Received: 26 April 2024 | Accepted: 6 September 2024

DOI: 10.1097/HEP.0000000000001105

ORIGINAL ARTICLE

Exchangeable copper for patients with Wilson disease at follow-up: Rethinking normal ranges or changing methodology

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Abstract

Background and Aims: Determining suitable copper parameters for monitoring Wilson disease remains a topic of ongoing discussion. International recommendations currently rely on the combination of urinary copper excretion and nonspecific liver markers when considering therapy and time elapsed since diagnosis. The emergence of exchangeable copper (CuEX) as a novel measurement reflecting the "free copper pool" held promise as a valuable target to ensure metabolic stability during follow-up, although the validation of target ranges remains unknown. We aimed to evaluate CuEX quantification in repeated samples from 92 real-world patients with Wilson disease during a 2-year period.

Approach: Patients were classified as "stable" if a diagnosis had been made more than 1 year before and were compliant with stable anti-copper drug and dose. Otherwise, patients were classified as "nonstable."

Results: Two hundred and thirteen CuEX samples were obtained per clinical practice. Overall, 57% of CuEX measurements fell below the reference "range of normality," whereas only 34% were within and 9% were above normal levels. There was no association of CuEX levels with therapy, elapsed time from diagnosis, or clinical stability, although most of the samples above normality corresponded to nonstable patients. Only 23.4% of the CuEX samples were aligned with data obtained from concomitant urinary copper excretion.

Conclusions: Our findings suggest that CuEX is a suboptimal tool for assessing copper homeostasis when used alone and should be used with caution if no additional information is available. Normal reference intervals for Wilson disease–treated patients should be redefined, as most CuEX quantifications fell in the lower range, with no sign of overtreatment in these patients.

Abbreviations: Cal-NCC, calculated non–Cp-bound copper; Cp, ceruloplasmin; CuEX, exchangeable copper; NCC, non–Cp-bound copper; P1, time point 1; P2, time point 2; P3, time point 3; P4, time point 4; REC, ratio of exchangeable copper to total copper; UCE, 24-hour quantification of urinary copper excretion; WD, Wilson disease.

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Supplemental Digital Content is available for this article. Direct URL citations are provided in the HTML and PDF versions of this article on the journal's website, www.hepjournal.com.

Keywords: exchangeable copper, free copper, monitorization, non–ceruloplasmin binding copper, REC

INTRODUCTION

Wilson disease (WD) is a rare and genetically heterogeneous disease characterized by copper overload and variable phenotype predominantly affecting the liver and/or central nervous system. This condition arises from mutations in the *ATP7B* gene, leading to dysfunction or absence of the ATP7B protein, which is considered critical for systemic copper homeostasis. Located primarily in the liver, hepatic ATP7B plays a key role in channeling copper into ceruloplasmin (Cp) and facilitating the biliary excretion of the metal. In WD, impaired ATP7B does not transfer copper to Cp and deficiently excretes copper through the bile, thus resulting in copper accumulation in the hepatocytes. At early stages, metallothioneins may offset copper effects, but eventually, tissue damage ensues. WD is characterized by progressive liver disease and potential extrahepatic involvement due to copper deposition, mainly in the brain and cornea.[1]

Once diagnosed, treatment is started with the aim of extracting copper from tissues and generating a negative copper balance. One of the main unmet needs in WD is the absence of accurate biochemical markers to ensure that an optimal copper status has been reached during follow-up. International guidelines recommend a combination of nonspecific parameters (ie, ALT, urinary copper, and/or "free copper") for assessing clinical stability in patients with WD.[2–5] However, these measurements have limitations, including interindividual and intraindividual variability, susceptibility to diet or copper intake, different results based on anti-copper treatments, and an inability to reflect precise copper status.

Serum copper comprises 2 pools, the majority bound to Cp ($>90\%$, considered nontoxic) and a smaller pool ($<10\%$) circulating as "free" or loosely bound to other transporting proteins (mainly albumin, but also alpha-2-macroglobulin and histidine), representing the non–Cp-bound copper (NCC) or labile fraction.[1] This global concept of NCC represents the copper available for mobilization or exchange measured by different approaches. In untreated patients with WD, NCC is typically elevated. Classically, NCC has been calculated in WD by subtracting Cp-bound copper from total copper (considering that the amount of Cu associated with Cp is about 3.15 μg per mg). However, this mathematical calculation may be imprecise depending on Cp determination methods, leading to interpretational challenges in a significant percentage of cases.[6,7] The introduction of the exchangeable copper (CuEX) methodology by El Balkhi et al in 2009,[8] as a direct measurement of NCC, was a significant breakthrough. Basically, this method is based on the properties of the chelator EDTA, which is able to extract (or exchange) copper from the non-Cp protein compartment under physiological pH and conditions while the Cp-bound copper remains intact. CuEX (also abbreviated as CuEXC, NCC-Ex, or ExCu elsewhere) was shown to be significantly increased among patients with WD when compared to heterozygous (carriers) or healthy individuals,[9,10] whereas its ratio to total copper (referred to as ratio of exchangeable copper [REC]) was shown to be an excellent tool for WD diagnosis, especially when it was above a certain threshold ($\geq 18\%$).[8,11] In addition, CuEX and REC were shown to be significantly elevated in patients with WD compared to healthy individuals, with slight differences between adults and pediatric patients[12] and proved to be useful in various clinical contexts.[13] Indeed, higher levels of CuEX and increased REC have been described among patients with neuropsychiatric phenotypes[14] or noncompliant patients.[15] Alternative NCC measurements, such as the speciation spectrometry method,[16] also showed high levels of this "exchangeable copper" among patients with WD presenting with acute fulminant presentations.[17]

It is globally assumed that the REC should only be used for diagnostic purposes as it might not reflect subtle changes that can happen over time when patients are treated.[13,18] Regarding CuEX quantification, no specific cutoff has been proposed to date for the identification of new WD cases, as it may differ among patients with WD with different clinical severity. Besides, data regarding its potential use and accuracy during follow-up is lacking, and uncertainties persist regarding the ideal target levels of CuEX for monitoring. However, a recent publication among WD-stable patients assumed NCC as the best primary outcome to define clinical and biochemical stability.[19] However, up to now, only one study has shown the progressive decrease of CuEX among a cohort of 36 pediatric patients with WD during the first 5 years of therapy after diagnosis.[20] To address this gap, we conducted a study assessing CuEX behavior in the clinical follow-up of a large cohort of real-world patients with WD in Spain, aiming to provide insights into these unknown therapeutic goals.

