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Relative Exchangeable Copper, Exchangeable Copper and Total Copper in the Diagnosis of Wilson Disease

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ABSTRACT

Background and Aims: Diagnosing Wilson disease (WD) remains challenging. The exchangeable copper (CuEXC) methodology measures the non-ceruloplasmin-bound copper fraction in serum. Relative exchangeable copper (REC), the ratio of CuEXC to total serum copper (Total Cu), has been proposed as a potential diagnostic biomarker. This study aimed to evaluate the diagnostic performance of these three copper biomarkers in WD.

Methods: CuEXC and Total Cu levels were measured in newly diagnosed treatment-naïve patients with WD ($n = 13$), treated WD ($n = 91$), non-Wilsonian hepatic disease ($n = 206$) and non-Wilsonian acute liver failure ($n = 22$). REC, CuEXC and Total Cu were compared among groups. Receiver-operating characteristic analyses were performed.

Results: Median REC was significantly elevated among patients with WD compared to all other groups combined (23.6% vs. 4.9%, $p < 0.001$). The opposite was found for Total Cu (3.5 μmol/L vs. 17.2 μmol/L, $p < 0.001$). In newly diagnosed patients with WD, median REC was significantly higher than in treated patients (29.1% vs. 21.6%, $p = 0.008$). The optimal diagnostic cut-off value for REC was ≥ 13.8% (sensitivity 100% and specificity 99.6%) for newly diagnosed patients versus those with non-Wilsonian hepatic disease. For Total Cu, the optimal cut-off was ≤ 7.1 μmol/L (sensitivity 61.5% and specificity 99.1%) for newly diagnosed patients with WD versus those with non-Wilsonian hepatic disease.

Abbreviations: AIH, autoimmune hepatitis; ALF, acute liver failure; AUC, area under the curve; Cp, ceruloplasmin; CRP, C-reactive protein; CuEXC, exchangeable serum copper; NCC, non-ceruloplasmin-bound copper; REC, relative exchangeable copper; Total Cu, total serum copper; WD, Wilson disease.

Conclusion: Our data support the diagnostic value of REC in WD. The more broadly available Total Cu also demonstrates a strong diagnostic performance and may be useful in initial work-up. We suggest including REC and/or Total Cu in a future revision of the Leipzig score.

1 | Introduction

Wilson disease (WD) is a rare autosomal recessive genetic disorder caused by variants in the *ATP7B* gene, encoding a copper-transporting P-type ATPase essential for copper homeostasis [1]. ATP7B dysfunction impairs hepatobiliary copper excretion and disables the incorporation of copper into ceruloplasmin (Cp) [2–4], which in turn causes copper to accumulate, particularly in hepatic and cerebral tissue [1, 5, 6].

Even though effective treatments exist, the condition can be fatal [6]. The diagnosis of WD can be challenging due to diverse symptoms and heterogeneous presentation [5], and diagnostic delay poses a serious concern, underscoring the urgent need for rapid and reliable diagnostic tests [7, 8].

Currently, several tests are available for the diagnostic work-up in a patient with suspected WD [1]. Still, no single test can confirm or exclude WD—each has its disadvantages preventing it from standing alone [7]. The Leipzig score is the pragmatic current gold standard for diagnosing WD, incorporating scores for hepatic copper content, serum ceruloplasmin and mutational analysis, among others. The Leipzig score does not include measurements of copper in serum [9].

In healthy individuals, the majority of copper in the blood is irreversibly bound to Cp, while a small fraction, the non-ceruloplasmin-bound copper (NCC), is loosely bound to other proteins and peptides. The NCC fraction is more bioavailable, elevated in WD, and believed to be responsible for the copper-induced organ damage observed in WD [10, 11]. The concurrent reduction of Cp explains why total serum copper (Total Cu) levels are low in WD despite elevated NCC.

El Balkhi and colleagues developed a method for direct measurement of the NCC fraction, called exchangeable serum copper (CuEXC). This method utilises the chelating ability of ethylenediaminetetraacetic acid, followed by ultrafiltration and copper quantification by flame atomic absorption spectrometry and subsequent direct measurement of the NCC fraction [12]. After separate measurements of Total Cu, relative exchangeable copper (REC) can be calculated as the ratio of CuEXC to Total Cu. Subsequent studies suggested that REC > 18.5% has 100% sensitivity and specificity in diagnosing WD, whereas CuEXC was more useful in monitoring treatment [13, 14]. These findings have only been partly validated; therefore, this study aimed to validate these findings in a larger independent cohort.

We examined the diagnostic value of REC, CuEXC and Total Cu in a combined cohort of Spanish and Danish patients with WD or non-Wilsonian hepatic diseases. Further, we examined potential correlations between these biomarkers and various demographic variables. As a subanalysis, we assessed the effects of inflammation and acute liver failure (ALF) on these biomarkers.

2 | Methods and Design

This is a cross-sectional study of two cohorts conducted at Aarhus University Hospital and Hospital Clinic Barcelona. The two cohorts were compared and also combined into a single cohort for analysis to achieve a more extensive study population. Furthermore, a longitudinal subanalysis was performed on a subset of the cohort.

In Denmark, the study was approved by the Central Jutland Regional Committee on Health Research Ethics (1-10-72-342-20). Patients were included after providing written informed consent. In Barcelona, Spain, the Hospital Clinic Ethics Committee approved the study, and patients were included after providing written informed consent for sample extraction for investigational purposes [HCB/2010/6144-R101116-039 and 2023-405-1]. All research was conducted following both the Declaration of Helsinki and Istanbul.

