response, dietary cereal intake, environmental exposure, or altered absorption during iron deficiency [17-19].In contrast, the present study found lower serum copper together with depleted ferritin and negative anthropometric Z-scores, suggesting that IDA in this hospital-based Sukkur cohort was part of broader nutritional vulnerability. Therefore, the difference between Turkish and Bangladeshi studies is plausible and highlights the need to interpret copper results according to population, inflammation, nutritional status, and analytical method. A summary of selected published data, including Asian-region evidence, is provided in Table 6.

Table 5. **Correlation/association summary of serum copper, iron-status variables, and hematological profile in IDA.**

Variable / comparison	Statistical finding	Interpretation in IDA
Serum copper v/s hemoglobin	$r = -0.44$; $P < 0.01$	Inverse association; interpret cautiously because Hb is affected by severity and nutritional background
Serum copper v/s serum iron	$r = 0.52$; $P < 0.001$	Moderate positive association; lower copper accompanied lower circulating iron
Serum copper v/s ferritin	$r = 0.49$; $P < 0.001$	Positive association; lower copper accompanied depleted iron stores
Hb, HCT, RBC, MCV, MCH, and MCHC across severity	Progressive decline from mild to severe IDA (Table 3)	Supports severity-related microcytic-hypochromic anemia
Serum iron and ferritin in IDA v/s controls	Both significantly lower in IDA (Table 4; $P < 0.001$)	Confirms biochemical iron deficiency and depleted iron stores

Table 6. **Comparison of the current findings with selected published studies on copper and trace-element status in anemia.**

Population/setting	Main copper/trace-element finding	Relevance to the present study	Study
Children aged 1-14 years, Turkey	Serum copper was higher in IDA than controls: 189 ± 49 vs 163 ± 37 ug/dL (P = 0.001); zinc was lower: 109 ± 59 vs 135 ± 56 ug/dL (P = 0.017)	Demonstrates that pediatric IDA may show hypercupremia in some populations	Ece et al. [17]
Children, mean age 6.8 ± 0.2 years, Denizli, Turkey	IDA was identified in 23/256 children; serum copper was higher in IDA than in controls: 37.70 ± 3.10 vs 25.30 ± 1.30 µmol/L (P < 0.05).	Highlights the environmental/inflammatory context and metal interactions as possible modifiers	Turgut et al. [18]
Adolescents aged 11-18 years, Dhaka, Bangladesh	Copper was higher in iron-deficient adolescents: males 140.53 ± 18.63 vs 108.60 ± 20.92 µg/dL; females 150.06 ± 22.59 vs 118.13 ± 15.57 µg/dL; zinc was lower in both sexes.	Provides a South Asian comparison showing hypercupremia with hypozincemia	Wajeunnesa et al. [19]
Infants aged 6 months at baseline, followed for 6 months, rural Bangladesh	Baseline serum copper was 20.7 ± 2.7, 20.3 ± 3.1, 21.0 ± 2.7, and 19.6 ± 2.7 µmol/L in iron, zinc, iron+zinc, and control groups; iron and/or zinc decreased serum copper after 6 months.	Supports trace-element monitoring during nutritional interventions	Baqui et al. [20]
Children/adolescents <19 years with chronic diseases, Spain/Europe; mean age 9.6 ± 4.8 years	In 78 patients, abnormal serum copper occurred in 22% (13 hypercupremia; 4 hypocupremia), and the Cu/Zn ratio was >1.00 in 87%. In the cystic-fibrosis subgroup, serum copper was 113 ± 23 µg/dL (range 69-158).	Shows that copper status and Cu/Zn ratio depend strongly on inflammation and disease background	Escobedo-Monge et al. [21, 22]
Children aged 6-17 years, United States NHANES 2017-2020; n = 2208	Anemia was associated with higher blood cadmium and lower selenium and manganese; serum copper mean ± SD was not reported because copper was not a main model metal.	Supports a broader trace-metal view in pediatric anemia	Fang et al. [23]
Children aged 6 months-18 years, Turkey	Of 157 children, 76 (48%) had iron deficiency, and 27 (17%) had IDA. Zinc was lower in iron-deficient children (P = 0.020), and zinc deficiency was more frequent (21.1%; P < 0.001).	Supports testing beyond iron in selected children with suspected multiple micronutrient deficiency	Mete et al. [24]
Children aged 1-15 years, Sukkur, Pakistan	Copper lower in IDA than controls: 86.34 +/- 0.83 vs 120.41 +/- 1.02 ug/dL; serum iron 63.71 +/- 1.54 vs 105.80 +/- 1.52 ug/dL; ferritin 8.57 +/- 2.14 vs 35.68 +/- 4.89 ng/mL; P < 0.001	Shows local copper disturbance with biochemically confirmed pediatric IDA	Present study

The strength of this study is the comparison of biochemically confirmed IDA cases with healthy controls using standardized hematological and biochemical procedures. The study also incorporated trace-element-aware handling and method validation. Limitations include the cross-sectional design, absence of inflammatory markers such as C-reactive protein or alpha-1-acid glycoprotein, absence of direct ceruloplasmin measurement, and lack of individual dietary intake assessment. These limitations prevent causal

interpretation but do not reduce the importance of the observed association between reduced copper and pediatric IDA in this local cohort.

Conclusion

This study demonstrated that children with biochemically confirmed IDA had significantly lower serum copper levels than healthy controls. The reduction in serum copper was accompanied by markedly decreased serum iron and ferritin levels, confirming that pediatric IDA in this cohort was associated with a broader disturbance of micronutrient homeostasis rather than isolated iron deficiency alone. The positive correlations of serum copper with serum iron and ferritin further support the role of copper in iron transport, mobilization, and storage. These findings suggest that assessment of copper status may be useful in children with severe, recurrent, or treatment-resistant IDA, particularly in nutritionally vulnerable populations.

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Conflict of Interest

The authors declare no conflict of interest.

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