Re-evaluation of the Diagnostic Criteria for Wilson Disease in Children With Mild Liver Disease

Emanuele Nicastro*, Giusy Ranucci*, Pietro Vajro, Angela Vegnente, and Raffaele Iorio

The diagnosis of Wilson disease (WD) is challenging, especially in children. Early detection is desirable in order to avoid dramatic disease progression. The aim of our study was to re-evaluate in WD children with mild liver disease the conventional diagnostic criteria and the WD scoring system proposed by an international consensus in 2001. Forty children with WD (26 boys and 14 girls, age range = 1.1-20.9 years) and 58 age-matched and sex-matched patients with a liver disease other than WD were evaluated. Both groups were symptom-free and had elevated aminotransferases as predominant signs of liver disease. In all WD patients, the diagnosis was supported by molecular analysis, the liver copper content, or both. A receiver operating characteristic (ROC) analysis of ceruloplasmin at the cutoff value of 20 mg/dL showed a sensitivity of 95% [95% confidence interval (CI) = 83%-99.4%] and a specificity of 84.5% (95% CI = 72.6%-92.6%). The optimal basal urinary copper diagnostic cutoff value was found to be 40 μg/24 hours (sensitivity = 78.9%, 95% CI = 62.7%-90.4%; specificity = 87.9%, 95% CI = 76.7%-95%). Urinary copper values after penicillamine challenge did not significantly differ between WD patients and control subjects, and the ROC analysis showed a sensitivity of only 12%. The WD scoring system was proved to have positive and negative predictive values of 93% and 91.6%, respectively. *Conclusion:* Urinary copper excretion greater than 40 μg/24 hours is suggestive of WD in asymptomatic children, whereas the penicillamine challenge test does not have a diagnostic role in this subset of patients. The WD scoring system provides good diagnostic accuracy. (HEPATOLOGY 2010;000:000-000.)

Wilson disease (WD) is an autosomal recessive disorder of copper metabolism caused by mutations in a gene [ATPase, Cu++ transporting, beta polypeptide (*ATP7B*)] encoding a copper-transporting, P-type ATPase.[1] This disease leads to progressive copper accumulation in the liver and subsequent deposition in other organs, such as the nervous system, corneas, kidneys, bones, and joints. The distribution of the metal in diverse organs over time accounts for the wide range of clinical manifestations.[2] In the pediatric age bracket, most cases have a hepatic presentation. In the available series, the percentage of WD children presenting with isolated elevated serum aminotransferases ranges from 14% to 88%; this depends on the health policy and the type of health care provided.[3-5] However, there is evidence that alterations in liver function tests may precede the onset of symptoms for a considerable time. Neurological symptoms are more frequent in adolescents and young adults[6-8] and are found in only 4% to 6% of pediatric cases with hepatic onset.[4,5,9]

If WD is not recognized and adequately treated, the progression of hepatic and neurological damage can be very rapid, and fulminant liver failure can occur. Therefore, the prompt detection of this condition is vital. Unfortunately, the diagnosis of WD is an especially challenging task in children because the conventional criteria established for adults are not always appropriate for children.[10] In particular, basal urinary copper excretion in most WD children is lower than the extensively accepted cutoff value of 100 μg/24

Abbreviations: A1AT, alpha-1-antitrypsin; AASLD, American Association for the Study of Liver Diseases; ACH, active chronic hepatitis; AIH, autoimmune hepatitis; ATP7B, ATPase, Cu++ transporting, beta polypeptide; C, cirrhosis; CDG, congenital disorders of glycosylation; CI, confidence interval; F, fibrosis; INR, international normalized ratio; KF, Kayser-Fleischer; NA, not applicable; NAFLD, nonalcoholic fatty liver disease; ND, not done; Neg, negative; NRH, nodular regenerative hyperplasia; NS, not significant; PCT, penicillamine challenge test; Pos, positive; PTT, partial thromboplastin time; r, Pearson correlation coefficient; ROC, receiver operating characteristic; S, steatosis; ULN, upper limit of normal; WD, Wilson disease.

From the Department of Pediatrics, University Federico II, Naples, Italy.

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**The first two authors contributed equally to this work.*

Address reprint requests to: Raffaele Iorio, M.D., Department of Pediatrics, University Federico II, Via Sergio Pansini 5, Naples, Italy 80131. E-mail: riorio@unina.it; fax: 0039 081 1464337.

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hours.[10] Additionally, the diagnostic accuracy of daily urinary copper measurements after chelation with penicillamine remains questionable.

From a genetic point of view, the diagnosis of WD is based on the identification of two disease-causing mutations or homozygosity for a single disease-causing mutation. However, according to the American Association for the Study of Liver Diseases (AASLD) guidelines, mutation analysis should be performed for individuals in whom the diagnosis is difficult to establish by clinical and biochemical testing.[2]

In order to obtain a more reliable diagnosis of WD, a scoring system was proposed by an international consensus of experts.[11] To date, this score has not been extensively evaluated in asymptomatic WD children.