2.1 | Study Population

2.1.1 | Cross-Sectional Section

A total of 332 individuals were enrolled in the cross-sectional part of the study, 227 Spanish and 105 Danish patients. In total, we included 104 patients with WD, 22 patients with ALF of non-Wilsonian causes and 206 patients with various non-Wilsonian hepatic diseases, distributed as follows: 42 alcohol-related liver disease, 40 autoimmune hepatitis (AIH), 22 primary biliary cholangitis, 8 primary sclerosing cholangitis, 65 metabolic dysfunction-associated steatotic liver disease, 10 viral hepatitis and 19 drug-induced liver disease. Cancer was an exclusion criterion. Overall, 329 of all enrolled patients were adults; the remaining 3 were under the age of 18. Furthermore, we included data from 120 healthy Danish blood donors, which has been previously published as part of the methodological development in Denmark [15]. Selected patient characteristics are presented in Table 1.

All patients with WD were diagnosed according to the Leipzig score (≥4) [9]. Patients with WD were divided into 'Newly diagnosed' treatment-naïve patients with WD with measurement of copper biomarkers available before initiation of treatment ($n = 13$) and patients with 'treated WD' ($n = 91$). Treated patients were further categorised as 'stable WD' ($n = 66$), defined as patients on the same treatment and dose for ≥ 1 year, or 'non-stable WD' ($n = 25$) either due to short-term follow-up (< 12 months), changes in treatment dose or type in the last year, or poor compliance.

For newly diagnosed patients with WD, the first available measurement was included in the data set. For patients with stable and non-stable WD, only the most recent measurement of copper markers was used, thus preventing the impact of repeated entries. Cirrhosis status was determined by

Summary

- We evaluated different copper markers for diagnosing Wilson disease.
- Relative exchangeable copper was found to be an accurate diagnostic tool.
- Total plasma copper, which is more widely available, was also surprisingly useful.

non-invasive elastography or liver histology and considered a binary yes/no variable.

2.1.2 | Longitudinal Section

The data set included multiple measurements over time for Danish newly diagnosed treatment-naïve patients with WD ($n = 8$). Further, for Danish patients with stable WD, the first and last available measurements of CuEXC and REC were used to assess changes over time.

Additionally, we included five Danish patients with non-Wilsonian hepatic disease and concurrent inflammation to assess the potential impact of inflammation on copper biomarkers. The inclusion criterion was C-reactive protein (CRP) ≥ 50 mg/L. Measurements of copper biomarkers were performed during (within 24 h of measuring CRP ≥ 50 mg/L) and after the resolution of inflammation (CRP < 50 mg/L).

2.2 | Analysis of Copper Biomarkers

In both Spain and Denmark, CuEXC measurement is based on the methodology previously described by El Balkhi et al. [12] Both the Danish and Spanish methodologies are elaborated in detail in previously published articles [15, 16]. Total Cu and CuEXC were determined in venous blood samples collected at the time of inclusion. All measurements were conducted using standardised procedures in the two laboratories, both of which meet international quality criteria. Further, both centres annually validate their assays through central laboratory validation conducted in France. There are slight differences in the methodology between Spain and Denmark (for further elaboration of the methodologies, see 'methods' in the Supporting Information Appendix).

2.3 | Statistical Analysis

The normality of the data was tested using histograms and QQ plots. As the data were generally non-parametric, continuous variables were presented using median and interquartile range. Group comparisons were analysed using the Kruskal–Wallis test and Mann–Whitney *U* test as appropriate. Correlations were tested using Spearman correlation analysis. For longitudinal data, repeated measures ANOVA was used.

Receiver-operating characteristic (ROC) curves were used to determine an optimal cut-off value for REC and to evaluate the diagnostic performance of REC, CuEXC and Total Cu. All comparisons were two-tailed, and the confidence intervals were fixed at 95%. Statistical significance was obtained if $p < 0.05$.

Data analysis was performed using STATA (StataCorp. 2023. *Stata Statistical Software: Release 17.0*. College Station, TX: StataCorp LLC.).

3 | Results

3.1 | Demographics

Table 1 presents selected WD patient characteristics. Of the 104 patients with WD, 13 were newly diagnosed treatment-naïve, 51 received a chelating agent monotherapy, 38 received zinc monotherapy and 2 received a combination of the two at the time of blood sampling. Demographic data and copper measurements on patients with non-Wilsonian hepatic disease are provided in the Supporting Information Appendix, Tables S1 and S2.

3.2 | Biomarkers of Copper

Median REC in the 104 patients with WD was 23.6% (IQR: 12.8–40.0) (Figure 1A). This value was statistically significantly higher than in all other groups combined 4.9% (3.9–6.3), $p < 0.001$, and considerably above the Danish reference interval (3.0%–9.7%) [15]. REC was also significantly higher in newly diagnosed patients with WD compared to treated patients with WD (stable and non-stable) (29.1% [23.6–59.0] vs. 21.6% [11.8–40.0]), $p = 0.008$. This was also true when newly diagnosed patients with WD were compared to both patients with stable WD (21.4% [10.8–40.0], $p = 0.007$) and patients with non-stable WD (22.9% [14.6–40.0], $p = 0.04$). No difference in REC was found between patients with stable WD and non-stable WD, $p = 0.45$.

