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Original Article

# Referral-spectrum Effect in Hypercupriuric Referrals for Suspected Wilson Disease: Diagnostic Implications for Ceruloplasmin and 24-hour Urinary Copper Excretion

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## Abstract

***Background and Aims:*** In contemporary practice, elevated 24-hour urinary copper excretion (24-h UCE) often triggers referral for suspected Wilson disease (WD). In this hypercupriuric referral setting, interpretation of 24-h UCE may be distorted by spectrum effects. In this study, we aimed to compare conventional copper biomarkers in hypercupriuric referrals and evaluate whether a Leipzig-aligned ceruloplasmin (Cp) framework could provide a clinically useful triage approach. ***Methods:*** We retrospectively studied consecutive, untreated patients evaluated for suspected WD with hypercupriuria between February 2017 and February 2025. The final diagnosis was established using a prespecified Leipzig-based algorithm, with *ATP7B* testing when indicated. Diagnostic performance of Cp and 24-h UCE was compared. A prespecified Cp three-zone framework was evaluated using <0.10 g/L, 0.10–0.20 g/L, and >0.20 g/L as high-probability, indeterminate, and low-probability zones, respectively. ***Results:*** Among 541 untreated hypercupriuric patients, 65 had WD and 476 had adjudicated non-WD liver disease. Cp outperformed 24-h UCE for diagnosing WD (AUROC, 0.988 vs. 0.762). The optimal Cp cutoff was 0.15 g/L, with 90.8% sensitivity and 97.7% specificity. Cp < 0.10 g/L defined a high-probability zone with 98.0% WD prevalence, whereas Cp > 0.20 g/L defined a low-probability zone with 0.5% WD prevalence. Among non-WD controls, higher urinary copper was independently associated with higher bilirubin, prolonged international normalized ratio, and lower albumin. ***Conclusions:*** In hypercupriuric referrals for suspected WD, Cp retained strong diagnostic performance and outperformed 24-h UCE. A Leipzig-aligned Cp three-zone framework may support probability-based triage in contemporary referral practice.

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## Introduction

Wilson disease (WD) is an autosomal recessive disorder of copper metabolism caused by pathogenic variants in *ATP7B* (ATPase copper transporting beta).[1] Owing to its marked clinical and biochemical heterogeneity, timely and accurate diagnosis requires integrated interpretation of clinical, biochemical, ophthalmologic, and genetic findings, most commonly within the Leipzig framework.[1–5] Serum ceruloplasmin (Cp) and 24-hour urinary copper excretion (24-h UCE) remain central components of routine diagnostic assessment.[1,3,6–8]

In contemporary referral practice, however, 24-h UCE has a dual role: it is both a diagnostic biomarker for WD and a trigger for referral to specialized centers. When the test under evaluation also functions as a referral trigger, its apparent diagnostic performance becomes susceptible to referral bias and spectrum effects.[9–11] Moreover, hypercupriuria is not specific to WD and may also occur in non-WD liver diseases, particularly in cholestatic disorders, severe acute liver injury, and states of hepatic synthetic dysfunction.[12–14]

We therefore hypothesized that the performance of 24-h UCE would be materially altered in hypercupriuric referrals, whereas Cp might retain stronger diagnostic value. To test this hypothesis, we compared Cp and 24-h UCE in a consecutive referral cohort and evaluated a Leipzig-aligned Cp three-zone framework for clinically pragmatic, probability-based triage.

## Methods

### Study design and patients

This was a retrospective, single-center observational study conducted at Nanjing Second Hospital. Consecutive patients undergoing evaluation for suspected WD between February 2017 and February 2025 were screened. Eligible patients had persistent hypercupriuria, defined as at least two consecutive 24-h UCE measurements above the upper limit of normal (ULN, 60 μg/24 h), obtained before final diagnostic adjudication and before initiation of any copper-directed therapy. The two qualifying measurements were required to be obtained during the same diagnostic episode, usually within the same hospitalization or outpatient diagnostic evaluation period and generally within 14 days. For diagnostic performance analyses, the first qualifying 24-h UCE measurement was used as the index urinary copper excretion (UCE) value, whereas the second consecutive elevated measurement was used to confirm persistent hypercupriuria for eligibility. Patients with discordant repeated 24-h UCE results were not considered to have persistent hypercupriuria unless an additional 24-h UCE measurement confirmed elevation above the ULN. Acute or subacute liver injury alone was not an exclusion criterion.

Prespecified exclusions included missing key diagnostic data, prior copper-directed therapy before the index 24-h UCE measurement, failure to confirm persistent hypercupriuria, and insufficient information for final diagnostic adjudication.

### Diagnostic criteria and clinical grouping

Final WD status was adjudicated using a prespecified Leipzig-based diagnostic workflow before biomarker performance analyses.[1,3,5,15] Patients with a Leipzig score ≥4 were classified as having WD. Patients with a Leipzig score of 3 or persistent diagnostic uncertainty underwent genetic testing whenever feasible.

