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ORIGINAL ARTICLE

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Benefits of using exchangeable copper and the ratio of exchangeable copper in a real-world cohort of patients with Wilson disease

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Abstract

Wilson disease (WD) is a complex disease in which diagnosis and long-term metabolic copper control remains challenging. The absence of accurate biomarkers requires the combination of different parameters to ensure copper homeostasis. Exchangeable copper and its ratio (REC) have been suggested to be useful biomarkers in this setting. We aimed at introducing these measurements and evaluate their performance and accuracy in our real-world cohort of WD patients. Exchangeable copper and REC were measured in 48 WD patients and 56 control individuals by inductively coupled plasma-mass-spectrometry. Demographic and clinical characteristics were collected. REC was shown to be significantly higher among WD patients compared to controls and useful for WD identification by using the previously established cutoffs: 71.4% of WD patients with a recent diagnosis had REC ≥18.5% and 95.1% of long-term treated WD had REC ≥14%; only four patients of the cohort presented discordant levels. Moreover, REC values were below 15% in all the control individuals. Exchangeable copper was significantly higher in WD patients compared to

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controls and tended to be reduced among WD patients who were compliant to medication. This real-life study confirmed that exchangeable copper and REC are useful serum biomarkers that can be used as complementary tests to ensure WD diagnosis (REC) and copper homeostasis whithin time (exchangeable copper). The desirable target levels for this last objective still needs to be validated in prospective cohorts.

KEYWORDS

exchangeable copper, labile copper, non-ceruloplasmin bound copper, REC, serum biomarkers, Wilson disease

1 | INTRODUCTION

Wilson disease (WD) is a rare monogenic and recessive disease of copper metabolism. It is based on mutations in the *ATP7B* gen (gene ID: 540), which codifies a dysfunctional copper-transporter protein called ATP7B. Because of this abnormal protein function, the excess of copper will not be physiologically excreted into the bile. Copper overload is first stored in the liver, but after exceeding its storage capacity, it flows out and may affect other tissues (especially central nervous system, or cornea). The second consequence of a dysfunctional ATP7B is the impairment for copper transference from apo to holoceruloplasmin (the main circulating copper-transporter in humans) which results in an unstable protein, and a reduced copper availability for cell metabolic processes.[1]

WD diagnosis is often complex in clinical practice. Although urinary copper and ceruloplasmin are well-established biomarkers of the disease, they are not specific and have frequent positive/negative false results. Except for the pathogenic mutations in *ATP7B*, usually not performed at the first patient assessment, physicians must rely on the combination of different clinical/biochemical and/or radiological signs. In 2003, the Wilson's disease Working Group proposed the use of Leipzig criteria[2] which were included in the European Guidelines for WD.[3] American Guidelines suggest the use of some of these parameters within a practical clinical approach.[4–6] However, diagnosis may not be reached unless a high suspicion of WD is raised, and these items become available. Otherwise, misdiagnosis or underdiagnosis may occur, especially among hepatic phenotypes, where patients may be diagnosed with more prevalent liver diseases instead (such as metabolic or alcohol-related liver disease). The diagnostic approach is especially difficult in those patients presenting with mild hepatic symptoms (mild/moderate ALT elevations, for example) with no clear WD signs (such as concomitant neuropsychiatric or corneal involvement, for instance). Diagnostic difficulties become more evident in pediatric patients, as most of them are clinically paucisymptomatic, and the conventional criteria used in adults may not be appropriate.[7] Liver biopsies (with copper quantification) or genetic testing are expensive and/or invasive, and physicians may be reluctant to use them as a first-line diagnostic approach. In practice, WD diagnosis is commonly delayed,[8] impacting on long-term survival of patients or more advanced involvement.[9]

Furthermore, once the diagnosis of WD is established, long-term follow-up of the disease is not straightforward. Although copper metabolic control needs to be assured, no current isolated biomarker has been shown to be accurate enough for this purpose and this is mainly based on the combination of copper excretion evaluation and liver tests.[3,4,6] "Free copper" is thought to represent the toxic fraction of the systemic copper and can be calculated with a mathematical formula (based on ceruloplasmin and total copper) and should be below 50 μg/dL in well-controlled WD individuals. However, this marker may lead to negative results in up to 20% of cases[10] and therefore, results are often not reliable. Exchangeable copper (ExchCo) was proposed as a useful biomarker of copper metabolism, representing the direct measurement of non-ceruloplasmin bound copper (NCC) with potential for cell toxicity, overcoming the difficulties and inaccuracy of the free copper calculation depending on ceruloplasmin (Cp).[11] During the last 10 years, ExchCo and the ratio of exchangeable copper referred to total copper (REC) have been shown to be useful in different clinical scenarios of WD.[11–17] Indeed, REC seems to be a reliable biomarker for WD diagnosis at the cutoff of ≥18.5% for new cases,[13] whereas all treated WD patients have been shown to have REC > 14% during follow-up.[12] Moreover, REC values <15% were proposed to exclude WD at diagnosis.[17] Whether these cutoffs are the same for symptomatic or presymptomatic WD patients remains unknown. Nevertheless, these biomarkers have not been validated yet, nor have been included in the current international

guidelines for WD and this might be partially related with the limited number of publications to date and the lack of real-world experiences with Wilsonian cohorts around the world. Therefore, we decided to introduce these measurements in our real-life practice, and to assess the usefulness of ExchCo/REC in our well-characterized, real-world, multicentric cohort of patients with WD in Barcelona, with special attention to their potential use in the follow-up phase as markers of copper metabolic control.