Further, median REC was 5.9% (4.8–6.5) in patients with non-Wilsonian ALF. This was statistically significantly higher than in patients with other non-Wilsonian hepatic diseases (4.5% [3.5–5.5], $p = 0.001$) but still lower than in the WD population, $p < 0.001$. Of note, one Spanish patient with non-Wilsonian ALF had a very high CuEXC and REC value, 4.91 μmol/L and 49.5%, respectively. This patient was admitted due to severe acute hepatitis and was subsequently diagnosed with AIH.

No significant differences in REC were observed between Spanish and Danish patients with WD across the three subgroups (i.e., newly diagnosed, stable and non-stable patients). REC correlated weakly and negatively with age at diagnosis, $p = 0.01$, Spearman's $r = -0.45$, but not with sex, genotype, phenotype, treatment type (i.e., chelating agent or zinc therapy), country, time since diagnosis, or cirrhosis.

CuEXC in newly diagnosed patients with WD was 2.59 μmol/L (1.87–3.42) (Figure 1B). This was statistically significantly higher than in treated patients with WD (0.63 [0.49–0.91], $p < 0.001$), regardless of clinical stability: 0.63 μmol/L (0.40–0.89) in patients with stable WD and 0.66 μmol/L (0.52–1.01) in patients with non-stable WD, both $p < 0.001$. Further, median CuEXC in newly diagnosed patients with WD was significantly higher

TABLE 1 | Demographic data on patients with Wilson disease.

	Wilson disease demographics									
	Danish			Spanish			Combined			All
	New	Stable	Non-stable	New	Stable	Non-stable	New	Stable	Non-stable	
n	8	35	1	5	31	24	13	66	25	104
Female	3 (38)	18 (51)	1 (100)	3 (60)	17 (55)	10 (42)	6 (46)	35 (53)	11 (44)	52 (50)
Age, years	23 (14–36)	38 (27–54)	45 (45–45)	29 (25–45)	40 (29–50)	39 (31–52)	26 (17–41)	40 (28–52)	39 (32–51)	38 (28–50)
Clinical presentation of WD										
Hepatic	5 (63)	21 (60)	—	3 (60)	22 (71)	16 (67)	8 (62)	43 (65)	16 (64)	67 (64)
Neurologic	2 (25)	8 (23)	1 (100)	1 (20)	6 (19)	7 (29)	3 (23)	15 (23)	8 (32)	26 (25)
Psychiatric	—	1 (3)	—	—	—	—	—	1 (2)	—	1 (1)
Asymptomatic	1 (13)	4 (11)	—	1 (20)	3 (10)	1 (4)	2 (15)	7 (11)	1 (4)	10 (10)
Age at diagnosis of WD, years	21 (10–36)	16 (11–22)	24 (24–24)	29 (25–45)	13 (8–30)[a]	17 (9–30)	26 (13–41)	16 (10–26)[a]	17 (9–30)	16 (10–29)[a]
Time since diagnosis, years	0 (0–0)	22 (12–28)	21 (21–21)	0 (0–0)	20.5 (16–31)[a]	24 (10–33)	0 (0–0)	21 (14–30)[a]	24 (10–33)	19 (9–29)[a]
Leipzig score	14 (8–6)	10 (8–12)	10 (10–10)	9 (7–11)	6 (5–8)[a]	8 (6–9)	8 (6–11)	8 (6–11)[a]	8 (6–9)	8 (6–10)[a]
Cirrhosis	2 (25)	2 (6)	—	2 (40)	6 (19)	6 (25)	4 (31)	8 (12)	6 (24)	18 (17)

Note: Data are presented as count (%) or median (interquartile range). If patients with Wilson disease presented with both hepatic and neurologic symptoms, they are categorised as neurologic.
Abbreviation: WD, Wilson disease.
[a]Data on one patient N/A.

