Review Article

New tools for Wilson's disease diagnosis: exchangeable copper fraction

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Abstract: Wilson's disease (WD) biochemical markers continue to evolve. Classical tests [serum copper, serum ceruloplasmin (Cp), urinary copper] have their own limits, and they are often insufficient to diagnose or exclude WD. So, calculated estimation of copper that is not bound to Cp has been proposed, but it is flawed. Therefore, we focused our research on a direct measurement of serum copper labile fraction. Exchangeable copper (CuEXC) offers a correct view of the free copper overload. It provides information on the spread and severity of WD. Relative exchangeable copper (REC) (percentage of exchangeable to total serum copper) that appreciates the toxic fraction of copper in blood is an excellent biomarker for WD diagnosis. These two tests are reliable and non-invasive. They give rapid answers for an appropriate diagnosis and make possible to start the treatment quickly without waiting for the result of the genetic tests. As early diagnosis and treatment are the keystones of successful management of patients with WD, different teams have already applied these tests in a routine framework to a large number of patients.

Keywords: Wilson's disease (WD); diagnosis; relative exchangeable copper (REC); exchangeable copper; cupric markers

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In Wilson's disease (WD), irreversible and serious clinical injuries and even death could be consequences of misdiagnosis or treatment delay (1). The diagnosis is evoked on clinical symptoms and several biological tests: ceruloplasmin (Cp), serum and urinary copper concentrations, and sometimes calculated estimation of copper that is not bound to Cp. These determinations are often insufficient to diagnose or to exclude WD. Although hepatic copper evaluation is rather discriminatory and helps for diagnosis, invasive sampling, false negative results and inhomogeneous liver copper distribution moderate its usefulness (2). Molecular genetic diagnosis confirms the diagnosis in 98% of cases; the result is that 2% of patients with proven WD have a single heterozygous mutation or even no mutation in the *ATP7B* gene (3); furthermore, this diagnosis can be time-consuming because of more than 600 mutations have been described. However, early diagnosis and treatment are the keystones of the successful management of patients with WD. But thinking of a WD diagnosis is challenging as many patients have non-specific symptoms that precede those leading to the diagnosis. So, it is important to have a rapid, reliable, non-invasive and discriminative tool for the diagnosis of WD. It is why we developed the exchangeable copper (CuEXC) assay that leads to the calculation of the relative exchangeable copper (REC).

The limits of the traditional and cupric parameters

The traditional and non-invasive cupric parameters used

for WD diagnosis are serum Cp and copper concentrations, calculated non-Cp-bound copper and 24 h-urinary copper excretion.

Serum Cp determination

Serum copper is mostly carried by Cp. This protein is synthesized in hepatic microsomes as an inactive, unstable non-copper-bound apoprotein called apo-Cp. Charged with six copper atoms, it is excreted in the circulation as a holoprotein with acts as a ferro-oxidase (4). In normal subjects, 90% of plasma Cp circulates as holo-Cp and therefore contains copper.

The determination of serum Cp is essentially performed by immunological method (as radioimmunoassay, radial immunodiffusion or nephelometry) which simultaneously measures inactive apo-Cp and active holo-Cp. This method currently used by automated clinical laboratories is rapid but tends to overestimate active Cp. The only method available to determine the copper-dependent oxidative activity of Cp (active holo-Cp) is an enzymatic determination that is not performed routinely.

Due to *ATP7B* defect in WD, apo-Cp copper incorporation is stucked, and holo-Cp serum concentration is decreased while apo-Cp and free copper are released from liver. Serum Cp is typically decreased in WD, lower than 0.1 g/L. But diagnosis couldn't be excluded in front of normal Cp observation, more than half of patients presenting severe liver disease (5), 25–36% of children with WD (6) and few patients with neurologic presentation of WD (7) have normal serum Cp measured by immunologic assays. Patients with sub-normal levels of Cp during the acute phase are likely to synthesize apo-Cp essentially. Cp enzymatic activity determination has then been proposed (8,9). These enzymatic determinations detect only active holo-Cp and are the reflect of what *ATP7B* does in hepatocytes. Methods for Cp enzymatic activity determination, instead of immunological antigenic properties, rely on functionally active Cp catalytic oxidation, towards different substrates. ρ-phenylenediamine (PPD) was the first described (10). Ortho-dianisidine dihydrochloride (OD) was also used in numerous methods (11,12). The use of ferric iron, considered as the only biological substrate, allows to determine Cp ferroxidase activity (13,14). These assays, even applied to automated analyzers, are not yet available in routine, but will certainly be more informative for WD diagnosis than immunological assay.