2 | PATIENTS AND METHODS

This is a multicentric transversal non-interventional study performed among three Hepatology units in hospitals in Barcelona following patients with WD (Hospital Sant Joan de Deu, Hospital Vall d'Hebró and Hospital Clínic). We included patients with a confirmed WD diagnosis (defined by Leipzig ≥4)[2] who had serum samples collected. We analyzed single serum samples for each patient, that had been previously extracted and stored at −80°C during the clinical course of the disease. Seven patients of the cohort had sequential serum samples that were used for descriptive kinetics. All patients (or legal tutors-parents if pediatric individuals) had signed a global informed consent for investigational purposes and data analysis [HCB/2010/6144-R101116-039].

WD patients were further divided into two groups for analytical purposes: "recent diagnosis group" ($n = 7$, including naïve WD patients and those receiving therapy for <3 months together) and "treated WD group" ($n = 41$, including those patients treated for >3 months). A control group of healthy pseudo anonymized non-WD individuals ($n = 56$) was also included. These individuals were previously healthy subjects with trivial gastrointestinal conditions and/or submitted for minor surgical interventions together with voluntary adult participants with no analytical nor clinical suspicion of liver disease.

Total plasma copper (μg/dL), ExchCo (μg/dL), and REC (%) were analyzed on a Agilent 7500 CE (Agilent technologies, Germany) ICP Mass Spectrometer. For optimal performance, we have used the collision method and in addition, Germaniun (Merck) was used as internal standard to correct for changes in instrument operating conditions and sample-specific matrix effects which may enhance or suppress the analyte signal. The clinical diluent used for pretreatment was a mixture of 2% propanol, 0.7 mmol/L EDTA (Merck), 0.07% Triton X-100, 0.5% ammonium, 10 μg/L of Germanium and purified water. All reagents were purchased from Merck. The accuracy and precision of the method were below 8%. The limit of quantification obtained was 11.5 mg/L, and the limit of detection was 3.3 mg/L. For ExchCo separation, serum was diluted with EDTA 3 g/L (1:1) and incubated for 1 h before ultrafiltration on Amicon® Ultra-4® (Merck/Sigma Aldrich UCF803096), following a validated procedure described elsewhere.[11,18]

Normal levels of ExchCo were established between 3.9 and 7.3 μg/dL and were in agreement of those reported by El-Balkhi's paper in non-wilsonian individuals.[13] Cp was measured by nephelometry by standard automated procedures and expressed in g/L; limit of detection was 0.02 g/L. Free copper was calculated considering the usual mathematical formula (free copper = total copper [μg/L]–[3.15 × Cp (mg/L)])[3] and also expressed in μg/dL.

Demographic, clinical, and laboratory data were collected at the time of the WD diagnosis and at the time of serum extraction. Compliance to medication (dichotomized as poor or adequate) was extracted retrospectively from medical records and based on the patients' self-report plus the physician's observations based on urinary copper/zinc and aminotransferases values, based on current international guidelines.[3,6] Basically, patients were considered "non-compliant" if urinary copper was persistently >100 μg/24 h and urinary zinc constantly lower than 2 g/24 h in zinc-treated patients, or urinary copper was persistently >500 μg/24 h or highly fluctuant (some of them being >900 μg/24 h) among those treated with stable doses of chelators. ALT levels were classified as "normal" when below the upper limit of normal (considered as ≤40 IU/L).

Quantitative variables were expressed as median and interquartile ranges ($IQR_{25–75}$), whereas categorical variables were reported in number (n) and percentages (%). Comparisons between groups were done by Chi-Square or U-Mann Whitney/Kruskal–Wallis as appropriate, and correlations were assessed by Spearman test. ExchCo/REC accuracy (sensitivity, specificity, positive, and negative predictive values) was evaluated by considering the predefined cutoffs for REC: ≥18.5% in new WD diagnosis and > 14% when patients were already under anti-copper-therapies, whereas REC < 15% was considered for exclusion. Statistical significance was set whenever a p-value was <0.05. All the analysis was performed with SPSS Statistics v25.

3 | RESULTS

3.1 | Baseline characteristics

Fifty-four WD patients were initially included in this exploratory study, although six cases were excluded from the analysis due to unclear WD diagnosis (see Table S1 for details). Therefore, 48 WD patients were finally

analyzed (39 adults, 9 children). Half of them were female and the mean age at WD diagnosis was 13.5 (9.2–26.5) years old. Up to 72.9% of the patients had presented with a liver phenotype[2] (acute presentation, n = 8 [16.7%]); chronic presentation, n = 27 [56.3%]). Genetic analysis could be performed in all patients: 75% of the cohort was compound heterozygous, highly enriched with the p.*Met645Arg* (37.5%) and p.*His1069Gln* (22.9%) mutations. Only 2 cases had a single identified pathogenic mutation (p.*Met645Arg/WT* and p.*His1069Gln/WT*, respectively). Most of the samples had been obtained during the follow-up phase of the disease and constituted the "treated WD group" ([$n = 41$, 85.4%; median time from diagnosis: 13 years [5.7–23]). Seven remaining samples (14.5%) were extracted at diagnosis or soon (<12 weeks) after specific anti-copper medication had started ("recent diagnosis group"). The global mean age at sample extraction was 28.5 years (21–42). The pediatric population (<18 years old) was composed of 9 patients (21.4%) with a mean age of 15 (11–16.5) years old. Up to 54.2% of the whole cohort ($n = 26$) was treated with zinc salts in monotherapy (see Table 1 for further clinical details).

