Correlation Factors and a Predictive Model of Serum Ceruloplasmin Normalized Wilson's Disease

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Research

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Abstract

Background: The false increase of ceruloplasmin (Cp) in some Wilson's disease (WD) patients, which overlaps with those in non-WD liver disease patients, decrease the diagnostic accuracy. The aims of our study was to understand the factors affecting WD patients' Cp normalization, and develop a model using routine predictors to identify WD patients with ambiguous serum Cp.

Results: The mixed effects model analysis which executed in longitudinal study revealed that the WD patients' Cp normalization were significantly associated with the copper burden and liver function indexes, like urinary copper treated with dimercaptopropansulfonate sodium (*P*=0.000), aspartate aminotransferase (*P*=0.011), γ-glutamyltransferase (*P*=0.000), albumin (*P*=0.000). Multivariate logistic regression analysis in case-control study showed age (P=0.000) and serum creatine (*P*=0.000) were independent risk factors associated with WD. Based on their regression coefficients, a simplified WD index was derived: *0.001 × age [yr] × Creatine [umol/L]*. The AUC value of WD index in total cohort were 0.923 (*P*=0.000). At a WD index cutoff value of ≤ 1.9 and ≤ 2.5, the positive and negtive predictive value are 88.2% and 89.9% for WD, respectively.

Conclusions: The increase of serum Cp in WD patients is related to their excessive copper burden and hepatic injury, common tests can effectively foretell those WD patients with nearly normal serum Cp from other liver injury patients.

Background

Wilson's Disease (WD) is an autosomal recessive copper metabolism disease caused by mutations in the ATP7B gene which codes for a copper-transporting P-type ATPase to convey copper for synthesizing ceruloplasmin (Cp).[1,2] The decrease of serum Cp concentration is a useful diagnostic hallmark of WD.[2] An epidemiological investigation revealed that the incidence and prevalence of WD in China were approximately 1.96/100,000 and 5.87/100,000 respectively.[3] It is estimated that 50% of patients with active Wilson's liver disease and 15-36% of child with WD may have Cp levels in the low normal range.[4–6] Serum Cp is mainly produced by hepatocytes, in addition, cells in several other secretory organs, like kidney, mammary gland, placenta and the choroid plexus of brain, also produce Cp.[7,8] As well as, macrophages and mononuclear cells in the blood during inflammation also have the above function.[9,10] So, the active liver disease, pregnancy, inflammation will push the Cp to increase, on the contrary, decrease in the kidney failure and end-stage liver disease and so forth. The normal concentration of Cp measured by the enzymatic assay is with a lower limit 200 mg/L.[6] The diagnostic accuracy for WD using a cutoff value of 140 mg/L had 100% positive predictive values and 97.1% negative predictive values[11]. In WD, it is usually lower than 100mg/L, especially in neurologic WD. But it may be in the low normal range in hepatic WD with active liver disease,[12–14] and correlated with liver histologic activity.[15] Because these WD patients' manifestations and serum Cp overlap with those of other liver diseases, their diagnosis may be missed.

Some laboratory findings and index are proved to be helpful in identifying the WD with acute liver failure (ALF) from non-WD ALF, including increased aspartate aminotransferase:alanine aminotransferase (AST:ALT) ratios and low serum alkaline phosphatase (AP) activity, a decreased AP to total bilirubin (TB) ratio.[13,16,17] But, in the non-ALF WD patients with nearly normal Cp, how to use easily available indicators to identify them from other hepatopathy patients has not yet been studied.

Here, we retrospectively enrolled 86 WD paitents who had nearly normal serum Cp levels (≥140 mg/L), analyzed their multiple consecutive medical records, in addition, compared to control group, according to logistic regression analysis, and then developed a simple model, to differentiate WD from hepatopathy patients with normal Cp too.

Methods

WD patients cohort

We retrospectively investigated 1032 WD patients who were firstly hospitalized in the affiliated hospital of the neurology institute of Anhui University of Chinese Medicine (Hefei city, eastern China) from March 2010 to July 2013. Enrolled criteria included (1) meeting Leipzig criteria for WD diagnosis;[16,26] (2) serum Cp concentration were ≥140 mg/L;[11] (3) less than 65 years old. The following cases were excluded: (1) pregnancy, liver[27] or renal failure,[28] and chronic kidney disease, infection, cancer and abusing alcohol; (2) other suspicious hepatitis such as viral or autoimmune hepatitis and drug-induced liver ingury; (3) incomplete medical records.

Finally, 86 WD patients were enrolled in this study. We followed up all of their electronic medical information as far as December 2019, and got their 352 records. In the light of the serum Cp concentration, their 352 medical records were classified as group A1 (Cp ≥140 mg/L, n=231) and group A2 (Cp <140 mg/L, n=121), no matter which patient they belong to. The flow chart is shown in Fig. 1.