Genetic evaluation included targeted *ATP7B* sequencing, *ATP7B* multiplex ligation-dependent probe amplification for exon-level deletions or duplications, or whole-exome sequencing when *ATP7B* variant information was available.[16–18] Patients with biallelic pathogenic or likely pathogenic *ATP7B* variants were classified as genetically confirmed WD.[1,3,5] In patients without biallelic *ATP7B* confirmation—including those without *ATP7B* testing, those with only monoallelic pathogenic or likely pathogenic *ATP7B* variants, and those without detected pathogenic *ATP7B* variants—WD classification was not based on clinical impression alone. Classification required fulfillment of the Leipzig diagnostic threshold using available non-genetic criteria, an integrated clinical assessment compatible with WD, and absence of a more plausible alternative diagnosis explaining the copper abnormalities. Missing or unavailable diagnostic items were conservatively assigned 0 points.

Patients were classified as non-WD when comprehensive evaluation did not provide sufficient Leipzig-based evidence for WD and an alternative liver disease diagnosis was established. In genetically tested patients, absence of biallelic pathogenic or likely pathogenic *ATP7B* variants was considered supportive evidence against WD but was not independently exclusionary.

### Prespecified threshold framework and subgroup definitions

Cp was evaluated using a prespecified Leipzig-aligned three-zone framework: <0.10 g/L as the high-probability threshold, 0.10–0.20 g/L as the indeterminate zone, and >0.20 g/L as the low-probability threshold supporting de-prioritization of WD rather than definitive exclusion. Patients in the indeterminate zone underwent integrated Leipzig-based assessment rather than classification by Cp alone. This assessment included reassessment of all available Leipzig components, ophthalmologic evaluation for Kayser–Fleischer rings, neurologic assessment and brain MRI when clinically indicated, repeat biochemical testing when appropriate, evaluation for alternative liver diseases, and *ATP7B* testing when the Leipzig score remained equivocal, diagnostic information was incomplete, clinical suspicion persisted, or no alternative liver disease adequately explained the copper abnormalities.[1,3,5,19]

To explore referral-spectrum effects, non-WD controls were stratified into predefined severity subgroups: severe/coagulopathic non-WD (international normalized ratio (INR) ≥ 1.5),[20,21] jaundiced non-WD (INR < 1.5 and total bilirubin ≥ 5 mg/dL), and mild non-WD (INR < 1.5 and total bilirubin < 5 mg/dL). Additional analyses were performed in referral subsets with higher urinary copper burden, defined as 24-h UCE ≥ 100 μg/24 h and ≥200 μg/24 h.[1,19]

### Data collection and laboratory measurements

Baseline demographic, biochemical, and hematologic data were retrospectively extracted from hospital and laboratory information systems. All analyzed measurements were obtained before copper-directed therapy. For 24-h UCE, the first qualifying measurement obtained during the diagnostic episode was used as the index value for diagnostic performance analyses. For other laboratory parameters, when multiple pretreatment results were available, the value closest to the index UCE measurement or baseline diagnostic assessment was used. 24-h UCE was measured by inductively coupled plasma mass spectrometry, and serum Cp was measured by immunoturbidimetry.

### Statistical analysis

Continuous variables are presented as median (IQR) or mean ± standard deviation, as appropriate, and categorical variables as counts (percentages). Between-group comparisons were performed using the Mann–Whitney U test or Student's t-test for continuous variables, as appropriate, and the chi-square test or Fisher's exact test for categorical variables. Diagnostic performance was assessed using receiver operating characteristic analysis with areas under the receiver operating characteristic curve (AUROCs) and 95% confidence intervals (CIs). AUROC 95% CIs were estimated using DeLong's method. Exploratory single-cutoff analyses used the Youden index. Threshold-based diagnostic indices included sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR), negative likelihood ratio (NLR), and diagnostic accuracy. CIs for sensitivity, specificity, PPV, NPV, and diagnostic accuracy were calculated using the exact binomial method. CIs for PLR and NLR were calculated using the log method.

Among non-WD controls, determinants of 24-h UCE were examined using multivariable linear regression with log10-transformed 24-h UCE and heteroscedasticity-consistent robust standard errors using the HC3 estimator. To account for potential confounding by liver injury severity, multivariable-adjusted ROC analyses were performed for Cp and 24-h UCE. Logistic regression models were constructed with final WD status as the dependent variable. Cp or 24-h UCE was entered as the primary diagnostic marker, together with age, total bilirubin, INR, and albumin as covariates. Adjusted AUROCs with 95% CIs were generated from the predicted probabilities of each model. In an extended liver-injury–adjusted model, age, alanine aminotransferase (ALT), aspartate ami-