METHODS

Patients

Patients aged ≥18 years old with a diagnosis of WD (Leipzig ≥4) followed as per real-world clinical practice at 2 large reference centers in Spain (Hospital Clínic in Barcelona and Hospital La Fe in Valencia) were included. Demographic, clinical, and analytical variables were obtained retrospectively from clinical records. Samples were obtained at different clinical time points, as required per standard clinical practice and following recommendations from the international guidelines.[1–3] Stable patients were checked every 6 months, while a more frequent evaluation (every 1–3 months) was recommended in uncontrolled conditions. All patients had consented to the use of their clinical characteristics as part of the Wilson Spanish National Registry AEEH (HCP/2021/1099) and had signed a specific informed consent for sample extraction with investigational purposes (HCB/2010/6144-R101116-039 and 2023-405-1). All research was conducted in accordance with both the Declarations of Helsinki and Istanbul.

For the purpose of the study, patients were divided into 2 groups: Group 1 included the so-called "stable patients," whereas group 2 incorporated the "nonstable" individuals. This definition was adapted from the previously published study on stable patients with WD.[19] Patients were considered clinically stable when they fulfilled 3 simultaneous conditions: (1) WD diagnosis had occurred more than 1 year before, (2) the patient had been on stable drug and dose for at least 12 months, and (3) the patient was compliant to medication. Nonstable patients were those with a recent WD diagnosis (in the last 12 months) and/or were noncompliant to medication and/or in which therapy had been changed either in dose or compound during the previous year. The same definition was used to classify patients at the end of their follow-up. Compliance with medication was defined as adequate or inadequate/not constant according to clinical reports, urinary copper excretion (UCE), and subjective self-reporting from patients at their medical visits.

Samples

Samples were obtained from November 2021 to November 2023; CuEX/REC quantification was centralized at Hospital Clínic Barcelona to reduce the potential methodological bias. Samples obtained from patients in Valencia were frozen at −80°C and sent to Barcelona. Our toxicology lab in Hospital Clinic Barcelona has extensive experience in the field of metals and is annually certified by the Spanish and European Biochemistry societies (see Supplemental Appendix 1 for details, http://links.lww.com/HEP/I694).

The baseline was defined as the date of the first sample with CuEX determination (point 1, P1). Successive time points of follow-up were defined as point 2 (P2), point 3 (P3), and point 4 (P4), respectively. Raw differences between CuEX time points were defined as "delta."

Total plasma copper (μg/dL), CuEX (μg/dL), and REC (%) were analyzed by inductively coupled plasma mass spectrometry, using a Perkin-Elmer NEXION 2000 Mass Spectrometer (Perkin- Elmer). The limit of quantification obtained was 0.053 μg/dL, and the limit of detection was 0.016 μg/dL (see Supplemental Appendix 2 for additional details, http://links.lww.com/HEP/I694). Normal ranges of CuEX were established between 4.1 and 7.1 μg/dL (equivalent to 0.64–1.12 μmol/L); these "reference ranges" had been previously set by the evaluation of 44 healthy subjects in El Balkhi's initial paper.[8] REC values were expressed as a percentage of the total copper (%); an REC value above 18% for new WD diagnosis and above 14% in patients with WD on follow-up were considered as characteristic of the disease, according to previous publications.[8–11,13] Classical NCC calculation (Cal-NCC) was obtained as Total-Cu (μg/L) − 3.15 × Cp (mg/L).[3] Quantification of the 24-hour UCE was done at each center by flame atomic absorption spectroscopy using a Perkin-Elmer AA200 atomic absorption spectrometer (Perkin-Elmer). The coefficient of variation between the series was 5.9%, and 2% within the series. According to the current guidelines,[2,3] UCE was considered "adequate" when copper quantification was within 200–500 μg/24 h in patients receiving chelators and <100 μg/24 h in patients receiving zinc salts.[21] Cp was measured by nephelometry by standard automated procedures and expressed in g/L; the limit of detection was 0.02 g/L.

Statistics

Quantitative variables were expressed as the median and interquartile range (IQR: 25th, 75th percentiles or expressed as IQR 25–75). Categorical variables were expressed as absolute frequencies and percentages. Comparisons between groups were made by the Mann-Whitney U or Fisher exact test, and lineal associations between CuEX/REC and other copper/liver parameters (liver transaminases, Cp and UCE) were assessed by the Pearson correlation coefficient. Dynamic changes in CuEX according to time were assessed among patients with at least 2 CuEX determinations. The fact that CuEX determination times were not standardized constituted a challenge to the correct estimation of the observed analytical changes from a statistical point of view. To overcome this limitation, we estimated the slope of change over time[22] by means of the following calculation:

$$\text{Daily slope} = \frac{\left(\frac{\text{Present value}}{\text{Index value}} \times 100\right) - 100}{\text{Time from Index value}},$$

where the *Index value* is the first CuEX determination at P1, and the determinations up to the current value are the subsequent results from P2 until the end of each patient's follow-up (*Present value*). *Time from index value* is the number of days from P1 to the day of determination of the CuEx result during follow-up. This statistical solution minimizes the impact of having different time intervals.

All the analyses were performed using commercially available software (IBM SPSS Statistics software version 26; IBM Corp.) with a 2-sided *p* value for significance set at ≤ 0.05.