A control group of 56 non-Wilsonian individuals was used as a comparison for measurement of these biomarkers; the mean age of this cohort was significantly lower than the WD cases (16.5 [9–40.2] years old, $p = 0.022$), as 53.6% of them were children.

3.2 | Usefulness of REC and exchangeable copper for the identification of WD patients

Total serum copper (μg/dL) was significantly lower in the WD cohort compared to control individuals (20.9 μg/dL [5.5–54.3] vs. 105.2 μg/dL [87.8–123.5], respectively, $p < 0.05$). Regarding ExchCo and REC, they were significantly higher in the WD cohort compared to controls: ExchCo 6.7 μg/dL (3.3–10.1) versus 4.1 μg/dL (2.9–5.5), $p < 0.001$, and REC values of 31% (19.5–43.2) versus 3.8% (2.6–5.2), $p < 0.001$, respectively. When considering WD patients in two different groups (according to recent diagnosis vs. follow-up) and compared to controls, these differences remained clear ($p < 0.001$) (Figure 1A,B). This difference might have been greater if all patients included in the "recent diagnosis group" had all been real naïve participants. These differences were also maintained when considering pediatric or adult patients with WD, compared to controls (data not shown).

By using the previously suggested REC cutoff value for WD diagnosis (≥18.5% in recently diagnosed patients),[12,13] 5 out of the 7 patients (71.4%) of this Wilsonian cohort could have been correctly identified. If we

TABLE 1 Baseline characteristics of the Wilson disease cohort ($n = 48$).

Variables	Values (n, %); (mean, IQR $_{25–75}$)
Main characteristics at Wilson disease presentation	
Age at diagnosis (years)	13.5 (9.2–26.5)
Sex (female)	24 (50%)
Genotype	
• Homozygous	10 (20.8%)
• Compound heterozygous	36 (75%)
• Simple heterozygous	2 (4.2%)
Most prevalent mutations in *ATP7B* gene:	
• *p.Met645Arg*	18 (37.5%)
• *p.His1069Gln*	11 (22.9%)
• *IVS4-1G > A*	9 (8.7%)
• *Exon 1 deletion*	4 (3.8%)
Phenotype at presentation	
• Acute hepatic presentation (H1)	8 (16.7%)
• Chronic hepatic presentation (H2)	27 (56.3%)
• Neurological + liver involvement (N1)	8 (16.7%)
• Asymptomatic/ presymptomatic	5 (10.4%)
Main characteristics at the moment of sample extraction	
Type of patient	
• Adult (≥18 years old)	39 (81.2%)
• Pediatric (<18 years old)	9 (18.7%)
Phase of the disease	
• Diagnosis (new or < 12 weeks since diagnosis)	7 (14.6%)
• Follow-up (≥12 weeks from diagnosis)	41 (85.4%)
Time from WD diagnosis (years)	11.5 (4.2–20)
Age (years)	28.5 (21–42)
Platelet count (x10^9/L)	187 (128–249)
Ceruloplasmin (g/L)	0.05 (0.03–0.11)
• <0.14 g/L	• 37 (77.1%)
• 0.14–0.19 g/L	• 8 (16.7%)
• ≥0.20 g/L	• 3 (6.3%)
Total serum copper (μg/dL)	20.9 (9.4–52.4)
Calculated free copper (μg/dL)	19.7 (7.9–49)
Urinary copper (μg/24 h)	
• All patients ($n = 48$)	122 (59–456)
• Under chelators ($n = 15$)	474 (323–676)

(Continues)

TABLE 1 (Continued)

Variables	Values (n, %); (mean, IQR $_{25–75}$)
• Under zinc salts ($n = 26$)	65.5 (41–119)
ASAT/ALAT (IU/L)	34.5 (22–50.7)/50 (31–82)
Normal liver tests (defined as ALAT ≤ 40 IU/L)	17 (35.4%)
Therapy at sample extraction	
• D-Penicillamine	11 (22.9%)
• Trientine	3 (6.3%)
• Zinc salts	26 (54.2%)
• Combined therapy (zinc + chelator)	3 (6.3%)
• Biscoline tetratiomolibdate	1 (2.1%)
• None	4 (8.3%)[a]
Reported good compliance to medication (%)[b]	27 (56.3%)
Exchangeable copper (all)	6.7 (3.3–10.1)
• Recent diagnosis ($n = 7$)	7.5 (6.9–18.6)
• Follow-up ($n = 41$)	5.7 (3.1–10)
REC (%) (all)	31 (19.4–43.2)
• Recent diagnosis ($n = 7$)	30.8 (15.8–41.3)
• Follow-up ($n = 41$)	33.2 (19.6–46)

Note: Quantitative variables were expressed as median and interquartile ranges ($IQR_{25–75}$); categorical variables were reported in number (n) and percentages (%).