In addition to these methodological problems, there are physiological variations in Cp levels that can mislead to diagnosis. Inflammation states, pregnancy or estrogen supplementation can lead to serum Cp elevation (15). On the other hand, low serum Cp levels are observed in newborns or during Menkes disease, aceruloplasminemia, nephritic syndrome, copper deficiencies, severe chronic liver disease or malabsorption syndromes (16) and, about 20% of heterozygous subjects for the WD gene have reduced levels (17). Therefore, serum Cp interpretation is not easy and insufficient for WD diagnosis.

Serum copper

Circulating copper is partly inextricably bound within metalloprotein (Cp essentially) or loosely bound to proteins such as albumin, amino acids or peptides. Physiologically, nearly 70% of copper is bound to Cp and less than 20% is bound to albumin. Transcuprein (αmacroglobulin) and amino acids binds 7–15% and 2–5% of serum copper, respectively (18). Total serum copper determination measures copper incorporated in Cp and non-Cp bound copper. It is usually made by either atomic absorption or emission spectrometry or by inductively coupled plasma mass spectrometry (ICP-MS) methods (19). Normal range of total serum copper is estimated around 12.7–22. 2 µmol/L.

Although considered as a copper overload, total serum copper and Cp are usually decreased in WD. Indeed, the absence of holo-Cp that carries copper atoms, leads to dramatical reduction of total serum copper. However, normal serum copper concentration can be observed in WD. Acute hemolysis or hepatitis can lead to important release of copper from liver tissue stores. Dissociation between normal to increased total copper and decreased Cp levels could indicate an increase in the non-Cp-bound-copper. So, it's important to differentiate copper highly bound to Cp that is not bioavailable for tissues and organs from labile or free copper pool which, in case of WD, is thought to be responsible of organ damage.

Calculated non-Cp-bound copper concentration (NCC)

By estimating the toxic unbound (or "free") copper, NCC was proposed as a diagnostic test for WD (20,21). NCC is calculated from Cp and total serum copper concentrations and the adequacy of both measurements influences results of the formula used for calculation (22). In fact, this determination's limitations in WD context are due to very low values of total copper and Cp which sometimes do not

reach analytical method detection limits. Furthermore, this calculation is not valid when immunological assay is used for serum Cp determination, because of simultaneous detection of inactive apo-Cp and active holo-Cp. Oxidase activity measurement of Cp could resolve these difficulties but this enzymatic determination is not performed routinely.

Several authors showed that NCC is not a good test in WD. Twomey *et al.* showed large overlapping of this parameter between non WD subjects and WD patients. Moreover, due to Cp overestimation, nearly 20% of normal subjects present negative values (23). Our team showed also that 10.4% of WD patients have negative values of NCC at diagnosis (24). European Association for the Study of the Liver (EASL) guidelines did not recommend NCC for diagnosis of WD (25).

24 h-urinary copper excretion

Basal 24-h urinary copper excretion reflects, in non-treated patients, the amount of non-bound circulating copper. In symptomatic, non-treated patients, a threshold of 100 µg (1.6 µmol)/24 hours urinary copper excretion, has been retained for WD diagnosis (26). In practice, knowing the exact 24 hours urine volume can be difficult in young children and in patients with neurological symptoms. In addition, care should be taken to avoid external contaminations, which are very common for urine collections (27). At last, this determination is not applicable in case of renal impairment (28). Interpreting 24-h urinary copper excretion may be difficult. At the time of diagnosis, especially in children and asymptomatic siblings, more than a quarter of WD patients have 24-h urinary copper levels below the threshold described (29). So, the lower cut-off of 0.6 µmol/24 hours is considered to be more sensitive (30,31). Furthermore, high urinary copper excretion may be difficult to interpret, particularly because of the increasing observed in different liver diseases (32). Heterozygotes subjects on *ATP7B* gene may also present increased urinary copper level (5). Urinary copper excretion measurement under D-penicillamine treatment as a diagnosis test has been proposed but reference values have only been validated in children with liver symptoms (29).

In summary

Each isolated traditional biochemical marker is insufficient to set WD diagnosis. The association of the three biochemical markers (low serum Cp concentration, low serum copper concentration and high urinary copper excretion) is highly predictible of WD diagnosis. However, this classical triad is present in 15% of heterozygote carrier and 3% of WD patients with confirmed mutations have normal copper balance (personal data, Lariboisiere registry). Calculation of NCC is flawed. So, we focused our research on a direct determination of the labile fraction of serum copper.