The aim of our study was to re-evaluate in WD children with mild liver disease the conventional diagnostic criteria and the WD scoring system proposed by Ferenci et al.[11]

Patients and Methods

Patients We collected data for all patients with WD who were referred to the Department of Pediatrics (University Federico II, Naples, Italy) between 1984 and 2009 for the diagnostic investigation of elevated serum aminotransferases or for familial screening for WD. The diagnosis of WD was initially established with at least two of the following features: a low plasma ceruloplasmin level (<20 mg/dL), an increased basal urinary copper level (>100 μg/24 hours), an increased urinary copper level after the penicillamine challenge test (PCT; >1575 μg/24 hours), an increased liver copper level (>250 μg/g of dry weight), a positive family history, the presence of Kayser-Fleischer (KF) rings, and Coombs' negative hemolytic anemia.[3] Furthermore, genetic testing results, when available, were considered.

In order to re-evaluate the accuracy of the diagnostic criteria for WD in children with mild liver disease, we decided to analyze only patients for whom the diagnosis of WD was supported by the presence of an abnormal liver copper value, the identification of two disease-causing mutations or homozygosity for a single disease-causing mutation, or both. Therefore, of the 43 patients (28 males and 15 females) who fulfilled the aforementioned diagnostic criteria, 40 (26 males and 14 females, median age at diagnosis = 6.1 years, range = 1.1-20.9) were selected for the study.

We recruited for the control group patients with a liver disease other than WD who were being investigated for elevated serum aminotransferases and siblings of WD patients who were referred to our center in the same period to exclude a diagnosis of WD. Patients were included in the control group if, on at least one occasion, the levels of ceruloplasmin, basal urinary copper, and urinary copper after penicillamine challenge were tested.

Patients were considered to be affected by cryptogenic liver disease when the following entities were ruled out: WD, alpha-1-antitrypsin deficiency, infectious hepatitis, autoimmune hepatitis (AIH), biliary system disorders, drug-induced liver disease, nonalcoholic fatty liver disease (NAFLD), celiac disease, cystic fibrosis, congenital disorders of glycosylation (CDG), and extrahepatic causes of elevated serum aminotransferases.

The following data at diagnosis were analyzed: age, sex, reason for referral, clinical symptoms, laboratory tests (e.g., levels of serum ceruloplasmin, basal urinary copper, urinary copper after PCT, and hepatic copper), and molecular analysis for *ATP7B*. The WD diagnostic scores were calculated in accordance with Ferenci et al.[11] and are shown in Table 1. The diagnosis of WD was considered certain if the final score was 4 or more and probable if it was 2 to 3. Assigning points for urinary copper, we considered as upper limits of normal (ULNs) both 100 and 40 μg/24 hours. We initially calculated the score without taking into account the mutation analysis.

Among the enrolled WD patients, 34 were referred for raised serum aminotransferases, and 6 were referred for familial screening. Twenty-three subjects (57.5%) presented with hepatomegaly at the clinical examination, ultrasound examination, or both. In five patients (12.5%), neurological signs were highlighted after a detailed neurological examination when the WD diagnosis was already known, and in two of these five patients, KF rings were detected. Molecular analysis for the *ATP7B* gene was performed for 36 patients, and disease-causing mutations were found in 34 (26 homozygotes and 8 heterozygotes). The characteristics of the WD patients are shown in Table 2.

Fifty-eight patients (36 males and 22 females, median age at diagnosis = 7.1 years, range = 1-20) were enrolled as control subjects. Among them, 52 patients who were referred for elevated serum aminotransferases had a liver disease other than WD: 17 (29.3%) had NAFLD, 13 (22.4%) had chronic cryptogenic liver disease, 5 (8.6%) had AIH, 5 had nodular regenerative hyperplasia (NRH) of the liver, 4 (6.8%) had CDG, 2 (3.44%) had congenital hepatic fibrosis, and 2 (3.44%) had Klippel-Trénaunay-Weber syndrome with hepatic vascular malformations. Furthermore, celiac

Table 1. Diagnostic Scoring System for WD

Biochemistry	Score	Clinical Symptoms and Signs	Score
Liver copper content (in the absence of cholestasis)		KF rings	
Normal (<50 μg/g of dry weight)	−1	Absent	0
<5 times ULN (50-250 μg/g of dry weight)	+1	Present	+1
>5 times ULN (>250 μg/g of dry weight)	+2	Coombs' negative hemolytic anemia	
Rhodanine stain*		Absent	0
Absent	0	Present	+1
Present	+1	Neuropsychiatric symptoms suggestive of WD	
Serum ceruloplasmin		and/or typical brain magnetic resonance imaging	
Normal (>20 mg/dL)	0	Absent	0
10-20 mg/dL	+1	Mild	+1
<10 mg/dL	+2	Severe	+2
Daily urinary copper excretion		*ATP7B* genetic analysis	
Normal	0	No mutation found	0
1-2 times ULN	+1	Mutation on one chromosome	+1
>2 times ULN	+2	Mutations on both chromosomes	+4
Normal but >5 times ULN after PCT	+2		

This table was adapted from Ferenci et al.[11] A score $\geq$ 4 indicates that disease is highly likely, a score of 2 or 3 indicates that disease is probable and further investigations are needed, and a score of 0 or 1 indicates that disease is unlikely.