RESULTS

Baseline characteristics

A total number of 92 adult patients with WD were included in the study: 51% women, 63% with exclusively hepatic phenotype at diagnosis, and 19.2 median years from WD diagnosis (IQR: 11.9, 39.9). At baseline, 54.9% of patients were receiving therapy with chelators (see Table 1 for details). Up to 39 patients (42%) were classified as "clinically stable" (group 1) whereas 53

TABLE 1 Baseline characteristics of the cohort (n = 92)

	All patients (n = 92) N (%) median ($IQR_{25–75}$)	Group 1 (n = 39) Stable patients	Group 2 (n = 53) Unstable patients	*p* (group 1 vs. group 2)
Age (y)	39 (28.5; 48)	40 (29; 50)	39 (26; 47)	0.57
Sex (female)	47 (51.1)	20 (51.3)	27 (50.9)	1
Time from WD diagnosis (y)	19.2 (11.9; 39.9)	19.6 (12.5; 30.7)	18.3 (11.1; 28.9)	0.53
Time from WD diagnosis (groups)				
Short-term (≤ 1 y)	5 (5.4)	0	5 (9.4)	
Median-term (>1 but ≤ 5 y)	6 (6.5)	3 (7.7)	3 (5.7)	
Long-term (>5 y)	81 (88)	37 (92.3)	45 (84.9)	0.21
WD phenotype at diagnosis (hepatic)[a]	58 (63)	26 (66.7)	32 (60.4)	0.49
Leipzig score at diagnosis	7 (5; 9)	8 (5; 9)	6 (5; 8)	0.40
Cirrhosis at diagnosis (yes)	22 (23.9)	6 (15.4)	16 (30.1)	0.13
Current therapy (chelator-based)[b]	50 (54.9)	17 (43.6)	33 (63.5)	0.047
Adequate compliance (subjective, yes)[c]	70 (76.9)	37 (94.9)	33 (63.5)	<0.001
Baseline fibrosis (elastography, kPa)[d]	5.1 (4.6; 7)	4.9 (4.4; 6.1)	5.8 (4.7; 7.1)	0.17
Baseline ASAT (IU/L)	26.5 (20; 37)	27 (21; 35)	25 (19.5; 38)	0.85
Baseline ALAT (IU/L)	34.5 (23; 53.7)	39 (23; 55)	31 (21.5; 49.5)	0.41
Baseline ceruloplasmin (g/L)	0.05 (0.05; 0.12)	0.05 (0.05; 0.11)	0.05 (0.05; 0.13)	0.91
Baseline total copper (μg/dL)	11 (7; 37)	11 (5.75; 22)	12 (8; 39)	0.29
Baseline CuEX (μg/dL)	3.8 (2.2; 4.9)	3.4 (2; 4.8)	3.8 (2.4; 5.7)	0.35
Baseline REC (%)	22.9 (14.5; 40)	20.9 (12.5; 41.5)	25 (16.6; 37.5)	0.47
Baseline Cal-NCC[e]	−67 (−101.45; 4.05)	−82 (−113.7; −8.05)	−64.9 (−91.2; 32.3)	0.22
Baseline UCE (μg/24 h)[f]	262 (98; 741)	127 (66.5; 770)	272 (118; 725)	0.09
Zinc-based treatment	98 (58–127)	86 (48–144)	98 (65–156)	
Chelator-based treatment	520 (266–1096)	498 (303–835)	535 (252–978)	
Sample number ≥ 3	36 (39.1)	11 (28.2)	25 (47.2)	0.051

Note: Baseline corresponds to the time for the first sample extraction (P1).

[a]Additional phenotypes at diagnosis were asymptomatic (n = 12, 13%), neurological (n = 8, 8.8%), mixed (n = 10, 11%), and others (n = 3, 3.3%).

[b]Treatment with chelators (n = 50) includes patients on DPEN (n = 32, 34.8%), TRI (n = 8, 8.7%), TTM (n = 1, 1.1%), or combination therapies with chelators and zinc (n = 9, 9.8%); otherwise, patients were treated with zinc salts only (n = 41) and 1 patient was not receiving any therapy at the time of sample acquisition (recent diagnosis, group 2).

[c]Adherence was registered in 87 patients.

[d]Elastography available in 55 patients at baseline.

[e]Calculated NCC (Cal-NCC) was performed among 86 patients as Total-Cu (μg/L) − 3.15·Ceruloplasmin (mg/L) according to Schilsky et al[3]; 62 (72.1%) cases resulted in negative uninterpretable results.

[f]UCE was available in 78 patients only: 35 from patients treated with zinc salts, 42 from those treated with chelators, and 1 patient with no therapy (not considered for these mean values).

Abbreviations: Cal-NCC, calculated non–ceruloplasmin-bound copper; CuEX, exchangeable copper; REC, ratio of exchangeable copper; UCE, urinary copper excretion in 24 hours; WD, Wilson disease.

patients (58%) were classified as "clinically nonstable" (group 2) due to recent diagnosis (n = 2), recent changes in therapy (n = 34), or noncompliance (n = 17). Two additional new WD diagnoses were detected once our database had been closed. Their CuEX data were only used for descriptive results (Supplemental Table S1, http://links.lww.com/HEP/I695 and Figure 1). No statistical differences were observed between both groups (stable vs. nonstable) in terms of sex, age, and time from WD diagnosis or therapy (chelators vs. zinc salts).

All patients had at least 1 CuEX or REC evaluation during the 2-year study, accounting for a total of 213 samples: 76 samples (35.7%) from group 1 and 137 (64.3%) from group 2. Sixteen patients (17%) had only 1 evaluation, 40 (43%) had 2 evaluations, 27 (29%) had 3 evaluations, and 9 patients (10%) had up to 4 determinations. The number of patients with 3 or 4 CuEX measurements available was significantly higher among those from group 2 (nonstable patients) compared to group 1 (stable patients): 25 (47.2%) versus 11 (28.2%), respectively ($p < 0.01$) (Table 1).

CuEX and REC quantification at baseline

The median CuEX and REC concentrations at baseline were 3.8 μg/dL (IQR: 2.2, 4.9) and 22.9% (IQR: 14.5; 40), respectively with no significant differences between stable (group 1) and nonstable (group 2) patients. When exploring the adequacy of all samples (n = 213) compared to the predefined normal ranges for CuEX (4.1–7.1 μg/dL),[8] we observed that only 73 (34.3%) were within range, with 121 (56.8%) below and 19 (8.9%) above the normal (Figure 2A). At baseline, the distribution of CuEX among stable and nonstable patients was very similar (Figure 2B), with the proportion of samples below normal ranges for both groups being high and comparable (64.1% and 56.6%, respectively). Importantly, no signs of copper deficiency were observed in individuals with low CuEX levels (Table 2). Only 35.9% and 30.3% of the samples fell within normal ranges for both groups, whereas the 7 samples (13.2%) above normality at baseline all belonged to nonstable patients: 2 due to recent changes in therapy, 3 reporting low compliance, and 2 patients with a recent diagnosis of WD (Figures 2B, C).