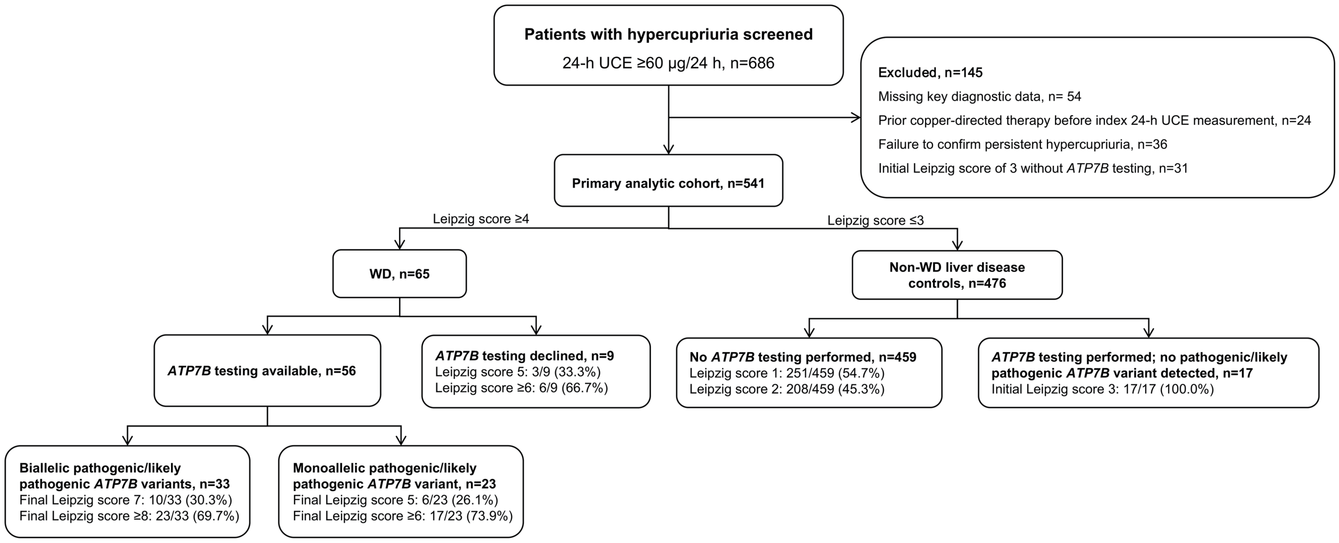


**Fig. 1. Flow chart of patient selection and final diagnostic classification.** Among 686 patients screened for hypercupriuria, defined as 24-h UCE ≥ 60 μg/24 h, 541 were included in the primary analytic cohort after prespecified exclusions. The final cohort comprised 65 WD cases and 476 adjudicated non-WD liver disease controls. Exclusions were due to missing key diagnostic data, prior copper-directed therapy before the index 24-h UCE measurement, failure to confirm persistent hypercupriuria, or an initial Leipzig score of 3 without *ATP7B* testing. Final diagnostic classification followed a predefined Leipzig-based algorithm, with *ATP7B* testing incorporated when indicated or available. Patients with an initial Leipzig score of 3 were considered diagnostically indeterminate; those who underwent *ATP7B* testing but had no pathogenic or likely pathogenic *ATP7B* variant detected and had an established alternative liver disease diagnosis were classified as non-WD controls, whereas those without *ATP7B* testing were excluded from the primary analytic cohort. Among the 9 WD patients who declined *ATP7B* testing, all fulfilled the Leipzig diagnostic threshold based on integrated non-genetic evidence, with final Leipzig scores ranging from 5 to 7 and a median score of 6. Individual non-genetic diagnostic components supporting WD classification in these 9 patients are provided in Supplementary Table 1. Final Leipzig scores shown in *ATP7B*-tested WD subgroups include *ATP7B* genetic evidence. Abbreviations: 24-h UCE, 24-hour urinary copper excretion; *ATP7B*, ATPase copper transporting beta; WD, Wilson disease.

notransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total bilirubin, INR, and albumin were included as covariates.

As sensitivity analyses, diagnostic performance was re-evaluated after restricting the WD group to patients with biallelic pathogenic/likely pathogenic *ATP7B* variants and to those with at least one pathogenic/likely pathogenic *ATP7B* variant. These analyses were intended to examine whether the main findings were robust when WD classification was less dependent on Cp-containing Leipzig scoring. All analyses were performed using R version 4.3.0 and GraphPad Prism version 10.0.1. A two-sided *P*-value < 0.05 was considered statistically significant. This study is reported in accordance with the STROBE statement for observational studies.

## Results

### *Study population*

A total of 686 patients with hypercupriuria were screened between February 2017 and February 2025. After prespecified exclusions, 541 patients met the eligibility criteria and were included in the primary analytic cohort, comprising 65 untreated patients with WD and 476 adjudicated non-WD liver disease controls. Details of patient selection and final diagnostic classification are shown in Figure 1.