*When the quantitative liver copper content was not available.

disease, chronic hepatitis C, Alagille syndrome, and sclerosing cholangitis were each present in a single case. The remaining six patients were recruited after familial screening and did not carry any mutation according to the molecular analysis of *ATP7B*.

Methods Liver function tests and other routine laboratory data were obtained with standard methods. The ceruloplasmin concentration in serum was measured by radial immunodiffusion (NOR-Partigen Coeruloplasmin, Behring, Marburg, Germany; normal range = 20-60 mg/dL).[12] Urine samples (basal urinary copper and urinary copper after PCT) were collected in an acid-washed, plastic, metal-free container. PCT urinary copper was evaluated after patients ingested 500 mg of *D*-penicillamine at time zero and again at 12 hours while 24-hour urinary copper collection progressed.[13] Copper levels in urine were determined by flame atomic absorption spectrophotometry as previously described.[14] Liver biopsy was performed by the Menghini technique with a disposable biopsy set (Hepafix, Braun, Melsungen, Germany). Copper levels in dried liver tissue were determined by flame atomic absorption spectroscopy according to Kingston and Jassie[15] (normal range = 6-50 μg/g of dry weight). All slides were examined by the same pathologist, and lesions were evaluated according to the recommendations of Batts and Ludwig.[16]

For the molecular analysis of the *ATP7B* gene, DNA extraction and polymerase chain reaction were carried out with the standard methods by Dr. Georgios Loudianos (Ospedale Regionale per le Microcitemie, Cagliari, Italy). With single-strand conformational polymorphism and sequencing methods, patients were analyzed for the 12 exons (5, 6, 8, 10, and 12-19) on which most mutations reside according to previous studies of the Italian continental population. DNA samples not completely characterized by the first step of analysis or those found to have a new missense mutation were further analyzed for the remaining exons of the *ATP7B* gene by single-strand conformational polymorphism and sequencing analysis.[17]

Statistical Analysis Continuous variables (ceruloplasmin, urinary copper, and liver copper) were presented as numbers of patients, means, medians, and standard deviations, whereas discrete variables (clinical manifestations at presentation and the presence or absence of KF rings) were presented as percentages. Normally distributed continuous variables were presented as means and standard deviations and were compared between groups by analysis of variance with post hoc testing (Scheffe's test). Continuous variables that were not normally distributed in the analyzed population were presented as medians and ranges and were compared between groups by Kruskal-Wallis analysis of variance with post hoc testing (Mann-Whitney U test).

Receiver operating characteristic (ROC) analysis was performed to determine the sensitivity and specificity with 95% confidence intervals (CIs) for the following variables at different previously proposed cutoff values: ceruloplasmin (cutoffs of 20, 14, and 10 mg/dL were considered),[18] basal 24-hour urinary copper [cutoffs of 100 (1.6 μmol/24 hours) and 40 μg/24 hours (0.6 μmol/24 hours) were considered],[2] and 24-hour urinary copper after PCT [cutoffs of