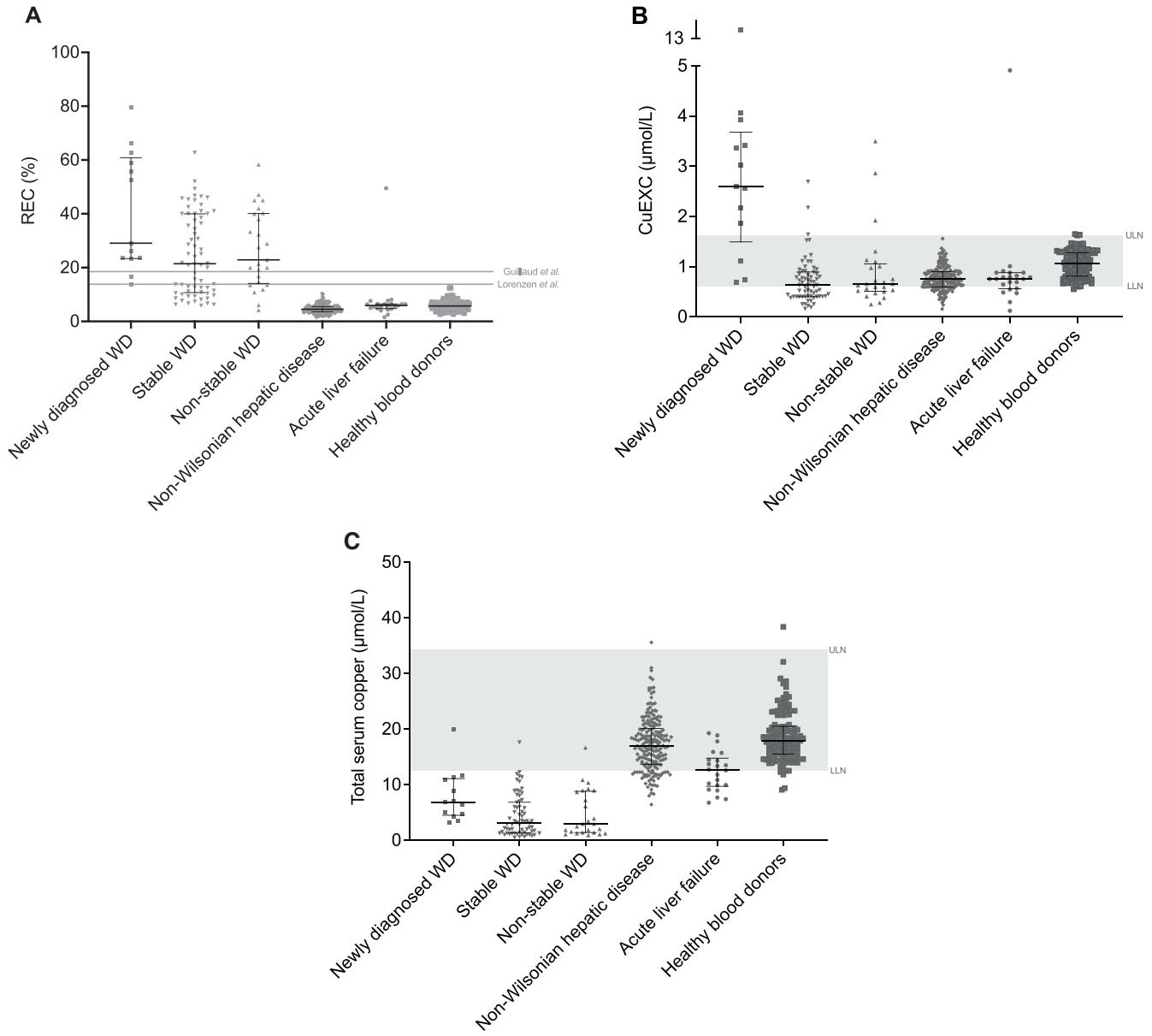

FIGURE 1 | Biomarkers of copper, distribution between groups. Median value with interquartile range is presented with black solid horizontal lines. (A) Relative exchangeable copper (REC) among groups. Grey lines represent suggested cut-off values for Wilson disease (WD) diagnosis (Guillaud et al., 18.5% [14]); (Lorenzen et al., 13.8%, from the current paper). (B) Exchangeable serum copper (CuEXC) among groups. The grey area represents the Danish adult reference interval (0.61–1.62 µmol/L), with the upper limit of normal (ULN) and lower limit of normal (LLN). (C) Total serum copper among groups. The grey area represents the Danish adult reference interval with ULN and LLN.

than in patients with non-Wilsonian hepatic disease and ALF combined (0.75 µmol/L [0.60–0.88], $p < 0.001$).

In treated patients with WD, CuEXC tended to be lower than the Danish reference interval (0.61–1.62 µmol/L) [15], $p < 0.001$. In stable patients with WD, CuEXC was slightly higher in the Danish group (0.70 µmol/L [0.41–1.00]) than in the Spanish group (0.50 µmol/L [0.31–0.79]), $p = 0.02$. No other differences were found in CuEXC when comparing Spanish and Danish patients with WD across the two other subgroups. No correlations were found between CuEXC and the aforementioned demographic variables.

Total Cu levels were 6.9 µmol/L (4.7–10.9) in newly diagnosed patients with WD, 3.2 µmol/L (1.4–6.8) in patients with stable WD and 3.0 µmol/L (1.4–8.7) in patients with non-stable WD (Figure 1C). In the WD population, Total Cu (3.5 µmol/L [1.5–7.6]) was significantly lower than in the group of patients with non-Wilsonian hepatic disease (17.0 µmol/L [13.7–20.1]), $p < 0.001$. Total Cu was significantly higher in the newly diagnosed compared to treated patients with stable and non-stable WD, $p = 0.003$ and $p = 0.01$, respectively. No significant differences in Total Cu were observed between Spanish and Danish patients with WD across the three subgroups. No correlations were found between Total Cu and the demographic variables.