Among the 65 patients finally classified as WD, 9 did not undergo *ATP7B* testing because testing was declined. All 9 patients fulfilled the Leipzig diagnostic threshold based on integrated non-genetic evidence, with final Leipzig scores ranging from 5 to 7 and a median score of 6. The individual non-genetic diagnostic components supporting WD classification in these patients are shown in Supplementary Table 1.

The non-WD control group represented a broad tertiary referral liver disease spectrum, with drug-induced or toxic liver injury, autoimmune or cholestatic liver disease, viral hepatitis–related liver disease, biliary obstruction or cholangitis, and alcohol-related or metabolic fatty liver disease being the main diagnostic categories (Supplementary Table 2).

### *Baseline characteristics*

Baseline characteristics are shown in Table 1. Patients with WD were younger than non-WD controls [28 (14–46) vs. 52 (40–60.25) years, *P* < 0.001], whereas sex distribution was similar [male sex: 49.23% vs. 48.95%, *P* = 0.966]. WD patients had higher 24-h UCE [201.1 (109.2–415.4) vs. 94.05 (72.3–148.18) μg/24 h, *P* < 0.001] and markedly lower Cp [0.08 (0.05–0.10) vs. 0.27 (0.22–0.35) g/L, *P* < 0.001]. In contrast, non-WD controls had higher total bilirubin, ALT, AST, ALP, and GGT, indicating more pronounced cholestatic and hepatocellular injury at baseline.

### *Diagnostic performance of Cp and 24-h UCE before and after covariate adjustment*

In the primary hypercupriuric cohort, Cp showed a higher AUROC than 24-h UCE for diagnosing WD (0.988 vs. 0.762; Table 2 and Fig. 2). After adjustment for age, total bilirubin, INR, and albumin, the AUROCs were 0.989 for Cp and 0.930 for 24-h UCE. In the extended liver-injury–adjusted model including age, ALT, AST, ALP, GGT, total bilirubin, INR, and albumin, the AUROCs were 0.992 for Cp and 0.950 for 24-h UCE (Supplementary Table 3).

The Youden-derived optimal cutoff for Cp was 0.15 g/L, which yielded a sensitivity of 90.8% and a specificity of 97.7%. By comparison, the optimal cutoff for 24-h UCE was 149.7 μg/24 h, corresponding to a sensitivity of 63.1% and a specificity of 75.6% (Table 2).

In sensitivity analyses according to *ATP7B* variant status, Cp consistently outperformed 24-h UCE among WD patients

**Table 1. Baseline characteristics of the hypercupriuric study cohort (24-h UCE ≥ 60 μg/24 h)**

| Characteristic | Non-WD (n = 476) | WD (n = 65) | *P*-value |
|---|---|---|---|
| Demographics | | | |
| Age at diagnosis, years | 52 (40, 60.25) | 28 (14, 46) | <0.001 |
| Male sex, n (%) | 233 (48.95) | 32 (49.23) | 0.966 |
| Copper indices | | | |
| 24-h UCE, μg/24 h | 94.05 (72.3, 148.18) | 201.1 (109.2, 415.4) | <0.001 |
| Cp, g/L | 0.27 (0.22, 0.35) | 0.08 (0.05, 0.1) | <0.001 |
| Liver biochemistry and synthetic function | | | |
| Total bilirubin, mg/dL | 4.52 (1.37, 12.84) | 1 (0.65, 1.82) | <0.001 |
| ALT, U/L | 140.2 (51.33, 452.2) | 50.8 (26.7, 123.9) | <0.001 |
| AST, U/L | 115.8 (52.75, 329.15) | 63.5 (32.7, 91.5) | <0.001 |
| ALP, U/L | 150.1 (106, 237.5) | 123.4 (90, 199) | 0.020 |
| GGT, U/L | 153.2 (78.95, 300) | 102 (53, 199) | 0.001 |
| Total protein, g/L | 62.85 (57.5, 69.3) | 66 (61.8, 71.1) | 0.014 |
| Albumin, g/L | 35.95 (31.5, 39.6) | 40 (30.4, 44.1) | 0.049 |
| Platelet count, $\times 10^9$/L | 150 (103, 206) | 122 (71, 228) | 0.049 |
| Prothrombin time, s | 12.6 (11.38, 14.61) | 13.9 (11.98, 16) | 0.014 |
| INR | 1.12 (1.01, 1.31) | 1.25 (1.05, 1.46) | 0.002 |

Data are presented as median (IQR) unless otherwise indicated. Categorical variables are presented as n (%). *P*-values were calculated using the Mann–Whitney U test for continuous variables and the chi-square test for sex. Abbreviations: 24-h UCE, 24-hour urinary copper excretion; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Cp, ceruloplasmin; GGT, gamma-glutamyl transferase; INR, international normalized ratio; IQR, interquartile range; non-WD, non-Wilson disease; WD, Wilson disease.

with biallelic pathogenic/likely pathogenic *ATP7B* variants and among those with at least one pathogenic/likely pathogenic *ATP7B* variant (AUROCs: 0.992 vs. 0.696 and 0.987 vs. 0.746, respectively; Supplementary Table 4).