Table 2. Characteristics of 40 Children With WD

Patient	Sex	Age at Diagnosis (Months)	KF Rings	Serum Copper (μg/dL)	Ceruloplasmin (mg/dL)	Basal Urinary Copper (μg/24 Hours)	Urinary Copper After PCT (μg/24 Hours)	Hepatic Copper (μg/g of Dry Weight)	Liver Biopsy	*ATP7B* Genotype	WD Score*	WD Score†
1	Male	65	Neg	ND	16	270	ND	1129	S, F	p.H1069Q/p.H1069Q	5	5
2	Male	60	Neg	<40	3	139	517	532	S, F	p.T7858A/c.51+4A>T	6	5
3	Male	174	Neg	53	20	130	1452	390	S, C, ACH	c.2122-8T>G/p.T1288M	5	4
4	Male	73	Neg	<40	8	262	ND	1056	S, F	c.2447+5G>A/c.2447+5G>A	6	6
5	Male	58	Neg	<40	6	241	332	1048	S	c.2447+5G>A/c.2447+5G>A	6	6
6	Male	52	Neg	ND	2	40	302	1203	S, F	p.R1319X/unknown	5	4
7	Male	251	Pos	ND	2	413	1469	60	S, F	p.T1220M/unknown	7	7
8	Female	79	Neg	<40	2	119	ND	1041	S, F	p.T1220M/c.51+4A>T	6	5
9	Female	29	Neg	6	2	22	ND	1002	S, F	p.T1220M/c.51+4A>T	4	4
10	Female	16	Neg	<40	3	15	ND	ND	ND	p.P840L/p.N1270S	NA	NA
11	Male	87	Neg	<40	2	135	ND	919	S, F, ACH	p.P840L/p.N1270S	6	5
12	Female	92	Neg	64	18	108	872	714	S, F	p.H1069Q/p.R1041P	5	4
13	Male	74	Neg	<20	2	116	ND	260	S, F	p.R1319X/p.R1319X	6	5
14	Male	100	Neg	<10	2	236	ND	ND	S, F	p.S1369L/p.S1369L	NA	NA
15	Male	125	Neg	64	18.8	300	ND	300	F	p.H1069Q/H 1207 Pro	5	5
16	Male	72	Neg	50	12	228	ND	<50	S, F	p.H1069Q/p.T1220M	3	3
17	Male	36	Neg	ND	16	31	ND	ND	ND	p.H1069Q/p.T1220M	NA	NA
18	Female	80	Neg	25	2	117	969	ND	ND	p.P840L/p.N1270S	NA	NA
19	Female	78	Neg	71	15	180	378	514	S, F	p.H1069Q/unknown	5	4
20	Male	108	Neg	79	23	198	ND	750	S, F	p.H1069Q/p.H1069Q	4	3
21	Male	19	Neg	<40	6	15	580	ND	ND	c.2299insG/c.2299insG	NA	NA
22	Female	13	Neg	3	7	ND	ND	ND	ND	c.2299insG/c.2299insG	NA	NA
23	Female	48	Neg	<40	8	4	150	1060	F	No mutation found	4	4
24	Male	96	Neg	<40	5	14	402	250	F	No mutation found	5	3
25	Male	25	Neg	ND	10	ND	ND	ND	ND	p.H1069Q/p.H1069Q	NA	NA
26	Male	101	Neg	80	19	120	859	ND	ND	p.H1069Q/p.H1069Q	NA	NA
27	Female	70	Neg	<40	7	172	ND	676	S, F	ND	6	5
28	Female	84	Neg	11	3	145	1829	600	F	ND	6	5
29	Female	108	Neg	<40	4	91	281	1215	F	ND	6	4
30	Male	84	Neg	19	5	234	526	250	S	c.2447+5G>A/unknown	5	5
31	Female	72	Neg	19	20	78	630	1700	S, ACH, C	ND	4	5
32	Male	39	Neg	71	3	25	64	904	S, F	c.2304dupC/unknown	4	4
33	Male	56	Neg	<40	2	138	802	1660	S, F	c.2304dupC/unknown	6	5
34	Female	63	Neg	ND	38	66	944	785	S, F	c.3895delC/unknown	3	4
35	Male	72	Neg	<40	19	41	888	ND	ND	p.Y594X/p.G1061E	NA	NA
36	Male	251	Neg	ND	19	223	143	ND	ND	p.Y594X/p.G1061E	NA	NA
37	Male	192	Pos	<40	6	165	1646	970	S, F	p.H1069Q/p.N1270S	8	7
38	Male	90	Neg	ND	3	141	1600	1350	C	c.2532delA/unknown	6	5
39	Female	72	Neg	ND	8	157	397	1449	S	p.I899F/p.N1270S	6	5
40	Male	73	Neg	ND	18	150	1251	620	ND	p.H1069Q/p.G711E	5	4

The WD scores were calculated under the assumption of urinary copper ULNs of
*40 and
†100 μg/24 hours.
Abbreviations: ACH, active chronic hepatitis; C, cirrhosis; F, fibrosis; NA, not applicable; ND, not done; Neg, negative; Pos, positive; S, steatosis.

1575 (25 μmol/24 hours), 500 (8 μmol/24 hours), and 200 μg/24 hours (3.2 μmol/24 hours) were considered].[9,11]

Linear regression analysis was applied to assess the dependence of urinary copper excretion and liver copper contents on age, and the Pearson correlation coefficient (r) was defined.

All P values were based on two-tailed comparisons, and those less than 0.05 were considered to indicate statistical significance.

All statistical analysis was performed with GraphPad Prism 5.00 for Mac (GraphPad Software, San Diego, CA).

Results

In Figure 1, WD patients and control subjects are plot-scattered with respect to the results for each diagnostic test for WD. In Fig. 2, ROC curves for ceruloplasmin, basal 24-hour urinary copper, and 24-hour urinary copper after PCT are shown.