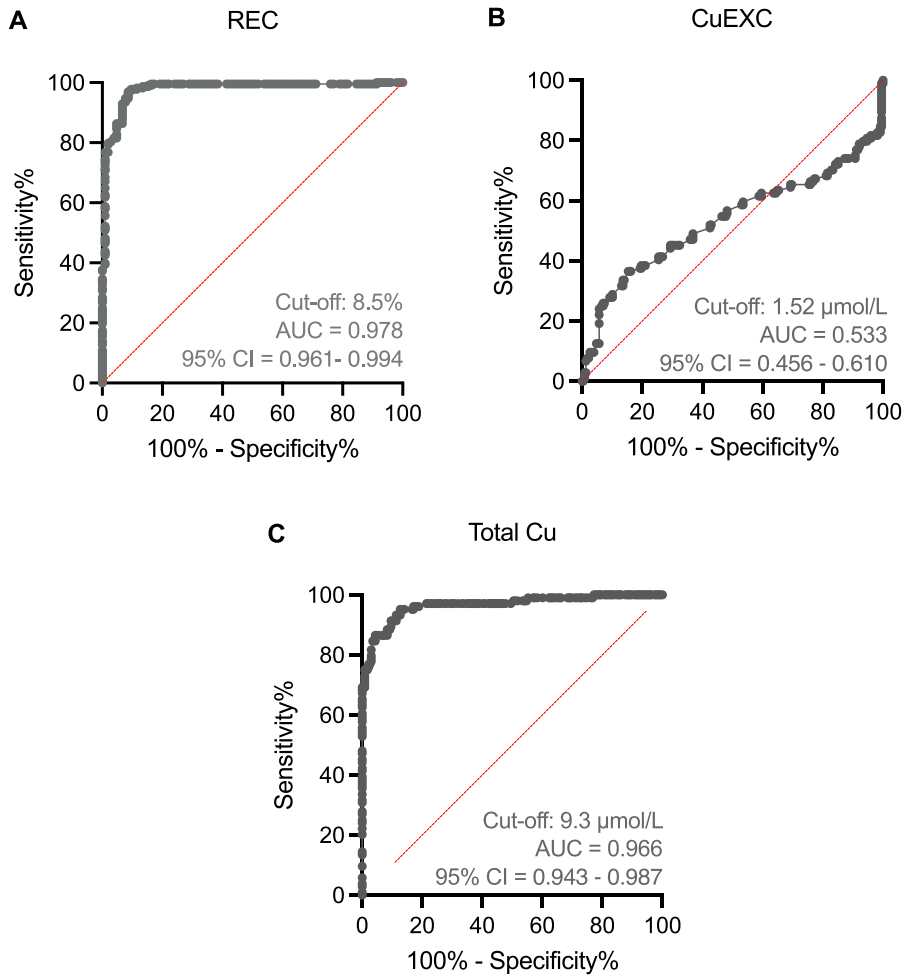

FIGURE 2 | Receiver-operating characteristic curves for all patients with Wilson disease versus patients with non-Wilsonian hepatic disease and acute liver failure. (A) Relative exchangeable copper (REC). (B) Exchangeable serum copper (CuEXC). (C) Total serum copper (Total Cu).

In patients with non-Wilsonian hepatic disease and concurrent inflammation ($n = 5$), CuEXC was slightly but statistically significantly lower during inflammation than after inflammation resolved (0.56 vs. 0.59 µmol/L, $p < 0.05$), whereas no significant change was observed in REC and Total Cu (see Supporting Information Appendix, Table S3).

3.3 | Receiver-Operating Characteristic Analyses

An ROC analysis for REC was performed for all patients with WD versus those with non-Wilsonian hepatic disease and those with ALF combined. The optimal diagnostic cut-off was ≥ 8.5%, with an area under the curve (AUC) of 0.978 (sensitivity 90.4% and specificity 97.8%) (Figure 2A). Of the treated patients with WD, 90% had a REC of ≥ 8.5%; i.e., the cut-off of ≥ 8.5% yielded nine false negatives. For the remaining groups, 2% were categorised as false positives. When analysing only newly diagnosed patients with WD versus those with non-Wilsonian hepatic disease and those with ALF, the optimal cut-off for REC was ≥ 13.8% (sensitivity 100% and specificity 99.6%) with an AUC of 0.998, i.e., all newly diagnosed patients with WD had a value of REC above 13.8% (Figure 3A). An ROC analysis conducted on the newly diagnosed patients with WD versus those with non-Wilsonian hepatic disease excluding ALF also yielded an optimal diagnostic cut-off of ≥ 13.8% (100% sensitivity and specificity) with an AUC of 1.000 (Table 2).

CuEXC, with an AUC of 0.533, was not discriminatory for WD diagnosis (Figure 2B). When considering only newly diagnosed patients with WD versus those with non-Wilsonian hepatic disease and those with ALF, the optimal cut-off for CuEXC was ≥ 1.87 µmol/L (sensitivity 76.9% and specificity 99.6%) with an AUC of 0.905 (Figure 3B). The same ROC analysis, excluding patients with ALF, also identified an optimal cut-off of ≥ 1.87 µmol/L (sensitivity 76.9% and specificity 100%) with an AUC of 0.908 (Table 2).

The ROC analysis for Total Cu demonstrated an optimal cut-off value of ≤ 9.3 µmol/L (sensitivity 86.5% and specificity 95.6%) with an AUC of 0.966 (Figure 2C). When analysing only newly diagnosed patients with WD versus those with non-Wilsonian hepatic disease and those with ALF, the optimal cut-off for Total Cu was ≤ 7.1 µmol/L (sensitivity 61.5% and specificity 99.1%) with an AUC of 0.911 (Figure 3C). Applying the ≤ 7.1 µmol/L cut-off would yield five false negatives in the group of newly diagnosed patients with WD. The same ROC