Direct plasma unbound copper determination

We distinguish ultrafiltrable copper (CuUF) and CuEXC. The results of recent studies with quantification of CuUF and CuEXC in WD animal models and human populations are summarized in *Table 1*.

Plasma CuUF

This fraction of copper which is bound to low molar mass molecules is obtained by ultrafiltration of plasma through a membrane with a cut off retaining albumin (67 kDa), Cp (132 kDa), and transcuprein (270 kDa). CuUF represents less than 1% of total copper in healthy subjects and is supposed to be constituted by copper released from liver that is bound to proteins such as albumin. CuUF is an unstable fraction that changes with copper movements between free form and plasma proteins binding (33). Moreover, CuUF have not demonstrated a great value in the diagnosis of WD patients (34).

CuEXC, a marker of the dissemination and severity of WD

This other fraction is obtained after albumin and amino acids copper complexation. Heavy extraction procedures have sometimes been used (40-42). However, easier complexation procedures using chelators such as ethylenediaminetetraacetic acid (EDTA) are able to mobilize this exchangeable fraction. Retained procedure involves serum ultrafiltration after 1-hour EDTA incubation. CuEXC includes both CuUF and copper loosely bound to albumin and other amino acids and then constitutes non-Cp-bound copper.

This determination has shown a good analytical reliability and a 24 hours or 7 days stability at room temperature or in frozen serum respectively (34). Thus, CuEXC determination requires an immediate serum freezing after sampling centrifugation if conservation exceed 24 h. Reference values for CuEXC are between 0.62

Table 1 Recent studies with quantification of CuUF and CuEXC in WD animal models and human populations

Reference	Population	Tested parameters				Observations
		CuUF	CuEXC	REC	Other parameters	
El Balkhi 2009 (33)	❖ 44 healthy adult volunteers ❖ 3 WD patients	0.071–0.153	Controls: 0.64–1.12 µmol/L		Total serum Cu	Extreme instability of CuUF *in vitro*
			Patients without treatment: CIEXC elevated		Serum Cp Calculated NCC	Negative values of NCC for the patients
El Balkhi 2011 (34)	❖ 62 healthy adult volunteers ❖ 25 wild type homozygous ❖ 45 heterozygous ❖ 16 newly diagnosed WD patients before treatment initiation	Above RV for 9 patients Irrelevant in diagnosis of WD (poor Se and Sp)	Cut Off for WD diagnosis =1.53 µmol/L (Sp =99%; Se =88%)	Cut Off for WD diagnosis =18.5% (Sp =100%; Se =100%)	Total serum Cu Serum Cp Calculated NCC Urinary Cu	CuEXC stability: ❖ 24 h at room temperature ❖ Frozen: 7 days
Schmitt 2013 (35)	❖ Control group: 6 LE rats (without *Atp7b* mutation and with normal food) ❖ WD animal model group: 24 LEC rats (with an *Atp7b* mutation: 15 normal food and 9 poor copper food)		LE: constant over time	Higher in all diseased rats than in controls at every time point of the study (P<0.01)	STB/ALT/AST	REC discriminating and independent of all confounding factors tested
			LEC: higher than controls (P<0.001) and dependent on liver failure	Cut off 19.0%: Se =100% and Sp =100% (only adult rats)	Serum Cu	Necessity of establishing reference values in young population
			CuExC lower in LEC with poor copper food than in LEC with normal food		Serum Cp oxidase activity (o-dianisidine dihydrochloride)	
Trocello 2014 (36)	126 clinically asymptomatic subjects in context of family screening for WD classified in 3 groups: ❖ WD (n=5)/Htz (n=87)/NoM (n=34)		Studied but data not available	Significantly different for: ❖ WD *vs.* Htz (P=0.016) ❖ WD *vs.* no (P=0.015)	*ATP7B* gene analysis Total serum Cu Serum Cp Urinary copper	
Poujois 2017 (24)	48 new WD patients ❖ 3 pre-symptomatic ❖ 17 H ❖ 28 EH		Significantly higher in EH than H patients (P<0.0001)	REC >25.2% in all patients	*ATP7B* gene analysis	1 patient in H group (haemolytic anaemia + liver failure) presented high CuEXC value
			Threshold for an extra-hepatic diagnosis >2.08 µmol/L	No statistical difference between H and EH groups (P=0.09)	Total Cu Serum Cp Calculated NCC Urinary Cu	