Cp also outperformed 24-h UCE in referral subsets with higher urinary copper burden, including 24-h UCE ≥ 100 μg/24 h and ≥ 200 μg/24 h (AUROCs: 0.987 vs. 0.710 and 0.978 vs. 0.763, respectively; Supplementary Table 5).

### *Performance of the prespecified Cp three-zone framework*

We evaluated a prespecified Leipzig-aligned Cp three-zone framework using thresholds of <0.10 g/L, 0.10–0.20 g/L, and >0.20 g/L (Fig. 3). In the overall cohort, Cp < 0.10 g/L identified 48 of 65 WD patients and only 1 of 476 non-WD controls, corresponding to a WD prevalence of 98.0% (48/49) in this high-probability zone. Cp 0.10–0.20 g/L represented an indeterminate zone, in which 15 of 90 patients had WD, corresponding to a WD prevalence of 16.7%. Cp > 0.20 g/L represented a low-probability zone, including 2 of 65 WD patients and 400 of 476 non-WD controls, with a WD prevalence of 0.5% (2/402) (Table 3).

Supplementary binary threshold analyses showed that Cp < 0.10 g/L had high specificity and PPV, whereas Cp > 0.20 g/L had high NPV (Supplementary Table 6). Similar patterns were observed across non-WD severity strata, although WD prevalence among patients with Cp > 0.20 g/L was higher in the severe/coagulopathic subgroup.

Three discordant Cp cases were identified (Supplementary Table 7). Two WD patients had Cp > 0.20 g/L and therefore fell into the low-probability zone; one had biallelic pathogenic *ATP7B* variants, whereas the other had a monoallelic patho-

**Table 2. Diagnostic performance of Cp and 24-h UCE in hypercupriuric referrals for suspected WD**

| Marker | N, WD/ non-WD | AUROC (95% CI) | Optimal cutoff | Sensitivity, % (95% CI) | Specificity, % (95% CI) | PPV, % (95% CI) | NPV, % (95% CI) | PLR (95% CI) | NLR (95% CI) | Diagnostic accuracy, % (95% CI) |
|---|---|---|---|---|---|---|---|---|---|---|
| 24-h UCE, μg/24 h | 65/ 476 | 0.762 (0.693–0.828) | 149.70 | 63.1 (50.2–74.7) | 75.6 (71.5–79.4) | 26.1 (19.4–33.7) | 93.8 (90.8–96.0) | 2.59 (2.03–3.30) | 0.49 (0.35–0.67) | 74.1 (70.2–77.8) |
| Cp, g/L | 65/ 476 | 0.988 (0.976–0.997) | 0.15 | 90.8 (81.0–96.5) | 97.7 (95.9–98.8) | 84.3 (73.6–91.9) | 98.7 (97.2–99.5) | 39.28 (21.79–70.80) | 0.09 (0.04–0.20) | 96.9 (95.0–98.2) |

The optimal cutoff was determined using the Youden index. Diagnostic accuracy was calculated as (true positives + true negatives) divided by the total number of patients. AUROC 95% CIs were estimated using DeLong's method. Confidence intervals for sensitivity, specificity, PPV, NPV, and diagnostic accuracy were calculated using the exact binomial method. Confidence intervals for PLR and NLR were calculated using the log method. Abbreviations: 24-h UCE, 24-hour urinary copper excretion; AUROC, area under the receiver operating characteristic curve; CI, confidence interval; Cp, ceruloplasmin; NLR, negative likelihood ratio; non-WD, non-Wilson disease; NPV, negative predictive value; PLR, positive likelihood ratio; PPV, positive predictive value; WD, Wilson disease.