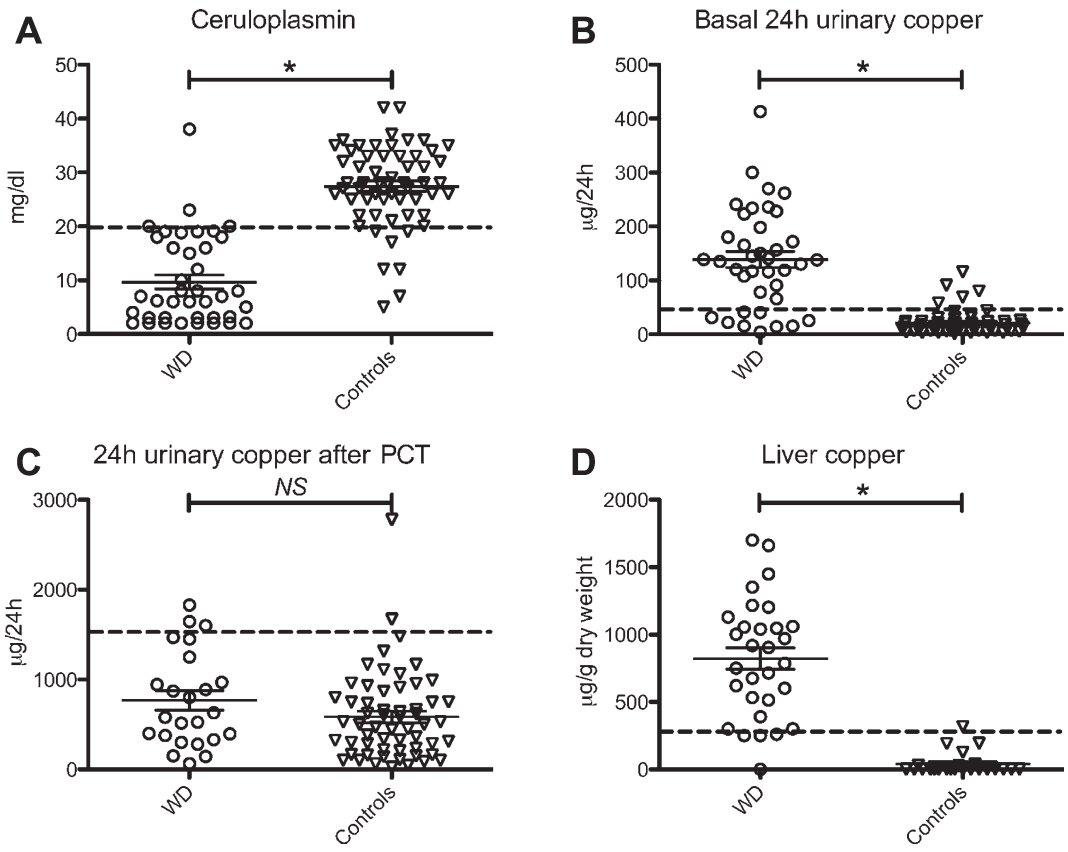

Fig. 1. Results of diagnostic tests for patients with WD and control subjects: (A) ceruloplasmin, (B) basal 24-hour urinary copper, (C) 24-hour urinary copper after PCT, and (D) liver copper. *$P < 0.0001$. The broken lines represent the best cutoff for each test. NS, not significant.

The serum ceruloplasmin concentration was significantly lower in children with WD (9.6 ± 1.3 mg/dL) versus controls (27.45 ± 0.9 mg/dL, $P < 0.0001$). Notably, only 2 of 40 WD patients (5%) had serum ceruloplasmin levels > 20 mg/dL, whereas 13 (32.5%) had values between 10 and 20 mg/dL. Among control subjects, 10 of 58 (17.24%) had ceruloplasmin levels ≤ 20 mg/dL: 4 had CDG, 3 had NAFLD, 2 were picked up by familial screening and did not carry any mutation, and 1 had congenital hepatic fibrosis. It is remarkable that all children with CDG had hypoceruloplasminemia. We performed an ROC analysis of ceruloplasmin for 40 WD patients and all 58 control subjects. The analysis suggested that the most useful cutoff value was 20 mg/dL, which had a sensitivity of 95% (95% CI = 83%-99.4%) and a specificity of 84.5% (95% CI = 72.6%-92.6%).

Basal 24-hour urinary copper excretion was significantly higher in patients with WD (138.9 ± 15.1 µg/24 hours) versus controls (20.9 ± 2.9 µg/24 hours, $P < 0.0001$). Among WD patients, 12 of 38 (31.5%) and 7 of 38 (18.4%) had basal urinary copper levels < 100 µg/24 hours and < 40 µg/24 hours, respectively. Among seven children with urinary copper levels < 40 µg/24 hours (four males and three females, median age = 3 years, range = 1.3-8), five were picked up with familial screening. In the control group, 4 of 58 patients (6.8%) had urinary copper levels ≥ 40 µg/24 hours: 2 had NAFLD, 1 had NRH, 1 had AIH type 2, and all had urinary copper levels < 100 µg/24 hours. An ROC analysis of 38 WD patients and 58 controls confirmed that a threshold of 40 µg/24 hours (sensitivity = 78.9%, 95% CI = 62.7%-90.4%) provided acceptable diagnostic accuracy in identifying WD with respect to 100 µg/24 hours for basal urinary copper (sensitivity = 65.8%, 95% CI = 48.6%-80.4%). Moreover, basal urinary copper was directly correlated with the age at diagnosis ($r = 0.58$, $P < 0.0001$) in children with WD but not in the control group.

The daily urinary copper level after PCT did not statistically differ between patients with WD (771.3 ± 103.3 µg/24 hours) and controls (585.5 ± 63.8 µg/24 hours, $P = 0.69$). Among WD patients, only 3 of 25 (12%) presented values > 1575 µg/24 hours: all of them had fibrosis at liver biopsy and basal copper excretion > 100 µg/24 hours. Among controls, 3 of 58 (5.2%) had PCT cupriuria > 1575 µg/24 hours, and they presented with NASH, NRH, or AIH type