[a]Referred to three patients at diagnosis with no medical therapy at sample extraction and one patient with an early liver transplantation (the sample was extracted before LT took place).

[b]Adequate compliance to medication was defined retrospectively from medical records and based on the patients´ self-report plus the physician's observations in urinary copper/zinc and aminotransferases values, according to international guidelines.

lower the cutoff to ≥14%, considering those WD patients on long-term follow-up (in whom copper metabolism would have been expected to be controlled), 39 out of 41 patients (95.1%) would had been identified. Overall, 44 WD patients in our cohort (91.6%) were detected by the specified diagnostic REC cutoffs. Only four WD patients were classified as "discordant" in terms of their REC levels: two patients at diagnosis (one adult and one child), and two adult patients during follow-up. Three of them showed REC levels below 15%. See Table 2 for individual details.

Conversely, 100% of the control individuals showed REC values below 15%.[12] That means that a REC value lower than 15% had a negative predictive value of 91.8% (and specificity of 100%). Positive predictive value for REC when the used cutoff was ≥18.5% was 100% in our hands (although sensitivity failed to 79.2%); when we lowered the cutoff to >14%, positive predictive value remained 100% and sensitivity was increased to 93.8%; this was explained by the highly enriched number of long-term treated WD patients.

3.3 | Assessment of ExchCo and REC among WD patients

No significant differences were observed on ExchCo levels among WD patients according to different explored variables: gender, clinical phenotype at diagnosis, age at sample extraction, or type of therapy (chelator vs. non-chelator). When the analysis was restricted to those WD treated patients for >3 months ($n = 41$), no differences were neither observed in ExchCo depending on the use of zinc or chelators. Although we could observe a tendency to present reduced levels of ExchCo among patients in the maintenance phase compared to patients at diagnosis (5.7 μg/dL vs. 7.5 μg/dL, respectively), this difference did not reach statistical significance ($p = 0.13$). Levels of ExchCo were neither significantly different among those patients classified as "poor compliant" to anti-copper medication, compared to patients with "adequate compliance" (6.7 μg/dL [4.0–11.4] vs. 5.1 μg/dL [3.0–7.6], $p = 0.2$), although a tendency to lower levels of copper were observed again in this last group. The same was observed in patients with abnormal ALT levels, in which ExchCo levels were higher than in those patients with normal liver tests (6.9 μg/dL vs. 5.7 μg/dL, $p = 0.5$), although no statistical significance was reached.

Five patients had been classified as presymptomatic at WD diagnosis (Table 1); exchangeable copper tended to be lower among these patients when compared to symptomatic patients (including hepatic, neurological, or mixed phenotypes, $n = 43$) although this difference did not reach statistical significance ($p = 0.09$), and it must be reminded that most of the samples had been taken during follow-up. Interestingly, REC values among presymptomatic patients were also significantly lower than in symptomatic WD patients (14.1% vs. 33.8%, $p < 0.01$), possibly identifying patients with the lowest copper overload and consequently, less clinical manifestations. The only presymptomatic case in the group of "recent diagnosis" was an atypical late-onset WD with low levels of ExchCo and low REC (included as discordant in Table 2).

Regarding REC, we were not able to find any other significant difference among groups of WD. Of note, REC was high but not different between patients at recent diagnosis versus patients at the maintenance phase (31% [13.7–43.8] vs. 31.4% [19.8–45], respectively) although the number of patients at diagnosis was very limited. The proportion of patients with REC values ≥18.5%, in the intermediate range of 18.4%–14%, or < 14% among