When patients were grouped according to the elapsed time from WD diagnosis to sample extraction (short term [≤1 year, n = 5], medium term [>1 and ≤5 years, n = 6], long term [>5 years, n = 81]), the majority of samples below and within the normal range at P1 were seen among patients on long-term follow-up (89.4%, n = 76). Samples above normality (>7.1 μg/dL, n = 7) were distributed both in the short-term (n = 2) and the long-term group (n = 5). No differences were observed at baseline in CuEX distribution according to the received treatment (chelators vs. non-chelator).

When using the predefined thresholds for the use of REC among naive WD[9–11] and treated patients[13,18] at P1, REC was shown to be above 18% in 61 (66.3%) patients (including the 2 patients with a recent diagnosis), between 14% and 18% in 10 (10.9%) and below 14% in 21 (22.8%) patients. This distribution was similar when comparing stable and nonstable groups at P1 (Table 3) regardless of the elapsed time from diagnosis or the received treatment (Kruskal-Wallis >0.05). REC distribution followed a similar pattern in consecutive

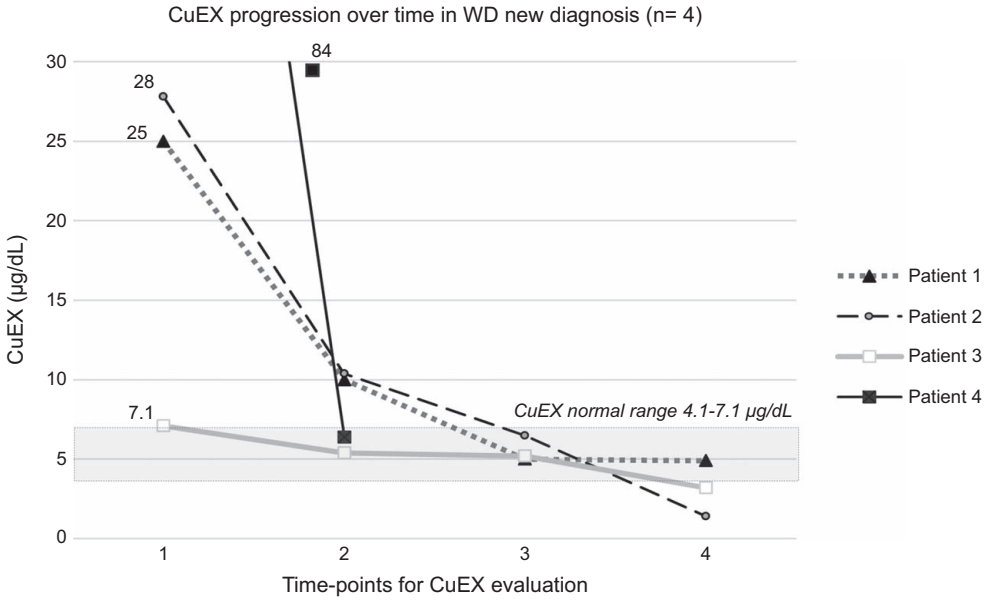

FIGURE 1 Dynamic changes in CuEX among WD recent diagnosis (n = 4). *Note*: Normal range for CuEX (4.1–7.1 mg/dL) is defined by the dotted box.