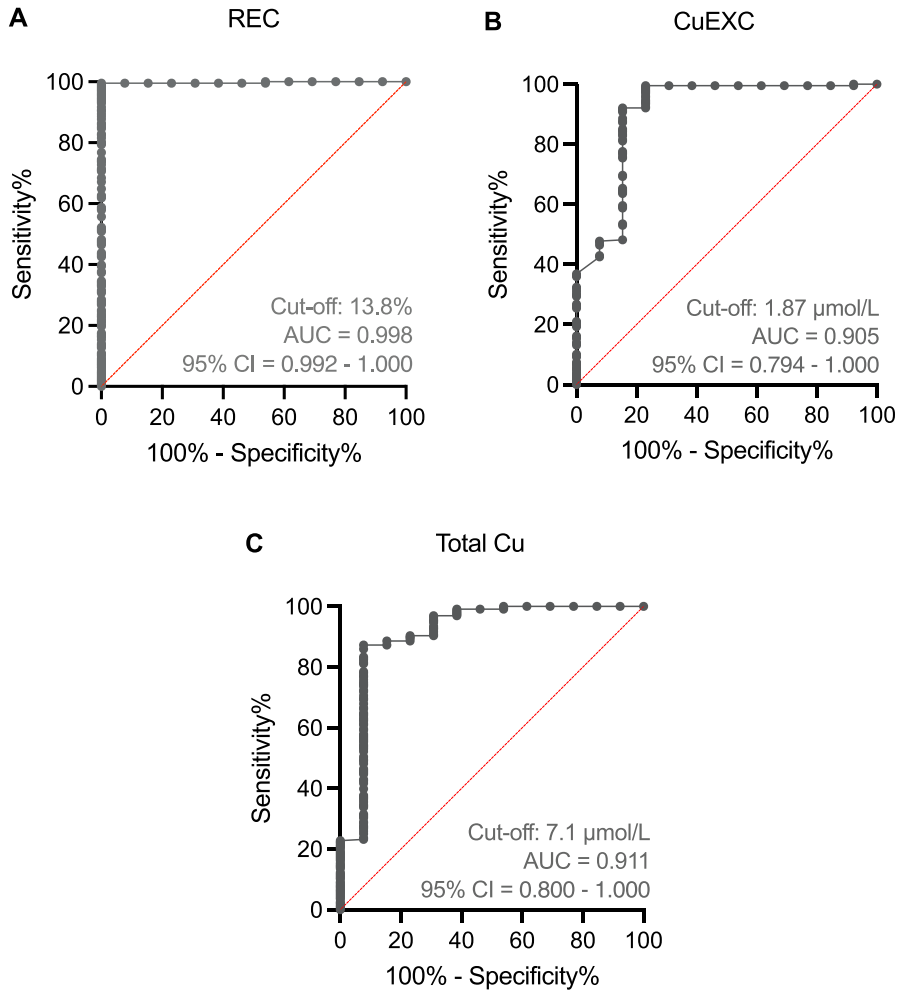

FIGURE 3 | Receiver-operating characteristic curves for newly diagnosed treatment-naïve patients with Wilson disease versus patients with non-Wilsonian hepatic disease and acute liver failure. (A) Relative exchangeable copper (REC). (B) Exchangeable serum copper (CuEXC). (C) Total serum copper (Total Cu).

analysis, excluding patients with ALF, also demonstrated an optimal cut-off of ≤ 7.1 µmol/L (sensitivity 61.5% and specificity 97.3%) with an AUC of 0.922 (Table 2).

3.4 | REC and CuEXC Over Time

In Figure 4, REC and CuEXC values are displayed over time for the eight Danish newly diagnosed patients with WD. Because of the significant difference in REC and CuEXC between newly diagnosed patients and patients with stable and non-stable WD, a repeated measures ANOVA was performed to compare the effect of time and/or treatment initiation. The analysis of REC showed no statistically significant change over time for Danish newly diagnosed patients with WD, $p = 0.32$ (Figure 4A). In contrast, CuEXC significantly decreased after treatment initiation, $p = 0.03$ (Figure 4B).

Further, we also performed a repeated measures ANOVA on REC and CuEXC, using the first and last available measurements from Danish treated patients with stable WD ($n = 35$); no statistically significant difference over time in REC or CuEXC was found, $p = 0.05$ and $p = 0.31$, respectively.

4 | Discussion

In this study, we examined the value of REC, CuEXC and Total Cu in the diagnosis of WD by comparison of measurements in patients with WD and patients with different non-Wilsonian hepatic diseases. Patients were recruited from Spain and Denmark to improve generalisability. The primary finding of this study was confirmation of earlier studies suggesting the potential of REC as a diagnostic tool for WD. A secondary finding was that Total Cu—with much broader availability than REC—had an unexpectedly good ability to identify patients with WD correctly. As in other studies, CuEXC seemed less suitable for diagnosing WD because elevated CuEXC is found in multiple hepatic diseases [13, 14]. These findings support the use of serum copper biomarkers during the diagnostic work-up in patients with suspected WD.

The diagnostic work-up for WD is initiated by either clinical suspicion or screening of family members. This study primarily relates to situations where otherwise unexplained hepatic disease raises suspicion of WD. Since WD can mimic almost any hepatic disease [17], the comparison groups included a wide range of other hepatic disorders.

TABLE 2 | Receiver-operating characteristic analyses.

Receiver-operating characteristic analyses						
	AUC	**Cut-off**	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**
All patients with WD (newly diagnosed and treated) versus those with non-Wilsonian hepatic disease and those with ALF						
Relative exchangeable copper (%)	0.978	≥8.5%	90.4	97.8	94.9	95.7
Exchangeable copper (μmol/L, μg/dL)	0.533	≥1.52 μmol/L ≥9.66 μg/dL	17.3	99.1	89.5	72.2
Total serum copper (μmol/L, μg/dL)	0.966	≤9.3 μmol/L ≤59.1 μg/dL	86.5	95.6	90.0	94.0
Newly diagnosed patients with WD versus those with non-Wilsonian hepatic disease and those with ALF						
Relative exchangeable copper (%)	0.998	≥13.8%	100	99.6	92.9	100
Exchangeable copper (μmol/L, μg/dL)	0.905	≥1.87 μmol/L ≥11.88 μg/dL	76.9	99.6	90.9	98.7
Total serum copper (μmol/L, μg/dL)	0.911	≤7.1 μmol/L ≤45.11 μg/dL	61.5	99.1	80.8	97.8
Newly diagnosed patients with WD versus those with non-Wilsonian hepatic disease						
Relative exchangeable copper (%)	1.000	≥13.8%	100	100	100	100
Exchangeable copper (μmol/L, μg/dL)	0.908	≥1.87 μmol/L ≥11.88 μg/dL	76.9	100	100	98.6
Total serum copper (μmol/L, μg/dL)	0.922	≤7.1 μmol/L ≤45.11 μg/dL	61.5	97.3	88.9	97.6

Note: Conversion factor from μmol/L to μg/dL = 0.1574.