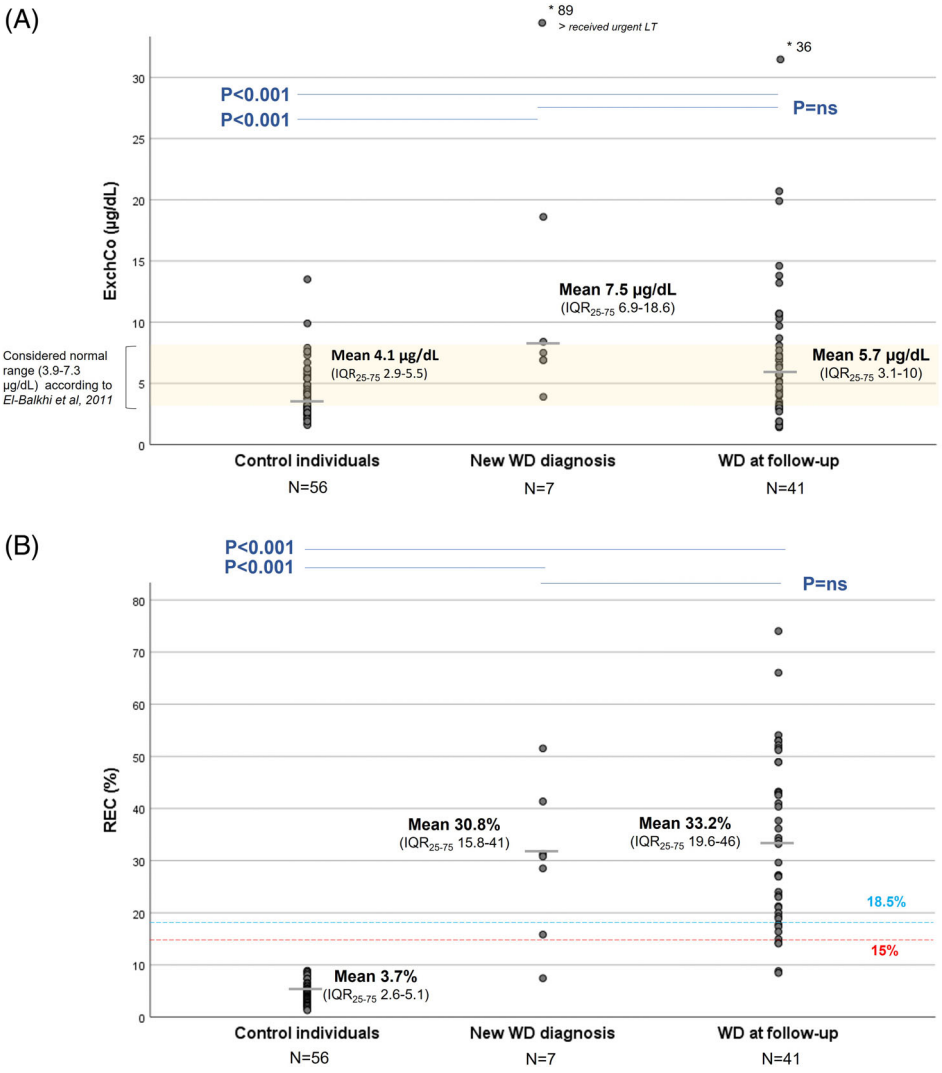

FIGURE 1 (A) Exchangeable copper levels (µg/dL) are represented in this figure for the three different groups of patients: control individuals, Wilson disease (WD) patients of new-recent diagnosis, and WD patients at long-term term follow-up. Normal levels were considered in the range of 3.9–7.3 µg/dL. Median ExchCo levels were significantly higher among the two groups of WD patients compared to controls (p<0.001). Differences between recent WD patients and long-term WD were not observed, probably due to limited samples. (B) Ratio of exchangeable copper (REC) values (%) are represented in this figure for the three different groups of patients. The previously proposed cutoffs for new WD diagnosis (≥18.5%) and previously treated WD (15%) are depicted by discontinuous lines in the graph. Most of the WD patients presented with REC values higher than 15%. REC values were significantly higher (p<0.001) among WD patients compared to control individuals. No differences could be observed between recent WD patients and long-term WD, probably due to the small sample size.

patients with recent diagnosis ($n = 7$) versus patients in long-term follow-up ($n = 41$) were: 71.4%, 14.3%, and 14.3% versus 80.5%, 9.8%, and 9.8%, respectively (p = ns). Seven cases (all from the treated-WD group) had one or two additional REC evaluations during follow-up (see Figure S1), showing no significant changes within time. The reason why REC persisted high despite long-term treatment is beyond the scope of this study, but suggests limited value of REC for copper monitoring purposes.

There was a good concordance between Cp levels and REC among our WD patients. Up to 38 patients of the cohort (79.2%) had Cp levels below 0.20 g/L and REC values ≥18.5% ($p < 0.001$). In our cohort, three WD cases had Cp levels above 0.20 g/L. Among them, REC was <14% in a 36-year-old female treated with zinc salts for more than a decade and between 14% and 18.5% in two additional patients treated with trientine and copper-free diet, respectively (see Table S1 for details). Only one case with intermediate levels of Cp (0.14–0.19 g/L) presented REC values below the 14% threshold.

Importantly, REC was shown to be significantly higher among patients with abnormal ALT when compared to patients with normal ALT levels at sample extraction [REC 40% (27–51) vs. 23% (14–28), respectively, $p = 0.008$].

3.4 | ExchCo and REC among the excluded patients

Importantly, REC and ExchCo were shown to be normal among six patients we had initially excluded from the comparative analysis and in whom we had a high suspicion of misdiagnosis: median REC 8.5% (range 5.8–11.3%) and median ExchCo of 7.2 µg/dL (range 4.3–7.8 µg/dL). These patients had been diagnosed a long time ago based on old reports (all with Leipzig scores ≥3), and were on long-term anti-copper therapies. In these cases, anti-copper treatment was withdrawal, with a close prospective follow-up showing no impact on the

TABLE 2 Main characteristics of the four WD patients with discordant REC values.