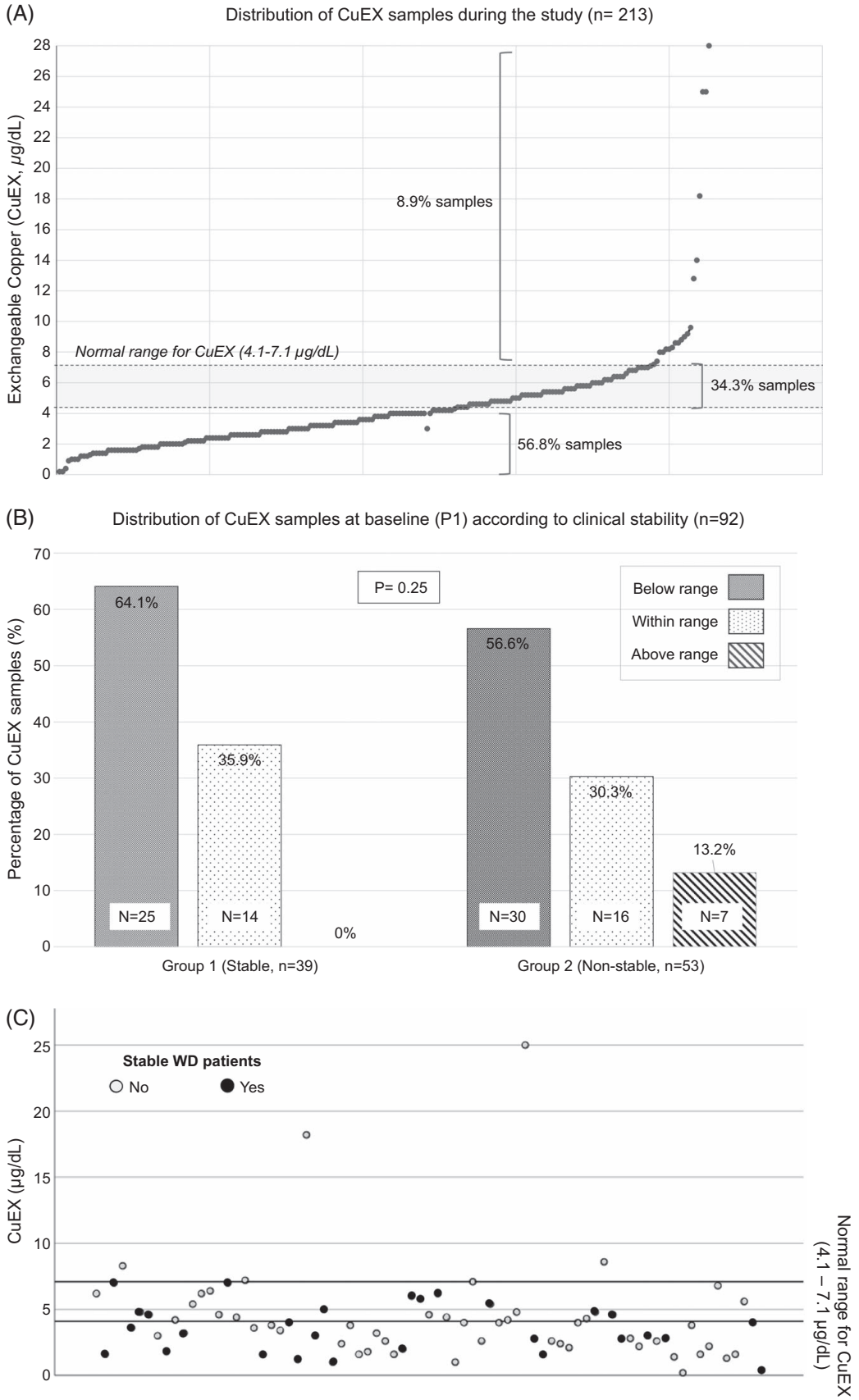

FIGURE 2 (A) Distribution of CuEX samples during the study (n = 213). (B) Distribution of CuEX samples at baseline (P1) according to clinical stability (n = 92). (C) Distribution of CuEX samples according to clinical stability (n = 92).

TABLE 2 Clinical and analytical evaluation of patients with CuEX levels below the established reference normal range (<4.1 mg/dL) at baseline (n = 55, 59.8%)

N (%) Median ($IQR_{25–75}$)	All patients (n = 55) (CuEX levels <4.1 mg/dL)	Group 1 (Stable pts, n = 25)	Group 2 (Nonstable pts, n = 30)	*p* (group 1 vs. group 2)
Sex (F)	33 (60)	15 (60)	18 (60)	0.608
Age (y)	40 (29–51)	40 (29–49)	41.5 (28–55)	0.388
Time elapsed from WD diagnosis (y)	19 (11.7–29.21)	18.6 (11.3–29.2)	19.9 (12–29.5)	0.685
Cirrhosis at diagnosis	16 (29.1)	5 (20)	11 (36.7)	0.145
Ceruloplasmin (g/L)[a]	0.05 (0.05–0.08)	0.05 (0.05–0.09)	0.05 (0.04–0.06)	0.094
Total serum copper[b](μg/dL)	9 (6–15.5)	10.5 (4.2–16.5)	8.5 (6.7–13)	0.902
CuEX (μg/dL)	2.6 (1.6–3.4)	2.8 (1.6–3.3)	2.5 (1.6–3.4)	1
REC (%)	22.9 (16.8–36.4)	20 (13.8–38)	26.9 (20–36.6)	0.130
Cal-NCC[c]	−86.3 (−117; −43.3)	−92 (−117; −40.8)	−77 (−109; −43.5)	0.268
UCE (μg/24 h)[d]	268 (95–651)	100 (55–780.75), n = 20	282 (121–651), n = 25	0.115
Zinc-based therapy	92 (49–135) (n = 21)	77 (43.2–118) (n = 14)	99 (67–265) (n = 7)	
Chelator-based therapy	627 (290–1550) (n = 24)	534 (246.5–775) (n = 18)	2017 (780.75–6593) (n = 6)	
WBC (10^9/L)	6.04 (1.89; 7.32)	6.49 (5.50; 7.48)	5.69 (4.82; 6.96)	0.146
Hemoglobin (g/L)	135 (125; 150)	143 (131; 154)	133 (123; 143.7)	0.062
Hematocrit (%)	41 (39; 46)	43 (40.7; 47.5)	40.95 (38; 44.20)	0.035
MCV (fl)	91 (88.6; 94)	90.3 (87.7; 94.2)	91.2 (88.9; 94.07)	0.571
Platelets (10^9/L)	223 (176; 277)	235 (175; 264)	220.5 (175.7; 284.2)	0.906
Neurological symptoms	1 (1.8)	1 (4)[e]	0 (0)	NA

[a]Cp available in 54 patients.
[b]Total serum copper available in 51 patients.
[c]Calculated NCC (Cal-NCC) was estimated in 50 patients as Cal-NCC = Total-Cu (μg/L) − 3.15·Ceruloplasmin (mg/L) according to Schilsky et al[3]; 46 (92%) resulted in negative and uninterpretable results.
[d]UCE was available in 45 patients (21 zinc-treated and 24 chelator-treated patients).
[e]One stable patient on long-term treatment with zinc salts (30 y from diagnosis) presented with atypical and fluctuating neurological signs and low levels of CuEX (2 μg/dL) and UCE (48 μg/24 h). The initial clinical suspicion was copper deficiency presenting as myelopathy, and treatment was withdrawn at this point. The patient did not improve clinically, and copper parameters did not change significantly after 2 months off-therapy (CuEX not available, UCE 70 μg/24 h), leading to retreatment. Unfortunately, the patient progressed and developed hepatic decompensation (ascites) despite therapy and was waitlisted for liver transplantation. In conclusion, the diagnosis of copper deficiency was not supported by our final data and/or clinical outcomes.
Abbreviations: Cal-NCC, calculated non–ceruloplasmin-bound copper; CuEX, exchangeable copper; MCV, mean corpuscular volume; NA, not available; REC, ratio of exchangeable copper; UCE, urinary copper excretion in 24 hours; WBC, white blood count; WD, Wilson disease.

time points (P2, P3, and P4), reinforcing the fact that this ratio has a reduced accuracy for WD diagnosis or follow-up when patients are receiving specific anti-copper therapy.