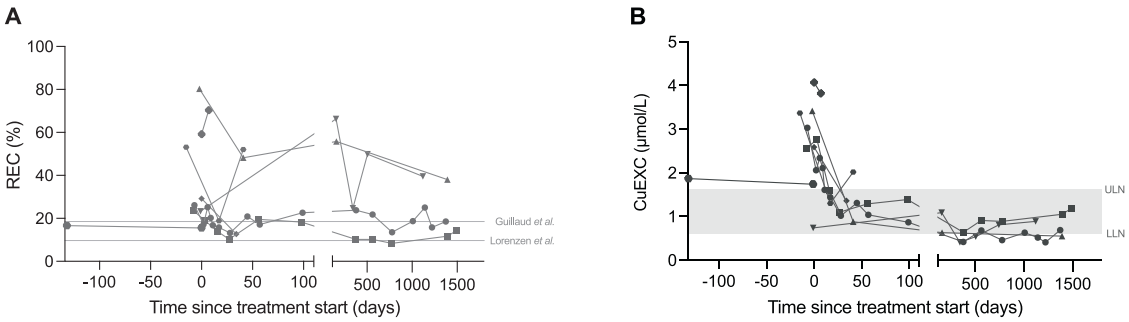

FIGURE 4 | Measurements of copper biomarkers since time of treatment initiation for Danish newly diagnosed patients with Wilson disease. (A) Relative exchangeable copper (REC) over time since time of treatment initiation. Lines represent suggested cut-off values. (B) Exchangeable serum copper (CuEXC) over time since time of treatment initiation. The grey area represents the Danish adult reference interval (0.61–1.62 μmol/L), with the upper limit of normal (ULN) and lower limit of normal (LLN).

When REC data from all 104 patients with WD were compared to all 228 patients with non-Wilsonian hepatic disease and those with ALF, the ROC analysis provided an optimal cut-off of ≥8.5%, whereas a cut-off of ≥13.8% was optimal when only newly diagnosed patients with WD were included (Table 2). This is in line with a smaller study [14] where the optimal cut-off was 14% when both untreated (n = 9) and treated (n = 40) patients with WD were combined and compared to 152 patients with other liver diseases, whereas a cut-off of 18.5% was optimal when only newly diagnosed patients with WD were included. In the clinical setting, the diagnostic work-up will usually be in treatment-naïve patients, and therefore, the cut-off for REC of ≥13.8% might be most appropriate.

This difference in optimal cut-off is probably because REC decreases slightly after treatment onset. In our cohort, REC was statistically significantly higher in newly diagnosed patients with WD (29.1%) compared to treated patients with WD (21.6%). This suggests a moderate reduction of REC during treatment, though we found no significant difference in our longitudinal

analysis of REC (Figure 4A), in line with previous results [16, 18, 19].

Our study did not examine the value of REC in family screening for WD. However, in one study including 16 newly diagnosed patients with WD, 45 heterozygotes, 25 wildtype and 62 unrelated controls [13], and another study with five newly diagnosed patients with WD, 87 heterozygotes and 34 wildtype individuals all identified by family screening [20], a REC of 18% provided total separation of patients with WD and the other groups. As seen in Figure 1, the use of this cut-off in our sample would have overlooked 2/13 (15%) of newly diagnosed patients with WD. Interestingly, these two cases presented as 'asymptomatic' and 'incidental findings' and are the only patients with asymptomatic presentations in the group of newly diagnosed patients with WD. Thus, this may suggest a potential trend of increasing REC levels over time among patients with WD whose disease has not yet manifested.

ALF due to WD may constitute a problem that deserves further studies. We have very limited data on REC in ALF due to WD. In a paediatric population, Sarma et al. reported a median REC of 21.0% in three patients with WD presenting with ALF. This was slightly lower than in 28 newly diagnosed patients with WD, who had other presentations than ALF, but still above the local cut-off [21]. The utility of REC in WD-induced ALF is still to be determined in a larger cohort of both children and adults.

Total Cu is usually not applied in the diagnosis of WD, although it tends to be decreased because of the typically reduced levels of ceruloplasmin [4, 22, 23]. In this study, the ROC analysis provided a surprisingly high AUC of 0.966 for Total Cu at a $\leq$9.3 µmol/L cut-off (sensitivity 86.5% and specificity 95.6%) (Figure 2C). Since the measurement of Total Cu is more widely available than the CuEXC method, it should be considered for diagnostic use but with caution in ALF, where Total Cu is lower than in other liver diseases [24]. In our WD population, Total Cu below the lower limit of normal (12.5 µmol/L) would identify 101/104 patients with WD (Figure 1C), i.e., sensitivity of 97%. Given the low Total Cu in some patients with non-Wilsonian hepatic disease, this could lead to approximately 25% false positives, requiring further investigation to rule out WD. Applying the $\leq$9.3 µmol/L cut-off would significantly reduce false positives but overlook 14% of patients with WD, in accordance with other reports [13, 14, 20].