WD subgroup	Patient: number, sex, age (years), genotype	REC (%) ExchCo (μg/dL)	Cp (g/L), Serum copper (μg/dL), Free copper[a] (Fco) (μg/dL), Urinary copper (UCo) (μg/24 h)	Main clinical characteristics	Additional comments
Recent WD diagnosis ($n = 2$)	No 1: Male, 80 (p.*Met665Val/E16-21 deletion*)	**REC: 7.4%**[a] ExchCo: 3.9 μg/dL	Cp 0.11 g/L; SerumCo: 47 μg/dL FCo: 17.7 μg/dL; UCo: 70 μg/24 h	Mild hepatic presentation (normal ALT). Incidental diagnosis at late stages, not receiving any therapy (other than copper-free diet).	*p.Met665Val* detected in *trans* and considered a possibly pathogenic VUS. *E16-21* deletion has been described as pathogenic according to database (loss of function).
	No 2: Female, 15 (*p.Gln111Ter/ p.Arg1319Ter*)	REC: 15.8% ExchCo: 7.5 μg/dL	Cp 0.03 g/L; SerumCo: 47 μg/dL; Fco: 37.9 μg/dL; UCo: 456 μg/24 h	Non-severe acute hepatic presentation.	Starts first line therapy with DPA.
WD at follow-up ($n = 2$)	No. 3: Male, 53 (*IVS4-1G > A/IVS4-1G > A*)	**REC: 8.8%**[a] ExchCo: 6.3 μg/dL	Cp: 0.16 g/L; SerumCo: 76 μg/dL; FCo: 21.1 μg/dL UCo: 547 μg/24 h	Positive family history, long-term therapy with DPA, excellent compliance, normal ALT.	Cirrhosis
	No. 4: Female, 36 (*p.His1069Gln/ IVS4-1G > 4*)	**REC: 8.5%**[a] ExchCo: 7.2 μg/dL	Cp: 0.22 g/L; SerumCo: 86 μg/dL FCo 15.9 μg/dL; UCo: 130 μg/24 h	Long-term therapy with Zinc, not compliant. ALT 145 IU/L, comorbid NAFLD.	

Note: Discordant values were considered among WD patients if REC values were < 18.5% in naïve/recent patients (<3 months) or REC values were below 14% in WD patients already under treatment, according to Guillaud O et al., *Liver Internat* 2018. Bold letter identifies the three patients with WD and discordant REC values, with REC < 15% (suggested REC cutoff for the exclusion of WD).

Abbreviations: ALT, alanine aminotransferase; Cp, ceruloplasmin; DPA, D-penicillamine; ExchCo, exchangeable copper; FCo, free copper; NAFLD, non-alcoholic fatty liver disease; No, number; REC, ratio of exchangeable copper; SerumCo, serum total copper; UCo, 24-h urinary copper; WT, wild-type.

[a]Free copper (FCo) was calculated according to the usual formula recommended in the European Guidelines[3]: *FCo = total copper* (μg/L) – (*3.15* x *Cp* [mg/L]).

hepatic tests/clinical course after 12 months of follow-up. See details in Table S2.

4 | DISCUSSION

WD has a very high clinical heterogenicity and variable penetrance. The diagnostic process is complex in the absence of high clinical suspicion and may lead to underdiagnosis. The diagnostic approach often requires invasive tests or expensive and long-lasting studies (such as *ATP7B* mutational analysis), usually not performed as first-line studies, especially among adults in which other liver diseases could justify a mild hepatic presentation. In real clinical practice, a high suspicion remains the clue to ensure a deep evaluation of copper metabolism, and therefore, the use and expansion of reliable, easy, and affordable diagnostic and follow-up biomarkers for WD still remains a need and a challenge.

Exchangeable copper (ExchCo)[11] was proposed as a direct measurement of NCC that could be easily measured in serum. REC is the fraction of this ExchCo (the "toxic fraction") referred to the total measurable systemic copper, usually reduced among WD patients. In the last years, the French scientific community involved in WD management has reported solid evidence for the use of these biomarkers in clinical practice within different scenarios of WD, with special value as a diagnostic tool.[11,13] Essentially, REC values equal or higher to 18.5% were proposed for naïve patients at diagnosis,[13] whereas those WD who were already on treatment were shown to have REC values above 14%.[12] These biomarkers were also shown to be useful for differentiating real WD from carriers of a single mutation and healthy controls in a family screening program, as all of them presented with REC values lower than 15%.[16] Moreover, ExchCo and REC were shown to be significantly higher in WD patients presenting with neuropsychiatric/extrahepatic phenotypes[15] and could also be useful in the differential diagnostic of WD with other chronic liver diseases.[12] In pediatrics, equivalent ranges of normality have also been described.[14] Finally, ExchCo has been recently shown to be higher in patients

with suboptimal compliance to medication during long-term follow-up, compared to compliant patients suggesting its additional usefulness in clinical practice for ensuring a good copper control.[17] However, even though their use has been extended to different countries and has been included in clinical practice, no external validation has been published yet from other groups out of France, and publication regarding real-life clinical experiences is scarce.[18,19] Moreover, no standard ranges of "normal exchangeable copper levels" have been settled for WD patients at the maintenance phase, despite the fact of being used for copper metabolic follow-up showing similar dynamics to other copper parameters.[18,19]

Attracted by their potential use in our patients, and the need for enlarging publicly data on their use among WD patients, especially as potential biomarkers during follow-up, in which metabolic homeostasis is assumed to be improved with specific therapy compared to diagnosis, we decided to perform this exploratory approach to assess the value of ExchCo and REC in a cohort of real-life patients with confirmed WD in Barcelona (all with Leipzig ≥4 points). Despite one could argue that this sample is small, 48 patients with such a rare disease as WD should be considered as significant and reflects a big effort of cooperation among centers.

Our study was understood as a pure transversal exploratory assessment, and data was therefore obtained at different time points of the natural history of the disease for each patient, although it was highly enriched with WD patients at follow-up (85.4%). Unfortunately, dynamic data on kinetics of these biomarkers at follow-up were only available in a reduced subgroup of seven patients, therefore hampering us to draw firm assumptions of their utility in this context.