Dynamic changes over time of copper-associated markers and other parameters

The median time between CuEX/REC determinations was 196 (137–322), 159 (113–213), and 143 (101–184) days for P2-P1, P3-P2 and P4-P3, respectively. Changes in copper-associated markers could be assessed in 77 patients (83.7%) in whom at least 2 time point measurements were available (54 patients from Barcelona and 23 patients in Valencia). During the study period, 57 patients remained in the same clinical group (either stable or nonstable), whereas 20 patients changed from baseline to the end of the evaluation: 14 (38.9%) nonstable patients at baseline were stable at the end of follow-up, and 6 (14.6%) stable patients at first evaluation changed into a nonstable condition.

Median CuEX concentrations at P1 (n = 92), P2 (n = 76), P3 (n = 36), and P4 (n = 9) were similar: 3.8 (IQR: 2.2; 4.9) μg/dL, 3.4 (IQR: 2.4; 5.6) μg/dL, 4.2 (IQR: 2.6; 5.4) μg/dL, and 4 (IQR: 3.5; 6.1) μg/dL, respectively, with no significant differences observed between stable (group 1) and nonstable (group 2) patients at any time point. REC values at the same time points were 22.9% (IQR: 14.5; 40) (P1), 26.7% (IQR: 13.9; 40) (P2), 24.4% (IQR: 15.6; 33.3) (P3), and 14.5% (IQR: 10; 33.9) (P4), again without differences between groups (Table 3). The median differential raw value for CuEX concentrations (Delta CuEX) among patients requiring treatment changes at baseline (either

TABLE 3 Copper and liver parameters over time

N (%) Median ($IQR_{25–75}$)	Point 1 (n = 92)		Point 2 (n = 76)		Point 3 (n = 36)		Point 4 (n = 9)	
Patient groups at baseline[a]	**Group 1 (n = 39)**	**Group 2 (n = 53)**	**Group 1 (n = 26)**	**Group 2 (n = 50)**	**Group 1 (n = 11)**	**Group 2 (n = 25)**	**Group 1 (n = 0)**	**Group 2 (n = 9)**
Total copper (μg/dL)[b]	11 (5.75; 22)	11.5 (8; 39)	13.5 (6.7; 24.5)	11.5 (8; 33)	18 (9; 40)	14 (8.5; 45)	NA	26.5 (9.7; 49.5)
CuEX[c]	3.4 (2; 4.8)	3.8 (2.4; 6)	3.4 (2; 5)	3.4 (2.4; 5.6)	4 (2.2; 5.2)	4.4 (2.8; 5.9)	NA	4 (3.5; 6.1)
CuEX range[c]								
Above	0	7 (13.2)	2 (7.7)	5 (10)	1 (9.1)	3 (12)		1 (11.1)
Within	14 (35.9)	16 (30.2)	8 (30.8)	17 (34)	4 (36.4)	11 (44)	NA	3 (33.3)
Below	25 (64.1)	30 (56.6)	16 (61.5)	28 (56)	6 (54.5)	11 (44)		5 (55.6)
REC (%)	20.9 (12.5; 41.5)	25 (16.6; 37.5)	25.2 (13.2; 40.6)	27 (14.3; 39)	21.7 (10; 30)	25.8 (17; 40)	NA	14.5 (10; 34)
REC ≥ 18%	22 (56.4)	39 (73.6)	16 (61.5)	34 (69.4)	7 (63.3)	17 (70.8)	NA	3 (33.3)
REC 14%–17.9%	6 (15.4)	4 (7.5)	3 (11.5)	3 (6.1)	1 (9.1)	3 (12.5)		2 (22.2)
REC < 14%	11 (28)	10 (18.9)	7 (26.9)	12 (24.5)	3 (8.6)	4 (16.7)		4 (44.4)
UCE (μg/24 h)[d]	127 (66.5; 770)	272 (118; 725)	90 (71; 234)	226 (72; 630)	84 (44; 209)	260 (102; 925)	NA	700 (64; 1875)
UCE (μg/24 h)[d]:								
Zinc-treated	86 (48; 144.5)	98.5 (64.7; 156.2)	84 (58.8; 91)	90 (52.5; 130)	72 (30; 84)	102 (51; 194)	NA	66.5 (61.3; 741.5)
Chelator-treated	498 (303–835)	535 (252.7; 978.5)	428 (111; 560)	368.5 (213.7; 763)	232 (188.75; 352)	554 (251; 1276)		1360 (390.9; 2112.7)
ASAT (IU/L)	27 (21; 35)	25 (19; 37)	28 (23; 35)	28 (20; 38.5)	24 (21; 47)	28 (20; 42)	NA	20 (18; 39)
ASAT < ULN[e] (%)	35 (89.7)	42 (79.2)	24 (85.7)	38 (77.6)	7 (63.6)	17 (68)		8 (89)
ALAT (IU/L)	39 (23; 55)	31 (21; 50)	38 (21; 57.5)	32 (22; 63.5)	34 (24; 75)	43 (27; 71)	NA	33 (22.7; 47)
ALAT < ULN[e] (%)	20 (51.3)	35 (66)	14 (50)	29 (59.2)	6 (54.5)	12 (48)		6 (67)
GGT (IU/L)	28 (18; 43)	30 (22; 54)	27 (18; 46)	32 (22; 56)	25 (15; 51)	33 (22; 58)	NA	32.5 (21; 69)

[a]No statistical differences were seen between group 1 (stable patients) and group 2 (nonstable patients) regarding all these quantitative measurements at any point during follow-up.
[b]Total copper normal levels at our institution are 70–140 μg/dL.
[c]CuEX range of normality was established according to El Balkhi et al[8] between 4.1 and 7.1 μg/dL (explored among 44 healthy individuals). Group 1 refers to clinically stable patients; group 2 refers to nonclinically stable patients (either due to noncompliance, recent diagnosis < 1 year, or change in dose or drug for WD).
[d]UCE was available for 78 patients only.
[e]ULN refers to ASAT or ALAT below 40 IU/L.
Abbreviations: CuEX, exchangeable copper; REC, ratio of exchangeable copper; UCE, urinary copper excretion; ULN, Upper limit of normal.

reduction, increase dose, or drug change) (n = 25, 27.2%) was different between those decreasing doses (n = 13, delta-CuEX +0.4 μg/dL), those increasing doses (n = 6, delta-CuEX −0.09 μg/dL), or those changing drug (n = 6, delta-CuEX -1 μg/dL). This analysis could not be replicated at successive time points due to the small sample size.