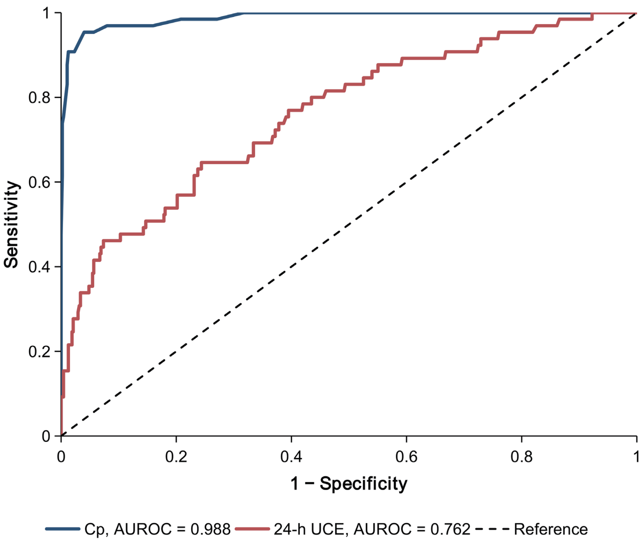


**Fig. 2. Receiver operating characteristic curves of Cp and 24-h UCE for diagnosing WD in the primary hypercupriuric referral cohort.** Receiver operating characteristic curves are shown for Cp and 24-h UCE in patients with hypercupriuria, defined as 24-h UCE ≥ 60 μg/24 h (N = 541). WD status was determined according to the final Leipzig-based diagnostic classification, with *ATP7B* testing incorporated when indicated. Cp showed markedly better diagnostic discrimination than 24-h UCE. Abbreviations: *ATP7B*, ATPase copper transporting beta; AUROC, area under the receiver operating characteristic curve; Cp, ceruloplasmin; 24-h UCE, 24-hour urinary copper excretion; WD, Wilson disease.

genic *ATP7B* variant but fulfilled the Leipzig diagnostic threshold based on integrated non-genetic evidence. Conversely, one non-WD patient with severe HBV-related liver injury had Cp < 0.10 g/L and fell into the high-probability zone.

### *Determinants of hypercupriuria among non-WD controls*

To characterize factors associated with urinary copper excretion outside WD, we examined determinants of 24-h UCE among non-WD controls. In multivariable linear regression adjusted for age and sex, higher 24-h UCE was associated with higher INR, higher total bilirubin, and lower albumin (Table 4). Per 0.5-unit increase in INR, 24-h UCE increased by 20.51% (95% CI, 10.34% to 31.61%; $P < 0.001$). Per doubling of total bilirubin, 24-h UCE increased by 5.91% (95% CI, 3.21% to 8.67%; $P < 0.001$). Per 5 g/L increase in albumin, 24-h UCE decreased by 6.17% (95% CI, −9.84% to −2.36%; $P = 0.002$).

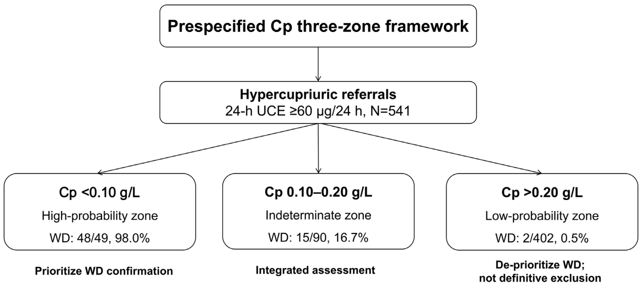


**Fig. 3. Prespecified Cp three-zone framework for hypercupriuric referrals undergoing evaluation for WD.** In the primary hypercupriuric cohort, defined by 24-h UCE ≥ 60 μg/24 h, prespecified Cp thresholds classified patients into high-probability, indeterminate, and low-probability zones. Observed WD prevalence was 98.0% for Cp < 0.10 g/L, 16.7% for Cp 0.10–0.20 g/L, and 0.5% for Cp > 0.20 g/L. Patients in the high-probability zone should undergo confirmatory Leipzig-based assessment, with *ATP7B* testing when clinically appropriate. Patients in the indeterminate zone should undergo integrated Leipzig-based assessment. Patients in the low-probability zone, defined as Cp > 0.20 g/L, should be evaluated for alternative causes of hypercupriuria; WD should be de-prioritized rather than definitively excluded, and further Leipzig-based evaluation remains warranted when clinical suspicion persists. Abbreviations: *ATP7B*, ATPase copper transporting beta; Cp, ceruloplasmin; 24-h UCE, 24-hour urinary copper excretion; WD, Wilson disease.

## Discussion

In this retrospective cohort of untreated patients referred for suspected WD with persistent hypercupriuria, Cp showed substantially better diagnostic performance than 24-h UCE. In routine referral practice, elevated urinary copper may prompt further evaluation for suspected WD; consequently, the present cohort was already enriched for hypercupriuria before the diagnostic performance of 24-h UCE was assessed. This referral-spectrum effect is consistent with the

**Table 3. Distribution of WD and non-WD patients across the three prespecified Cp probability zones**

| Cp zone | Clinical interpretation | WD, n/N (%) | Non-WD, n/N (%) | Total, n | WD prevalence in zone |
|---|---|---|---|---|---|
| <0.10 g/L | High-probability zone | 48/65 (73.8) | 1/476 (0.2) | 49 | 48/49 (98.0) |
| 0.10–0.20 g/L | Indeterminate zone | 15/65 (23.1) | 75/476 (15.8) | 90 | 15/90 (16.7) |
| >0.20 g/L | Low-probability zone | 2/65 (3.1) | 400/476 (84.0) | 402 | 2/402 (0.5) |

The Cp three-zone framework was defined using prespecified Leipzig-aligned thresholds. Cp < 0.10 g/L was defined as the high-probability zone, Cp 0.10–0.20 g/L as the indeterminate zone, and Cp > 0.20 g/L as the low-probability zone. WD prevalence in each zone was calculated as the number of WD patients divided by the total number of patients within that Cp zone. Abbreviations: Cp, ceruloplasmin; non-WD, non-Wilson disease; WD, Wilson disease.