In terms of WD identification, REC was shown to be useful in our hands, clearly identifying WD when compared to a control group of non-Wilsonian individuals. Indeed, most of our WD patients could be nicely identified by using the previously defined cutoffs (≥14% for previously treated and ≥ 18.5% for new diagnosis), with only four patients being considered as "discordant", two at diagnosis and two at follow-up. Therefore, the diagnostic accuracy of these biomarkers was globally good, as 91.6% were identified as WD according to the previously established cutoffs. Although this proportion was slightly lower than in the previously described series.[12,13] REC was shown to be a quick, helpful, non-invasive, and easy to use, and we therefore advocate for the global introduction of this complementary measurement at those medical centers taking care of patients with WD. Only two cases were not identified among the group of new WD cases, as they showed REC levels below 18.5%. One case was an adolescence with an acute non-severe presentation (REC 15.8%), whereas the other was an atypical late-onset presentation in an old male with a genetic confirmation of WD but with very subtle disturbances in copper metabolism. Whether this last case should be considered a real case of WD despite the fact of not presenting copper disturbances is arguable, but again suggests the usefulness of these biomarkers to detect those patients at a lower risk of copper overload, and to indicate or not more aggressive therapies in each case. In this patient, a copper-free diet was suggested and continuous copper monitoring is being performed.

On the contrary, REC turned to be high in almost all our patients (71.4% of new diagnosis had a REC ≥18.5% and 95.1% of long-term WD patients presented REC ≥14%). The median REC level among our WD patients was 31%. These high levels were high even after long-term periods of therapy, and regardless of the level of adherence to medical therapy and/or copper control, although significantly lower among those classified as presymptomatic at diagnosis. REC levels remained high among the seven cases in whom we had additional measurements within time. The reason of why these REC values remained high even after such long periods of therapy could not be answered by this study and will need to be defined in larger dynamic cohorts. Furthermore, REC behavior was not detailed in the only study assessing dynamic changes of copper parameters in a cohort of pediatric WD.[19] Taking all this data together, we would suggest that REC is a valuable tool for diagnostic purposes only, but it is not valuable enough for monitoring copper homeostasis once treatment has been started.

On the other hand, exchangeable copper levels tended to be lower in WD patients at the maintenance phase and under long-term therapy (compared to those new or recent WD diagnosis) and also among those patients with a better compliance, although no statistical significance was reached, probably due to the high heterogenicity and reduced size of our sample. Presymptomatic patients also showed slightly lower levels of exchangeable copper when compared to symptomatic patients, although statistical significance was not reached. However, this tendency is in line with other previous reports[12,19] and suggests it should be considered as a valuable tool for assessing dynamic copper homeostasis. Unfortunately, the reduced number of patients in whom we had ExchCo measurements during follow-up (only 7, being all treated patients) enabled us to draw any conclusion at this regard. To the best of our knowledge, only one recent paper performed in 36 WD children has shown the potential use of exchangeable copper to monitor therapy within the first 5 years of diagnosis.[19] In this work, the authors reported a significant decrease in ExchCo levels

starting from the third month of therapy and stabilizing at month 12 onwards. Nevertheless, the reference target of normality to be desirable under therapy in WD patients was not reported and it is still unknown and beyond the scope of this descriptive study.

Importantly, REC was also helpful in excluding the diagnosis of WD in six patients with an old diagnosis, who had been on anti-copper therapy for a long time, but in which we had important diagnostic doubts, despite all but one had presented with Leipzig scores >3 points at diagnosis. All six patients had REC < 15% (median: 8.5%, IQR_{25-75} 6%–10%) at evaluation. Indeed, all but one were carriers of one single mutation in *ATP7B* (the additional patient had no detected mutations), in which it is well known that copper disturbances can be detected at some level.[4,13,20] These normal REC and ExchCo results reinforced us to withdrawal anti-copper therapy. All of them remain completely asymptomatic with stable blood tests after a follow-up of at least 12 months. Importantly, however, these patients remain under long-term medical copper monitoring as changes in copper metabolism might take years to occur. This data reinforces the usefulness of performing such complementary diagnostic methods (ExchCo and REC), to identify/discard abnormal copper metabolism, even among some patients with a "confirmatory" Leipzig score ≥4 points. WD is a complex disease in which an abnormal copper metabolism and increase of the toxic fraction of copper should be proven for diagnosis, regardless of the diagnostic score of Leipzig values. Again, it should be remembered that some carriers of one mutation in the *ATP7B* gen may exert some abnormal values of Cp or urinary excretion, without being at risk of disease development/progression.[20]

We are aware of the important limitations of our study, which we believe that should be considered as an external validation of the French previous studies in our real clinical practice. The main limitation of this work is the limited size of our WD sample and the high clinical heterogenicity of WD patients, together with the retrospective nature of the design, which enabled us to obtain serum samples under controlled and similar circumstances. Noteworthy, and despite these important drawbacks, we were able to differentiate most of the WD patients from the healthy controls, and to show lower levels of exchangeable copper among patients during follow-up with adequate compliance to anti-copper medication, or among those patients classified as presymptomatic. We believe that the global implementation of these additional biomarkers would be extremely helpful for identifying and monitoring patients with WD in real life. In addition, we think they should be incorporated at the first-line study panel in patients with chronic liver diseases, even in the absence of clinical suspicion for WD (i.e., those patients with comorbidities and/or older than usual ages for WD debut). Prospective studies including sequential measurements during the disease, in real life settings, are expected and will probably be useful for defining normal reference ranges of copper homeostasis.