Dynamic changes of CuEX adjusting for time between samples (daily scope) were compared at the different time points (P2-P1, P3-P2, and P3-P1), but no differences were observed between patients classified as stable or nonstable at baseline. No differences were found for either REC, serum copper, Cp, or ASAT/GGT levels. Only delta ALAT P2-P1 was statistically different between stable versus nonstable patients (median change per day −1.1 IU/L (IQR −4.85; 0.13) versus +0.68 IU/L (−3.39; 5.61), respectively ($p = 0.04$). However, this change was not considered clinically relevant.

As mentioned, 2 additional patients were diagnosed with WD once our database had been closed, thus providing a total number of 4 naïve individuals. CuEX dynamics over time have been graphically represented in Figure 1, and descriptive analysis of copper parameters (total copper, CuEX, REC, and UCE) from these 4 special individuals is depicted in Supplemental Table S1, http://links.lww.com/HEP/I695. Over a variable period of time, a profound decrease in CuEX levels was observed for all cases once treatment was started.

Association between CuEX/REC and other biochemical parameters

To assess the potential additional benefits of CuEX/REC, we evaluated their association with the copper and hepatic measurements currently used for WD follow-up in clinical practice. No significant associations were observed with liver transaminases (ASAT, ALAT, GGT), Cp, serum total copper, or UCE at baseline. As expected, there was a statistically significant but negligible positive correlation between CuEX and its ratio ($r = 0.28$, $p = 0.008$). No correlation was observed between CuEX and UCE, and only a mild statistical association was seen between REC and UCE ($r = 0.25$ $p = 0.02$) (Supplemental Table S2, http://links.lww.com/HEP/I695).

UCE was available in 77 patients at P1 and was classified as "adequate" in only 32 patients (34.8%).[3] Interestingly, only 12 patients (16%) had both biochemical markers (UCE and CuEX) within the normal range at P1, 3 patients (4%) were below the normal range, and 3 (4%) were above for both measurements. Overall, the physician received similar information from both measurements in only 18 (23.4%) patients, whereas discrepancies about normality were provided for the remainder. In addition, only 6 out of the 12 patients with "normal" UCE and CuEX had been classified as patients with clinical stability (group 1).

A Kruskal-Wallis test was conducted to determine if there were differences between the median levels of transaminases at baseline according to CuEX [classified within (n = 30), above (n = 7), or below (n = 55) the established ranges for normality at baseline]. ALAT and ASAT levels were significantly lower among patients with CuEX below 4.1 μg/dL (median ASAT, 25 IU/L and ALAT, 30 IU/L) when compared to patients with CuEX within or above the normal range (ASAT, 32 and 34 IU/L; ALAT, 46.5 and 36 IU/L, respectively) ($p < 0.05$).

DISCUSSION

One of the main current unmet needs in WD relates to our inability as clinicians to ensure a patient is metabolically well-controlled with medication and reaches copper homeostasis. No isolated evaluation has proven to be sufficiently robust to guide therapy. International guidelines recommend different targets in UCE according to the phase of the disease (either recent diagnosis or maintenance phase) and treatment (either chelators or zinc salts).[2,3] However, urinary copper is not always stable and might suffer significant variability in our day-to-day practice.[21] Indirect mathematical calculations of "*free copper*" (Cal-NCC) showed to be noninterpretable in a high proportion of patients, and thus, it cannot be accepted as a reliable parameter for monitoring.[3]

The development of different NCC direct measurements[8,16] was an attempt to overcome these problems. This NCC was assumed to represent the labile fraction of the systemic copper and the best option to be reduced and stabilized in WD. EDTA-based CuEX was the first proposed direct measurement of NCC.[8,9] Different publications since then showed CuEX and REC to be excellent tools for WD diagnosis.[10,11] They were also shown to be useful for differential diagnosis with other liver diseases[13] and proved to be significantly elevated in patients with extrahepatic phenotypes[14] and patients with poor compliance to therapy.[15] However, their use as a longitudinal marker of copper stability during long-term follow-up has been seldom reported. In 2022, a single-center study performed among 36 pediatric patients in Lyon[20] showed a progressive decrease in CuEX, especially during the first 3 months of therapy, which then reached a *plateau* afterwards and slightly increased 10 years later. The authors suggested that this late increase could be explained by compliance problems, as they were used to seeing this pattern in regular practice. Even though broad experience has accumulated, no current recommendations have been published to date regarding the use of CuEX in WD monitoring.

The use of this CuEX methodology was incorporated into our hospital in November 2021. After an initial single-point evaluation,[18] we then wanted to assess its

utility for monitoring copper homeostasis over time. We therefore analyzed all the available CuEX quantifications during a 2-year period from 2 large, experienced centers in Spain. The most intriguing result was that more than half of our patients (57.8%) showed CuEX levels below the range of normality (even those considered as nonstable) and, most importantly, had no clinical or analytical signs of copper deficiency. These low levels of CuEX were not associated with other copper-associated parameters, clinical phenotype, time from diagnosis, or therapy, but patients with lower CuEX levels showed significantly lower levels of transaminases. Similar results were recently published by Ott et al,[23] where labile copper was measured both as CuEX and an alternative speciation method (NCC_Sp).[16] In this cohort, a significant number of individuals (32%) also presented with low CuEX levels, with no signs of copper deficiency and with lower levels of transaminases.