**Table 4. Multivariable determinants of 24-h UCE among hypercupriuric non-WD controls**

| Predictor | Scaling | % change in 24-h UCE (95% CI) | *P*-value |
|---|---|---|---|
| INR | per 0.5-unit increase | 20.51% (10.34 to 31.61) | <0.001 |
| Total bilirubin | per doubling ($log_2$) | 5.91% (3.21 to 8.67) | <0.001 |
| Albumin | per 5 g/L increase | −6.17% (−9.84 to −2.36) | 0.002 |

The model was adjusted for age and sex, and inference used heteroscedasticity-consistent robust standard errors with the HC3 estimator. Percent change in UCE was derived as $(10^{\beta} - 1) \times 100\%$ for a one-unit increase in the scaled predictor. Abbreviations: UCE, urinary copper excretion; 24-h UCE, 24-hour urinary copper excretion; CI, confidence interval; HC3, heteroscedasticity-consistent type 3; INR, international normalized ratio; non-WD, non-Wilson disease; WD, Wilson disease.

principle that diagnostic accuracy varies according to patient spectrum and referral pathway.[9–11] In this setting, 24-h UCE showed only modest standalone discrimination, whereas Cp remained highly informative.

The attenuated discrimination of 24-h UCE was closely related to the distribution of urinary copper among non-WD controls. Although WD patients were more frequently represented in higher 24-h UCE strata, non-WD controls were also present across all elevated UCE categories (Supplementary Fig. 1). This distributional overlap also motivated analyses in referral subsets with higher urinary copper burden, including 24-h UCE ≥ 100 μg/24 h and ≥ 200 μg/24 h (Supplementary Table 5). In non-WD controls, higher 24-h UCE was independently associated with higher total bilirubin, prolonged INR, and lower albumin (Table 4). Because biliary excretion is the major route of copper elimination, cholestasis and advanced liver injury may impair hepatic copper transport and excretion, thereby contributing to increased urinary copper excretion.[22–26] Thus, in a referral cohort already selected for hypercupriuria, urinary copper may reflect hepatic excretory and synthetic dysfunction as well as WD-specific copper dysregulation. This interpretation is consistent with the covariate-adjusted ROC analyses, in which adjustment for age, total bilirubin, INR, and albumin substantially increased the AUROC of 24-h UCE, whereas the AUROC of Cp changed little (Supplementary Table 3).

By contrast, Cp showed a more stable diagnostic profile. Despite the broad non-WD liver disease spectrum, Cp retained excellent discrimination. The Youden-derived cutoff of 0.15 g/L was close to values reported in previous studies.[14,27,28] However, a single ROC-derived cutoff is not necessarily the most clinically useful way to interpret Cp. Cp is a continuous biomarker, and dichotomizing it into a single positive or negative result may overinterpret borderline values.[29] Within the Leipzig framework, Cp < 0.10 g/L carries greater diagnostic weight, whereas Cp > 0.20 g/L generally makes WD less likely.[1,3,5] Therefore, the ROC-derived cutoff is best viewed as a summary of overall discrimination rather than as the preferred clinical decision threshold.

The prespecified Cp three-zone framework may be more clinically interpretable than a single dichotomous cutoff. In our cohort, Cp < 0.10 g/L defined a high-probability zone in which WD should be prioritized; Cp > 0.20 g/L defined a low-probability zone rather than a definitive exclusion zone; and Cp 0.10–0.20 g/L remained an indeterminate zone requiring integrated assessment. Patients in the indeterminate zone should be assessed within the full Leipzig-based framework rather than classified by Cp alone, because WD diagnosis requires integrated interpretation of clinical, biochemical, ophthalmologic, histologic, imaging, and genetic evidence.[1,3,5,15] *ATP7B* testing is not mandatory for every patient in the Cp indeterminate zone, particularly when integrated Leipzig-based assessment is not suggestive of WD and an alternative liver disease diagnosis is established. However, *ATP7B* testing should be strongly considered when the Leipzig score remains equivocal, especially with an initial Leipzig score of 3, when diagnostic information is incomplete, clinical suspicion persists, or no alternative liver disease adequately explains the copper abnormalities. Thus, the main value of the three-zone framework is to guide diagnostic prioritization and targeted genetic testing, not to replace comprehensive adjudication.