AUTHOR CONTRIBUTIONS

Zoe Mariño: design of the study, collection of data, statistical analysis, writing the manuscript, approved final draft. **Cristina Molera-Busoms**: design of the study, collection of data, writing the manuscript, approved final draft. **Celia Badenas**: critical review, approved final draft. **Jesús Quintero-Bernabeu**: collection of data, critical review, approved final draft. **Mercè Torra**: critical review, approved final draft. **Xavier Forns**: critical review, approved final draft. **Rafael Artuch**: design of the study, critical review, approved final draft.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ETHICS STATEMENT

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5).

INFORM CONSENT

Informed consent was obtained from all patients for being included in the study. Proof that informed consent was obtained will be available upon request.

ANIMAL RIGHTS

This article does not contain any studies with human or animal subjects performed by the any of the authors.

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REFERENCES

1. Członkowska A, Litwin T, Dusek P, et al. Wilson disease. *Nat Rev Dis Primers*. 2018;4:1-44.
2. Ferenci P, Caca K, Loudianos G, et al. Diagnosis and phenotypic classification of Wilson disease. *Liver Int*. 2003;23(3):139-142.
3. EASL. Clinical practice guidelines: Wilson's disease. *J Hepatol*. 2012;56(3):671-685.
4. Schilsky ML, Roberts EA, Bronstein JM, et al. A multidisciplinary approach to the diagnosis and management of Wilson disease:executive summary of the 2022 practice guidance on Wilson disease from the American Association for the Study of Liver Diseases. *Hepatology*. 2022;77(4):1428-1455. doi:10.1002/hep.32805
5. Roberts EA, Schilsky ML. Diagnosis and treatment of Wilson disease: an update. *Hepatology*. 2008;47(6):2089-2111.
6. Palumbo CS, Schilsky ML. Clinical practice guidelines in Wilson disease. *Ann Transl Med*. 2019;7(S2):S65.
7. Socha P, Janczyk W, Dhawan A, et al. Wilson's disease in children: a position paper by the hepatology committee of the european society for paediatric gastroenterology, hepatology and nutrition. *J Pediatr Gastroenterol Nutr*. 2018;66(2):334-344.
8. Ferenci P, Ott P. Wilson's disease: fatal when overlooked, curable when diagnosed. *J Hepatol*. 2019;71(1):222-224.
9. Beinhardt S, Leiss W, Stättermayer AF, et al. Long-term outcomes of patients with Wilson disease in a large Austrian cohort. *Clin Gastroenterol Hepatol*. 2014;12(4):683-689.
10. Duncan A, Yacoubian C, Beetham R, Catchpole A, Bullock D. The role of calculated non-caeruloplasmin-bound copper in Wilson's disease. *Ann Clin Biochem*. 2017;54(6):649-654.
11. El Balkhi S, Poupon J, Trocello JM, et al. Determination of ultrafiltrable and exchangeable copper in plasma: stability and reference values in healthy subjects. *Anal Bioanal Chem*. 2009;394(5):1477-1484.
12. Guillaud O, Brunet AS, Mallet I, et al. Relative exchangeable copper: a valuable tool for the diagnosis of Wilson disease. *Liver Int*. 2018;38(2):350-357.
13. El Balkhi S, Trocello JM, Poupon J, et al. Relative exchangeable copper: a new highly sensitive and highly specific biomarker for Wilson's disease diagnosis. *Clin Chim Acta*. 2011;412(23–24):2254-2260.
14. Yim J, Kwon SB, Han JS, Kim J-H, Lee EH, Lee S-G. Total and exchangeable copper assay using inductively coupled plasma mass spectrometry and establishment of a pediatric reference interval. *Arch Pathol Lab Med*. 2021;145(7):877-882.
15. Poujois A, Trocello JM, Djebrani-Oussedik N, et al. Exchangeable copper: a reflection of the neurological severity in Wilson's disease. *Eur J Neurol*. 2017;24(1):154-160.
16. Trocello JM, El Balkhi S, Woimant F, et al. Relative exchangeable copper: a promising tool for family screening in Wilson disease. *Mov Disord*. 2014;29(4):558-562.
17. Woimant F, Djebrani-Oussedik N, Poujois A. New tools for Wilson's disease diagnosis: exchangeable copper fraction. *Ann Transl Med*. 2019;7(S2):S70.
18. Jacquelet E, Poujois A, Pheulpin MC, et al. Adherence to treatment, a challenge even in treatable metabolic rare diseases: a cross sectional study of Wilson's disease. *J Inherit Metab Dis*. 2021;44(6):1481-1488.
19. Ngwanou DH, Couchonnal E, Parant F, et al. Long-term urinary copper excretion and exchangeable copper in children with Wilson disease under chelation therapy. *J Pediatr Gastroenterol Nutr*. 2022;75:e75-e80. doi:10.1097/MPG.0000000000003531
20. Tu J, Backwell R. Studies on levels of penicillamine-induced cupriuresis in heterozygotes of Wilson's disease. *Metabolism*. 1967;16(6):507-513.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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