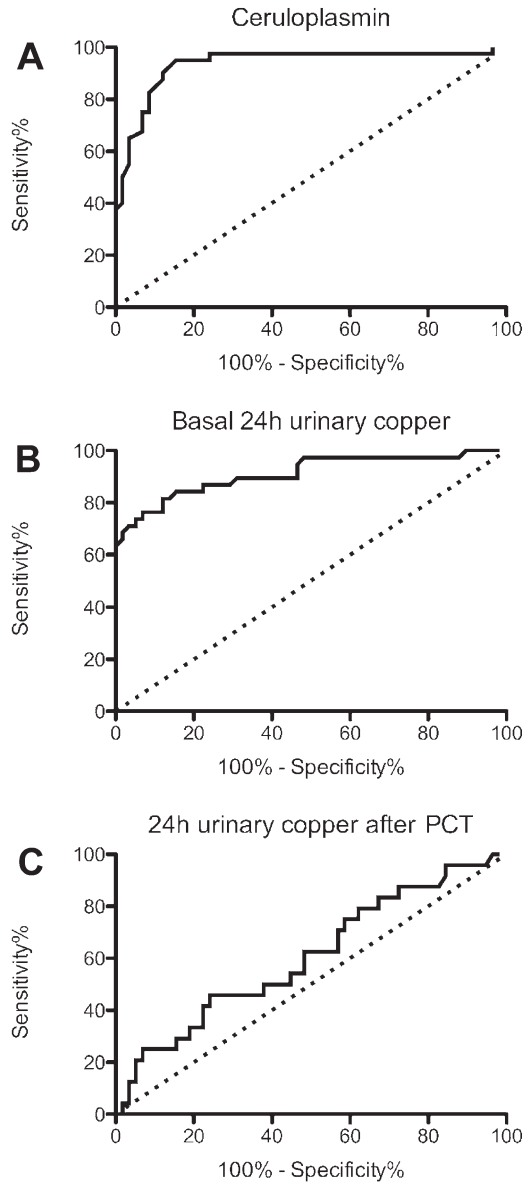

Fig. 2. ROC curves for different diagnostic tests for WD: (A) ceruloplasmin (area under the curve = 0.94, 95% CI = 0.88-0.99, $P < 0.0001$), (B) basal 24-hour urinary copper (area under the curve = 0.91, 95% CI = 0.85-0.97, $P < 0.0001$), and (C) 24-hour urinary copper after PCT (area under the curve = 0.61, 95% CI = 0.48-0.74, $P < 0.10$). The broken lines represent the identity.

1. The ROC analysis (area under the curve = 0.61, $P = 0.10$) of 25 WD patients and 58 controls showed that at the cutoff value of 1575 μg/24 hours, the sensitivity was only 12% (95% CI = 2.5%-31.2%); it was raised to 64% (95% CI = 42.5%-82%) and 88% (95% CI = 68.8%-97.4%) only when the threshold was lowered to >500 μg/24 hours and >200 μg/24 hours, respectively.

Liver copper levels were measured in 30 WD patients and 24 control subjects and significantly differed between the two groups (813.6 ± 81.7 versus 38.4 ± 17 μg/g of dry weight, $P < 0.0001$). Only 2 of 30 WD patients (7%) had a liver copper level < 75 μg/g of dry weight, which has been proposed as a novel diagnostic threshold[19]; the remaining 28 had values > 250 μg/g of dry weight. Liver copper levels in WD patients did not directly correlate with the severity of the histological picture (data not shown) or the age at liver biopsy ($r = 0.38$, $P = 0.03$). Among controls, 4 of 24 (6%) had liver copper levels > 50 μg/g of dry weight; 2 had CDG (318 and 250 μg/g of dry weight, respectively), 1 had NRH, and 1 had cryptogenic liver disease. The two patients affected by CDG also had low ceruloplasmin levels.

The sensitivity and specificity of ceruloplasmin, basal 24-hour urinary copper, and 24-hour urinary copper after PCT at different thresholds are summarized in Table 3.

An evaluation of all items of the WD scoring system proposed by Ferenci et al.[11] was possible in 30 patients with WD and in 24 control subjects. When the considered cutoff value for basal urinary copper was 40 μg/24 hours, only two patients with WD scored less than 4; when the cutoff value was 100 μg/24 hours, three patients did. Only two control subjects, both of whom had CDG, had a score of 4 regardless of the considered cutoff value (Fig. 3). When we considered 40 μg/24 hours instead of 100 μg/24 hours as the urinary copper ULN, the scoring system had the best diagnostic accuracy: a sensitivity of 93% versus 90%, a specificity of 91.6% versus 91.6%, a positive predictive value of 93% versus 93.1%, and a negative predictive value of 91.6% versus 88%.

It is remarkable that all the patients with WD were positive for at least ceruloplasmin or basal urinary copper excretion.

In Table 4, the characteristics of CDG and NRH patients (included in the control group) are summarized. These patients intriguingly shared some biochemical features with WD patients.

It is noteworthy that WD patients 23 and 24 (Table 2) were siblings who showed features very similar to

Table 3. Accuracy of Conventional Diagnostic Criteria for WD at Different Thresholds

Test	Cutoff	Sensitivity (95% CI)	Specificity (95% CI)
Ceruloplasmin (mg/dL)	<10	65% (48.3%-79.4%)	96.5% (88.1%-99.6%)
	<14	70% (53.5%-83.4%)	93.1% (83.3%-98.1%)
	<18	80% (64.3%-90.9%)	91.4% (81%-97.1%)
	<20	95% (83%-99.4%)	84.5% (72.6%-92.6%)
Basal 24-hour urinary copper (μg/24 hours)	>100	65.8% (48.6%-80.4%)	98.3% (90.8%-99.9%)
	>40	78.9% (62.7%-90.4%)	87.9% (76.7%-95%)
24-hour urinary copper after PCT (μg/24 hours)	>1575	12% (2.5%-31.2%)	96.5% (88.1%-99.6%)
	>500	64% (42.5%-82%)	51.7% (38.2%-65%)
	>200	88% (68.8%-97.4%)	24.1% (13.9%-37.2%)