Table 1 *(continued)*

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Reference	Population	Tested parameters				Observations
		CuUF	CuEXC	REC	Other parameters	
Heissat 2018 (37)	Genetically engineered rodent strain mice: ❖ 137 *Atp7b*$^{-/-}$ ❖ 101 WT (C57BL/6)		Lower in treated mice without reaching statistical difference	Significantly higher in *Atp7b*$^{-/-}$ Se and Sp =100%, with a 20% cut off in *Atp7b*$^{-/-}$ (irrespective of sex, age, stage or treatment)	STB/ALT/AST Total serum Cu Intrahepatic Cu Liver histological analysis	Age groups illustrate different stages of WD Effect of treatment (D-penicillamine) studied
Guillaud 2017 (38)	201 patients ❖ 9 WD at diagnosis or non-compliant (group 1) ❖ 40 WD treated (group 2) ❖ 103 adults non-WD hepatic diseases ❖ 49 children non-WD hepatic diseases		Significantly higher in group 1 Significantly lower in group 2	Significantly higher in group 1 and 2 *vs.* other liver diseases	*ATP7B* gene analysis (WD) Total serum Cu Serum Cp	1 non WD patient with a REC of 16.3% diagnosed with an autoimmune hepatitis responding to corticosteroid therapy
Lauwens 2018 (39)	21 middle-aged male volunteers: ❖ 14 alcoholic liver cirrhosis ❖ 7 assumed healthy individuals (reference population)	Determination of the sum: EXCH + UF	❖ EXCH + UF Cu of AC group higher than reference population (P=0.052) ❖ NEXCH + NUF: no significant difference between both populations	Significantly higher in AC population than reference population (P=0.048) ❖ 2/14 patients exceed 19%	Total serum Cu Bulk serum Cu isotopic composition Serum Cp Serum albumin	Significantly lighter isotopic composition of the EXCH + UF serum Cu and of total serum Cu in AC population than healthy individuals

CuUF, ultrafiltrable copper; CuEXC, exchangeable Cu; REC, relative exchangeable copper; WD, Wilson disease; CIEXC, cation exchange chromatography; Cp, ceruloplasmin; NCC, non-ceruloplasmin-bound copper concentration; RV, reference values; Sp, specificity; Se, sensitivity; LE, long-evans; LEC, long-evans Cinnamon; STB, serum total bilirubin; ALT, alamine aminotransferase; AST, aspartate aminotransferase; Htz, heterozygous carriers; NoM, no identified mutation in the *ATP7B* gene; H, hepatic; EH, extra-hepatic; EXCH + UF, exchangeable + ultrafiltrable Cu fraction; AC, alcoholic liver cirrhosis ;NEXCH+NUF: non-exchangeable +non-ultrafiltrable Cu fraction.

and 1.15 µmol/L (18).

Main advantage of this assay is that it doesn't depend on the dosage of Cp and it represents an exact estimation of copper overload. If CuEXC is over reference range then a blood and tissue copper overload are suspected as consequence of hepatocytes saturation and leakage of toxic free copper into the blood.

Studies in WD animal models showed that CuEXC increases with liver disease evolution. Long Evans Cinnamon (LEC) rats present, as a consequence of a loss of function mutation in the *ATP7B* gene a copper metabolism disorder. Schmitt *et al.* demonstrated that, in these rats, CuEXC levels were correlated with acute liver disease (35). In another WD animal model (mice from a genetically engineered rodent strain), *Atp7b*$^{-/-}$ mice have several features of WD. In these animals, hepatic lesions and CuEXC increase are observed, even if no acute liver failure was observed in this study (37).

We demonstrated that in case of WD affecting only the liver at diagnosis, CuEXC is normal or moderately increased except in case of acute liver failure associated with Coombs negative haemolytic failure. In these cases, the increase of CuEXC is owing to hepatic necrosis (24). Moreover, the level of CuEXC is shown to be statistically higher in WD patients with extrahepatic involvement (Kayser-Fleischer ring, neurological symptoms, pathological brain MRI) than in hepatic WD. A threshold of 2.08 µmol/L permitted to predict the presence of these extrahepatic lesions. Furthermore, CuEXC level at diagnosis time is predictive of extrahepatic involvement severity: a positive correlation is observed between CuEXC and the Unified Wilson Disease Rating Scale (UWDRS) (24). UWDRS neurological score, specific for WD has been developed for clinical studies and is widely used by WD expert teams. It evaluates consciousness (part 1), functional scale based on Barthel scale and activities of daily living (part 2) and neurological examination: speech, gait, and dystonic, ataxic, tremor and parkinsonian syndromes (part 3) (43). Interestingly, sensitivity to dietary copper has been demonstrated in LEC rats WD animal models (44) with lower CuEXC in low-copper diet rats (35). CuEXC is also interesting for WD treatment observance evaluation, with higher levels in non-compliant patients (38). In a population study of 100 WD patients, CuEXC abnormal increase was observed in 25 patients reflecting compliance/observance problems. Half of them (12/25) showed also liver enzymes increase. These observations highlighted the ability of CuEXC to reflect copper overload.