The predictive values observed in this study should be interpreted in relation to disease prevalence. Because PPV and NPV are prevalence-dependent, the values reported here primarily reflect the probability of WD within a high-risk hypercupriuric referral population rather than in an unselected screening population.[30–32] Sensitivity and specificity are less directly prevalence-dependent but may still vary across clinical spectra. In particular, the low-probability Cp threshold appeared more context-dependent in patients with severe hepatic dysfunction, whereas the high-probability threshold retained very high specificity even in this subgroup. Because Cp is synthesized in the liver and is also influenced by inflammatory and hepatic synthetic status, Cp levels may be altered in severe non-WD liver disease independently of WD.[12,19,33] This distinction supports the use of Cp thresholds as probability-based triage tools rather than absolute diagnostic rules.

The discordant Cp cases further support this cautious interpretation. Two WD patients had Cp > 0.20 g/L and would have been placed in the low-probability zone if Cp had been interpreted in isolation. One had biallelic pathogenic *ATP7B* variants and was genetically confirmed, whereas the other had a monoallelic pathogenic *ATP7B* variant but fulfilled the Leipzig diagnostic threshold based on integrated non-genetic evidence. Inflammatory activation, acute hepatic injury, or decompensation may increase measured Cp because Cp is an acute-phase reactant, potentially masking WD-associated hypoceruloplasminemia.[6,19,34,35] Immunoreactive Cp assays may also fail to fully reflect functional copper incorporation.[6] Conversely, one non-WD patient with severe HBV-related liver injury had Cp < 0.10 g/L, and *ATP7B* testing detected no pathogenic or likely pathogenic variants, consistent with secondary hypoceruloplasminemia in severe hepatic synthetic dysfunction. These discordant cases reinforce that Cp thresholds should guide diagnostic prioritization, but discordant Cp results should prompt integrated reassessment rather than automatic confirmation or exclusion of WD.

Several limitations should be acknowledged. First, this was a retrospective, single-center study and therefore remains susceptible to selection bias, although consecutive inclusion may have partly mitigated this concern. Second, patients with acute or severe non-WD liver injury were retained if they met the requirement for repeated 24-h UCE elevation and underwent final diagnostic adjudication. This was consistent with the referral-spectrum focus of the study, because such patients may be evaluated for suspected WD precisely because hepatic injury can be accompanied by increased urinary copper excretion.[3,34] Nevertheless, this design also increased clinical heterogeneity. Third, repeated 24-h UCE measurements were not obtained according to a prospectively fixed interval, even though repeated elevation during the same diagnostic episode was required before copper-directed therapy. Fourth, incorporation bias cannot be fully excluded because Cp and 24-h UCE are components of the Leipzig score. We addressed this by using an integrated diagnostic workflow and by performing sensitivity analyses restricted to WD patients with biallelic pathogenic/likely pathogenic *ATP7B* variants and to those with at least one pathogenic/likely pathogenic *ATP7B* variant. Fifth, not all patients in the indeterminate Cp zone underwent *ATP7B* testing, reflecting real-world practice. Sixth, the WD cohort was relatively small compared with the non-WD referral cohort, reflecting both the rarity of WD and the restriction to treatment-naïve patients undergoing initial diagnostic evaluation. Finally, the study population consisted exclusively of Chinese patients, and the proposed three-zone Cp strategy requires external validation in ethnically diverse multicenter cohorts.

## Conclusions

In patients referred because of hypercupriuria, the diagnostic interpretation of copper biomarkers is strongly context-dependent. In this referral setting, 24-h UCE had limited standalone discriminatory utility, whereas Cp retained strong diagnostic performance. Nevertheless, 24-h UCE remains clinically relevant when interpreted in biochemical context rather than in isolation. A Leipzig-aligned Cp three-zone framework may provide a practical basis for probability-based triage and targeted *ATP7B* testing, while preserving the need for integrated diagnostic assessment when clinical suspicion persists.

## Supporting information

Supplementary material for this article is available at https://doi.org/10.14218/JCTH.2026.00294.

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## Conflict of interest

The authors have no conflict of interests related to this publication.

## Author contributions

Study conception and design (YZ, YY, SL), data acquisition (YZ, YB, YW, YT, RL, HL, LW, WJ, YG, SZ, TM, KZ, XL), data interpretation (YZ, YB, YY, SL), drafting of the manuscript (YZ, YB), critical revision of the manuscript (SL, YY), and supervision (SL, YY). All authors reviewed the manuscript and approved the final version.

## Ethical statement

This study was conducted in accordance with the ethical principles of the Declaration of Helsinki (as revised in 2024) and its later amendments. Approval was granted by the Ethics Committee of Nanjing Second Hospital (Approval No. 2026-LS-ky036). Given the retrospective nature of the study and the use of de-identified data, the requirement for written informed consent was formally waived by the Ethics Committee of Nanjing Second Hospital.

## Data sharing statement

The data that support the findings of this study are not publicly available because of legal and ethical restrictions related to patient privacy. De-identified data may be made available from the corresponding authors upon reasonable request and subject to institutional approval.

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