Assessing why treated patients with WD frequently present lower CuEX levels than healthy non-WD individuals is beyond the scope of this analysis, but it has been replicated in both studies (ours and[23]), pointing toward different potential explanations. First, most patients were on long-term follow-up and had been de-coppered for many years; in this clinical scenario, CuEX changes might be subtle and difficult to quantify. Second, it is unknown whether chelators and zinc salts might have a real impact on the NCC pool and to what extent, due to their different mechanisms of action. However, the profound and accelerated decline in CuEX levels observed in our 4 new Wilsonian cases would suggest a very rapid effect of therapy in this NCC fraction. A similar slope was described by Ngwanou et al among pediatric naïve WD cases.[20] Third, it should be remembered that total serum copper in WD is typically very low when compared to healthy individuals, as it mainly depends on Cp levels. Keeping this in mind, it is not surprising that the CuEX levels were lower than expected, as these "normal levels" were extracted from non-Wilsonian healthy individuals with presumably normal copper levels.[8] Fourth and last, the possibility of CuEX not being an accurate marker for its use among WD-treated patients could also be real. This option was addressed by a recent publication,[16] postulating that this EDTA-exchange methodology has a significant lack of repeatability. In this work, the authors suggested that CuEX was significantly underestimated, as a significant part of the labile copper fraction was retained by the filter. To overcome these limitations, an alternative method for direct NCC measurement (NCC_Sp) was developed by using a novel speciation strategy. A direct comparison between both NCC methodologies (CuEX and NCC_Sp) was performed in the paper by Ott et al,[23] showing a positive correlation between methods, with a tendency of CuEX being higher than NCC-Sp when values were below 6 μg/dL, and lower above this cutoff. This is important if we consider the high percentage of patients with low CuEX in our hands. Nevertheless, further analysis will be required to assess comparability between methods.

What our work suggests is the need to redefine the "correct" CuEX target levels (if any) for patients with WD under treatment, instead of applying "normal reference ranges" from the general population, as they may not reflect the characteristic disturbed copper metabolism of WD. If we consider CuEX dynamics from the new WD diagnosis versus WD patients on follow-up from our cohort, we could additionally argue in favor of defining different "early" and "late" target ranges, acknowledging early changes might be much more informative for clinical guidance than those at later stages. Moreover, the potential clinical benefit of achieving low CuEX levels must be defined, as they were shown to be associated with significantly lower transaminases and no signs of copper deficiency. Interestingly, urinary copper evaluation was not optimal in our hands: only 34% were classified as "adequate" according to the current recommendations[2,3] and only 23% of the patients showed CuEX and UCE messages aligned. Altogether, we support the use of CuEX as a complementary tool to other biochemical parameters to guide clinical decisions on WD follow-up. Low CuEX levels should be considered cautiously before assuming patients are overtreated, whereas high or increasing CuEX levels (especially reaching the 7 μg/dL threshold) should prompt physicians to question treatment adequacy and/or adherence. Therefore, future research on defining the "target CuEX ranges" for WD-treated patients is highly needed.

Our work has some limitations that should also be considered. First, we artificially classified patients into 2 groups, considering recent diagnoses and drug changes in the last year as major reasons for nonstability. Whether this definition was adequate or not could be discussed, as there is no current standardized definition for clinical stability in WD. In fact, this is partially due to the lack of accurate biomarkers, which eventually was the main driver of our research. However, a similar classification was used in the phase III CHELATE study,[19] reinforcing our proposal. Unfortunately, "nonstability" could not be correlated in this work with WD clinical outcomes. This can be explained by the limited time of evaluation, the long-term maintenance phase for most of the patients, and the clinical heterogenicity of this WD cohort, which prevent us from identifying common endpoints. To partially overcome this limitation, nonstable patients were also considered if poor compliance with medication was observed. Unfortunately, adherence evaluation in this real-world setting was not validated nor quantified by objective measurements, and this might introduce some variability and subjectivity. However, differences observed in UCE among patients treated with chelators

or zinc supported our compliance evaluation. In addition, CuEX highest levels tended to be grouped within the nonstable group, aligned with what has been reported on higher CuEX among noncompliant patients.[15] Whether greater undetected differences between stable versus nonstable patients were due to the reduced sample size, the limited number of clinical events, the long-term follow-up of the cohort, or due to the copper assay itself is still unknown. Another limitation to be considered is the inherent fact of working with a real-world cohort in which CuEX was used as a complementary tool for clinical guidance, highly enriched by nonstable patients in whom samples were not obtained prospectively at specific time points. However, we introduced some statistical adjustments to minimize the absence of predefined schedules by considering CuEX changes related to a time unit (day) and thus avoiding the impact of different periods of time.

In conclusion, our real-world experience with CuEX during WD follow-up reveals that a large proportion of treated patients present this copper fraction below the expected ranges of normality. However, no sign of overtreatment was seen. On the other hand, high CuEX levels may reflect the uncontrolled copper status and may guide clinicians in increasing drug doses or improving adherence. Its use in clinical practice is clearly helpful but should still be combined with other copper evaluations until a better target range is defined. Whether CuEX needs a conversion factor or will be substituted by the more precise speciation method is still a matter of discussion and should be based on further direct comparisons between both methodologies in real-world practice.

AUTHOR CONTRIBUTIONS

Zoe Mariño, José Ríos, and Marina Berenguer: substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data. Zoe Mariño, José Ríos, Xavier Forns, Mercè Torra, and Marina Berenguer: drafting the article or revising it critically for important intellectual content. All authors: final approval of the version to be published.

ACKNOWLEDGMENTS

CERCA Programme/Generalitat de Catalunya. Zoe Mariño thanks Hospital Clínic for their Sabbatical Support, AEEH (Asociación Española para el Estudio del Hígado) for the Joan Rodés 2024 Grant, and CIBERehd.

CONFLICTS OF INTEREST

Zoe Mariño consults for, is on the speakers' bureau for, and received grants from Orphalan. She is on the speakers' bureau for and received grants from Gilead. She consults for Alexion and DeepGenomics. Anna Miralpeix is on the speakers' bureau for Orphalan. José Ríos received education and/or training fees from Boehringer Ingelheim, CZ Vaccines, Chiesi, GlaxoSmithKline, Grünenthal, MSD España S.A., Novartis, Lilly, and Vifor, unrelated to this manuscript. Xavier Forns consults for Gilead. Marina Berenguer consults for Alexion, Advanz, Abbvie, Chiesi, and Orphalan. She received grants from Gilead. The remaining authors have no conflicts to report.

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How to cite this article: Mariño Z, Garcia-Solà C, Ríos J, Bono A, García S, Miralpeix A, et al. Exchangeable copper for patients with Wilson disease at follow-up: Rethinking normal ranges or changing methodology. Hepatology. 2025;81:1728–1739. https://doi.org/10.1097/HEP.0000000000001105