CuEXC is not yet referenced in French bio-clinical analysis nomenclature. The analysis cost depends on the analytical methodology used for copper determination in the ultra-filtrate: Atomic Absorption Spectroscopy (AAS) or ICP-MS. It may, then, be slightly variable from a laboratory to another. In addition, the Ultra centrifugal filter (Amicon®) unit's cost has also to be included, and as REC determination also involves total serum copper determination, this parameter has to be taken in account for analysis billing.

REC: a specific and sensitive non-invasive tool for WD diagnosis

The percentage of exchangeable to total serum copper (e.g., copper incorporated in Cp and non-Cp-bound copper) called the REC appreciates toxic blood copper fraction. It is the most informative non-invasive WD diagnosis test. In an original work, El Balkhi *et al.* compared the REC to classical WD diagnosis tests (total serum copper, urinary copper excretion, Cp and NCC) in three populations (WD patients, heterozygous individuals and normal subjects). REC evaluation with an 18.5% cut off was shown to be more sensitive and more specific than other usual tests. REC test is then an excellent biomarker for the diagnosis of WD with 100% sensitivity and 100% specificity (34). These results were also confirmed in LEC rats; Schmitt *et al.* tested the validity of REC, in comparison with Cp oxidase activity and total serum copper, in different stages of liver disease. Using a cut off slightly higher than human threshold (19%), REC was the only parameter that permitted to distinguish between controls (Long-Evans rats) and WD models (LEC rats) with a 100% sensitivity and specificity. In addition, the authors demonstrated that liver failure stage didn't influence REC level (35). In another animal model, *Atp7b*$^{-/-}$ mice, discriminative power of REC was also demonstrated for WD diagnosis: diseased group mice presented an average REC statistically higher than wild type group. Here again, sensitivity and specificity of REC was fined to 100% using a cut off fixed at 20% (37).

We have also shown that REC is particularly efficient to distinguish individuals carrying one abnormal *ATP7B* allele from WD patients. This study was conducted in 127 asymptomatic siblings (up to the second-degree relatives) of index case WD patients presenting a genetically confirmed diagnosis. With a cut-off of 15%, REC determination significantly discriminated WD patients from individuals carrying one abnormal *ATP7B* allele and normal subjects (36). *REC* is then an important parameter

for WD family screening, that can also discriminate carriers (presenting heterozygous mutation in *ATP7B* gene) even in presence of copper biological abnormalities.

Moreover, REC performance in Wilson's disease diagnosis has been studied in a study group counting 103 adults and 49 children with various kinds of chronic liver disease (excluding WD) (38). The discriminative performance of REC, for WD diagnosis, has also been demonstrated among non-Wilsonian liver diseases patients (REC >18.5%, sensitivity and specificity of 100%) in addition to previously published discrimination among controls, heterozygous carriers, and family relatives (34,36), else more the authors noticed that in the other chronic liver diseases tested while liver function tests were abnormal REC remained normal and did not increase. In cirrhosis, because of frequent low serum Cp, REC is particularly interesting (45) and in icteric cholestasis because of basal 24-hour urine copper excretion frequently increased in this disorder (32). Nevertheless, in a recent study, 14 patients with alcoholic cirrhosis subjects have REC value ≤19% except two patients with REC at 21 and 25%. There is no information concerning *ATP7B* genetic testing for these patients (39).

Conclusions

CuEXC determination allows a direct an accurate measurement of copper overload. It provides, at diagnosis, information on the spread and severity of the disease. REC calculation (percentage of exchangeable to total serum copper) is a very valuable discriminative tool for WD diagnosis presenting excellent sensitivity and specificity. These two tests are rapid, reliable and non-invasive. Their routine uses, by different teams, in large cohorts of patients have been very successful and validated their important place as standard of care for WD patients. They give fast answers to an appropriate diagnosis and allow beginning quickly the treatment without waiting for genetic testing results. Furthermore, they proved a helpful contribution in initial treatment choice and dose progression rate determination.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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