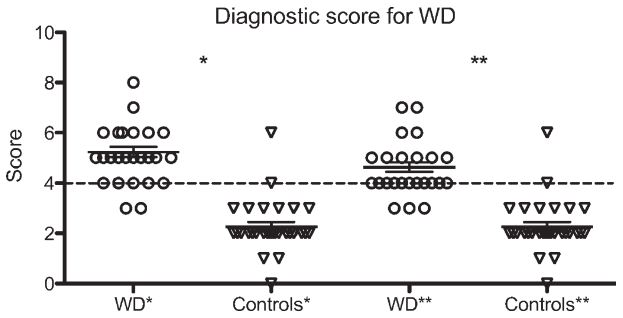

Fig. 3. WD diagnostic scores for WD patients and control subjects. The broken line represents the score of certain diagnosis. The scores were calculated under the assumption of basal urinary copper ULNs of *40 and **100 μg/24 hours.

those of CDG patients included in the control group, but in both CDG was excluded on the basis of a normal transferrin isoelectric focusing profile. Their serum aminotransferase levels normalized after 20 or 4 months of penicillamine treatment.

Discussion

The features of our series are remarkably different from those of other pediatric reports, which in most cases have included WD children with either acute or chronic symptomatic liver disease or liver failure.[3,6-9,13] In fact, all the WD patients evaluated in the present study were referred for raised aminotransferases and could be considered asymptomatic or presymptomatic. Therefore, this population represents a valuable specimen for assessing the appropriateness of the WD diagnostic criteria in children with mild liver disease. The present study has highlighted different peculiarities of these patients with respect to WD children reported elsewhere.[6-9,13]

The measurement of ceruloplasmin serum levels is also a first-step test for the diagnosis of WD in children with mild liver disease, as demonstrated by the good sensitivity and acceptable specificity of this test at the cutoff of 20 mg/dL in the studied population. Obviously, low levels of ceruloplasmin are not always indicative of a copper storage disorder because both heterozygotes for WD and patients with other disorders may share this feature.[20-23] Furthermore, as reported elsewhere, ceruloplasmin serum levels are also influenced by the *ATP7B* genotype.[24,25]

As for basal daily urinary copper excretion, on the basis of our results, the diagnosis of WD should be considered when this test produces a value $>$ 40 μg/24 hours. This cutoff value has also been recently stressed by AASLD guidelines,[2] although its diagnostic

Table 4. Characteristics of Control Patients With CDG and NRH

Case	Sex	Age at Diagnosis (Months)	KF Rings	Serum Copper (μg/dL)	Ceruloplasmin (mg/dL)	Basal Urinary Copper (μg/24 Hours)	Urinary Copper After PCT (μg/24 Hours)	Hepatic Copper (μg/g of Dry Weight)	Laboratory Abnormalities	Liver Biopsy	*ATP7B* Genotype	WD Score*	WD Score†
CDG	Female	40	Neg	30	6	11	173	318	Total cholesterol, 240 mg/dL (reference, <200 mg/dL); creatine kinase, 200 U/L (reference, 30-180 U/L)	S, F	Neg	4	4
CDG	Male	24	Neg	50	17	20	98	<50	INR, 1.54; PTT, 50 seconds; A1AT serum level, 0.76 g/L (reference, 0.9-2.0 g/L)	S, ACH	Neg	1	1
CDG	Male	18	Neg	77	19	7	190	<50	INR, 1.58; PTT, 44 seconds; A1AT serum level, 0.8 g/L (reference, 0.9-2.0 g/L); haptoglobin, 0.3 g/L (reference, 0.5-3.1 g/L)	S	Neg	1	1
CDG	Male	28	Neg	35	4	15	75	250	Total cholesterol, 220 mg/dL (reference, <200 mg/dL); creatine kinase, 210 U/L (reference, 30-180)	S, F	Neg	4	4
NRH	Male	96	Neg	11	21	91	1479	ND	Platelets, 70 $\times$ 10^9/L	NRH	Neg	1	2
NRH	Male	108	Neg	105	31	30	1315	194	–	NRH	Neg	3	3
NRH	Male	60	Neg	121	25	24	1666	ND	–	NRH	ND	2	2
NRH	Male	156	Neg	100	23	7	104	ND	–	NRH	ND	0	0
NRH	Male	192	Neg	90	22	20	1165	ND	–	NRH	ND	2	2

The WD scores were calculated under the assumption of urinary copper ULNs of
**40 and
†100 μg/24 hours.