From a clinical perspective, it is most relevant to apply the cut-off for newly diagnosed patients with WD compared to those with non-Wilsonian hepatic disease and those with ALF ($\leq$7.1 µmol/L) (Figure 3C). At this cut-off, the diagnostic performance of Total Cu is inferior to that observed at the $\leq$9.3 µmol/L cut-off, with AUC decreasing to 0.911, consequently increasing the percentage of false negatives. Thus, Total Cu can be valuable in initial diagnostics, with levels below the normal range indicating a very high likelihood of WD.

Patients with non-Wilsonian ALF are clinically very different from those with other non-Wilsonian hepatic diseases and are not encountered in daily clinical practice. Therefore, we also performed the ROC analyses comparing newly diagnosed patients with WD and those with non-Wilsonian hepatic disease but specifically excluding ALF. In summary, the optimal cut-off values for REC, CuEXC and Total Cu remained the same, with slight changes in sensitivity and specificity. REC remained the biomarker with the strongest diagnostic performance (Table 2).

A specific question was the effect of cirrhosis, which is known to affect copper biomarkers [25, 26]. In our large cohort, the presence of cirrhosis did not have a clinically relevant impact on REC, CuEXC or Total Cu. This conflicts with a small report on a cohort of 14 patients with alcohol-related cirrhosis, in whom a REC value of 13% and a non-significant trend towards higher Total Cu was observed [26]. However, the methodologies for CuEXC measurement are probably not directly comparable, but no details were given in the manuscript to refute or confirm this.

The subanalysis of the effect of inflammation demonstrated that CuEXC was statistically significantly lower during inflammation, though no difference was found regarding REC and Total Cu. This suggests the possible use of REC as a diagnostic tool even during inflammation, with reservations regarding the very small sample size. As for ALF due to WD, the influence of inflammation on copper markers in patients with WD is yet to be investigated.

An important limitation of this validation study is the small sample size. Particularly noteworthy is the small number of patients with measurements of REC prior to treatment initiation (newly diagnosed patients with WD). In the analysis of REC and CuEXC over time, with only eight Danish newly diagnosed treatment-naïve patients with WD, the small sample size considerably increases the risk of type 2 errors. However, the total number of included patients with WD is relatively large, given the rarity of WD. By including cohorts from both Spain and Denmark—from two specialised hepatologic departments—and nearly the entire Danish cohort of patients with WD (92%), we were able to expand our study population significantly. The larger sample size strengthens our confidence in the results.

All measurements of CuEXC and Total Cu were conducted using standardised procedures in the two laboratories [15, 16]. Although samples were not exchanged and directly validated between the two centres, both centres participate in the same international central laboratory validation programme to accommodate and minimise the risk of inter-assay variability. Regarding the determination of Cp, a turbidimetric method was used as per clinical practice, which is slightly more susceptible to measurement errors than a colorimetric method, and the results should be interpreted accordingly.

Despite the limitations, our study established significant differences in REC between patients with WD and controls, supporting its potential as a rapid tool in future WD diagnostics [13, 14, 19]. Before generalised implementation, it is imperative to establish an agreement regarding the CuEXC methodology, which is crucial for REC calculation.

REC may hold promise to be used in a broader perspective for both screening among children and across multiple medical specialties, for example, neurology and psychiatry, to hopefully reduce the diagnostic delay of WD. It remains to be confirmed whether a single REC measurement below the cut-off is

sufficient to exclude WD when screening in early life before the occurrence of pathogenic copper accumulation.

In conclusion, we found REC to be an accurate biomarker for diagnosing WD in concordance with previous studies [13, 14, 19, 20]. While our ROC analysis suggested a cut-off of ≥ 13.8% (sensitivity 100% and specificity 99.6%), other studies have proposed slightly higher cut-offs (14%–18.5%) [13, 14, 19, 20]. Given the efficiency, noninvasive nature, and superior discriminatory ability of REC, we propose that REC should be implemented in future clinical practice. Given its surprisingly strong diagnostic performance, Total Cu should be considered a useful tool for the initial diagnostic work-up, especially in places where the exchangeable copper analysis is not available. As with other markers before, Total Cu should not stand alone and will require further diagnostic work-up. Collectively, our findings support the inclusion of REC and/or Total Cu in a potential future revision of the Leipzig score.

Author Contributions

Authoring the manuscript: C.L. and K.D. Analysis and interpretation of results: C.L., K.D., F.T.K., L.D.C. and M.T. Substantial contributions to the development of the concept and design: F.T.K., E.M.L., T.D.S., P.O., M.T. and Z.M. Data collection: C.L., K.D., F.T.K., K.S.R., P.N.B., C.G.S., X.F., A.S., S.R.T., A.M., M.T. and Z.M. All authors reviewed and approved the final manuscript before publication.

Ethics Statement

In Aarhus, Denmark, the study was approved by the Central Jutland Regional Committee on Health Research Ethics (1-10-72-342-20). In Barcelona, Spain, the Hospital Clinic Ethics Committee approved the study [HCB/2010/6144-R101116-039 and 2023-405-1].

Consent

Patients were included after providing written informed consent.

Conflicts of Interest

Z.M.: Speaker fees from Orphalan, Gilead; consultancy fees from Orphalan, Alexion, DeepGenomics and Prime Medicine (grants from Gilead). The other authors have no conflicts of interest to declare.

Data Availability Statement

Data are available from the authors upon request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.