Abbreviations: A1AT, alpha-1-antitrypsin; ACH, active chronic hepatitis; F, fibrosis; INR, international normalized ratio; Neg, negative; ND, not done; PTT, partial thromboplastin time; S, steatosis.

accuracy has not yet been defined. There is only one report describing a sensitivity of 68% at the cutoff value of 40 μg/24 hours in an adult population.[26] Among the adult series, the sensitivity of basal urinary copper excretion at the cutoff value of 100 μg/24 hours is 59% to 88%.[7,26,27] As for the pediatric series, urinary copper levels have exceeded 100 μg/24 hours in 81% to 94% of cases.[5,9,28] In symptomatic and asymptomatic children, the sensitivity for basal cupriuria at the cutoff value of 63.5 μg/24 hours is approximately 95% and 70%, respectively.[3,9] No data are available about the specificity of this test because the cutoff value of 40 μg/24 hours has never been evaluated; our results suggest that this is the optimal threshold both as a single test and in the context of the WD scoring system in children with mild liver disease suspected of having WD. The fact that urinary copper levels are lower in very young children suggests an accumulation of the metal over time. However, it is not possible to exclude the idea that difficulties in daily urine collection in this age group may have played a role, even if in our population the parents underwent appropriate training and the daily urine volume was consistent with the age and weight.

Current recommendations by the AASLD conclude that PCT may be performed in symptomatic children if a diagnosis of WD is suspected but basal urinary copper excretion is normal.[2] Data about PCT sensitivity at the cutoff value of 1575 μg/24 hours are very heterogeneous; the sensitivity ranges from 69% to 88% in children with active liver disease and from 46% to 56% in asymptomatic siblings.[3,9] There is only one report showing a specificity of 93% at the proposed cutoff of 1575 μg/24 hours.[9] In this study, however, the group of asymptomatic children was small (only 13 patients) and was not well characterized with respect to liver enzymes. Our study provides further and stronger evidence that PCT should not be performed in children without symptomatic liver disease regardless of the presence of neurological symptoms. In our series, only patients with more severe liver damage according to a histological examination had a positive PCT in both the WD and control groups, and this suggests that copper excretion is influenced by the severity of the liver injury. Moreover, the suggestion of applying to children with basal urinary copper levels $<$ 100 μg/24 hours a test with a cutoff value established in a population of children with basal urinary copper levels $>$ 100 μg/24 hours[9,13] is controversial.

The present study has shown that CDG can mimic WD-related liver disease because patients with this disorder may present low serum levels of ceruloplasmin. A correct differential diagnosis of WD versus CDG may be challenging if we consider that the CDG patients included in our control group presented with an isolated liver disease in the absence of the typical CDG phenotype characterized by severe neurological involvement, dysmorphisms, and multiorgan impairment. In these patients, further investigations for CDG were triggered only by the presence of a mild coagulopathy not explained by the liver disease. It is noteworthy that this novel CDG phenotype with prevalent liver involvement has been recently recognized and characterized in asymptomatic children and young adults with cryptogenic elevated aminotransferases and/or liver steatosis with fibrosis.[23,29] The CDG patients, who were included in the control group and were diagnosed as being affected by a new phenotype called CDG-X, shared with the WD patients low levels of ceruloplasmin and high levels of liver copper, but in all of them, WD was ruled out because of negative *ATP7B* gene sequencing and spread haplotype analysis, low urinary copper excretion (both at the baseline and after PCT), and the absence of typical mitochondrial changes according to an electron microscopy examination. Tightly normal urinary copper excretion in these patients could be taken into account as an affordable tool for distinguishing them from patients with WD. We can hypothesize that this phenotype results from a disturbed redistribution of copper out of the liver via ceruloplasmin because of the disturbed biosynthesis of this glycoprotein in CDG, as observed in aceruloplasminemia. In aceruloplasminemic mice, the liver copper content is augmented, but normal copper absorption, transport, distribution, and excretion are observed.[30]

Furthermore, in our study, we found that patients with NRH of the liver also shared some features with WD patients. NRH is an uncommon benign condition characterized by diffuse transformation of the normal hepatic parenchyma into small, regenerative nodules without fibrosis; we found it to be associated with high copper urine excretion after PCT, but the latter finding is difficult to interpret. Records of CDG and NRH patients are displayed in Table 4.

Unlike urinary copper excretion, which was confirmed to be age-related as previously reported by our group,[24] the liver copper concentration did not seem to be influenced in the present study by the age of the patients, as documented by Ferenci et al.[19] This discrepancy remains unexplained. Studies of animal models, such as Rauch's toxic milk mice, Jackson's toxic milk mice, and Long-Evans Cinnamon rats, are likely to contribute to the clarification of the mechanism affecting the accumulation of copper in the liver over time. In these animals, with naturally occurring mutations in their WD homologue *Atp7b*, the copper

concentration in the liver increased with age in early life and then remained fairly constant during the progression of liver disease.[31-33] However, the results obtained from a rodent model of WD are not necessarily representative of the human mechanism of copper accumulation.

In conclusion, establishing the diagnosis of WD is problematic in children with mild liver disease. The 24-hour urinary copper excretion is highly informative when 40 μg/24 hours is considered the ULN. The WD scoring system proposed by Ferenci et al.[11] may be a reliable tool in this subset of patients if this limit is used for evaluating the 24-hour urinary copper excretion. PCT is of little value for diagnosis in these patients. Other rare diseases may display low ceruloplasmin levels and even elevated hepatic parenchymal copper levels; a genetic diagnosis remains critical for such patients.

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