Their usual informations and laboratory results were taken notes, including sex, age, duration of onset and follow-up, phenotype, Kayser-Fleischer ring (K-F ring), laboratory biochemical findings, serum Cu and Cp, serum ceruloplasmin oxidase (Sco), 24-hour urinary copper, Child-Pugh scores and classification, abdominal ultrasonographic or radiological findings, liver cirrhosis or not and mutational sites of ATP7B gene. Serum copper was determined by using flame atomic absorption spectrophotometry.[29] Serum Cp was measured by immuno-nephelometric assay with anti-serum to human Cp.[19] Sco activity with o-dianisidine dihydrochloride as substrate was determined by spectrophotometry.[19,30] The basic 24-hour urinary copper and with intravenous infusion of dimercaptopropansulfonate sodium (DMPS) or oral penicillamine were recorded.

Case-control cohort of non-WD liver disease patients

The control group patients came from the liver diseases department of the first affiliated hospital, Zhejiang University School of Medicine, in March 2021. Those patients were included if they met the

following criteria: (1) identifiable acute liver diseases and compensated or decompensated liver cirrhosis (LC) due to varied non-WD causes; (2) serum Cp concentration were ≥140 mg/L; (3) <65 years old. Exclusion criteria included pregnancy, liver or renal failure and chronic kidney disease, infection and cancer. Finally, 98 liver injury patients were included this control cohort, involving 23 nonalcoholic steatosis heptitis (NASH), 27 acute hepatitis (hepatitis B: 11, hepatitis E: 7, medicamentous: 5, autoimmune hepatitis: 2, Epstein-Barr virus infection and hyperthyreosis: 1 for each), 4 compensated LC induced by hepatitis virus B (HBV), and 44 decompensated LC (induced by CHB: 38, alcohol: 2, CHB+alcohol, medicamentous, primary sclerosing cholangitis and Budd-Chiari syndrome: 1 for each).

Statistical analysis

All statistical analyses were perfomed with SPSS version 21.0 (IBM, New York, USA). The chi-squared and non-parameters test, t-test and ANOVA were carried out respectively depending on different data types. Generalized linear mixed effect model (GLMM) was conducted on repeated measurement data (group A1 vs. A2) to seek the different indicators related to increase of Cp in two groups. WD and non-WD case-control cohort (group A vs. B) were divided into training and validation sets for conducting logistic regression analysis to develop a model and to validate it. Factors revealed significant on univariate analysis (P<0.5) were then involved in the multiple binary logistic regression analysis with backward method to find independent factors associated with WD. According to the independent factors of the final logistic regression model and the proportions of the corresponding regression coefficients, we derived a simplified formula. The area under the receiver operating characteristic curves (AUC) was used to evaluate the model's diagnostic value. The sensitivity, specificity, and positive predictive value (PPV), negative predictive value (NPV) of the model were calculated to determine the optimal and handy cutoff value.

Results

Baseline characteristics and laboratory findings of 86 WD patients

The baseline characteristics and laboratory findings of enrolled 86 WD patients (male:42, 48.8%) at the first admission are shown in Table 1. There were three phenotypes: H type (n=51, 59.3%), H-N type (n=27, 31.4%) and N type (n=8, 9.3%). All patients did not convert to other types during follow-up period. Comparing three phenotypes, there were no difference at age at presentation (P=0.091), but the age at diagnosis especially varied between them (P=0.005). So, the N type had the longest undiagnosed duration, duration of onset, 12.5±10.6 (mean±standard deviation, the same below) years (P=0.010), which is the time from onset to diagnosis. In addition, the duration and times of follow-up was 4.9 (1.8, 7.1) (median, quantile, the same below) years and 4 (2, 6) times, varying in 0.5-9.8 years and 1-10 times. 28 of their serum Cp concentration were ≥200 mg/L whose distrbution in each group had no different (P=0.784).

Table 1
Baseline characteristics of 86 WD patients with different phenotypes tested in the first hospitalization.

Variables	ALL	Heptic type	H-N† type	Neurologic type	*P*
Patients (n)	86	51	27	8	
Cp≥200mg/L (n, %)	28 (32.6%)	18 (35.3%)	8 (29.6%)	2 (25.0%)	0.784
Sex (male, %)	42 (48.8%)	27 (52.9%)	11 (40.7%)	4 (50%)	0.590
Age at presentation	18.8 (10.8, 28.3)	14.4 (8.0, 23.0)	22.3±9.3	22.5±11.4	0.091
Age at diagnosis	25.1±12.9	21.7±13.0	28.6±9.5	35.0±14.7	0.005
Duration of onset ‡ (years)	2.0 (0.5, 9.3)	1.0 (0.4, 6.2)	3.0 (1.0, 11.1)	12.5±10.6	0.010
Duration of follow-up (years)	4.9 (1.8, 7.1)	4.1 (1.5, 6.9)	5.9 (3.6, 7.5)	3.9±2.6	0.242
Rang (years)	0-9.8	0-9.8	0-8.2	0-7.0	N
Total records	352	194	126	32	N
Times per patient	4 (2, 6)	3 (2, 5)	5 (2, 7)	4.0±2.3	0.460
K-F ring (+, %)	67 (77.9%)	33 (64.7%)	27 (100%)	7 (87.5%)	0.001
Serum Cu (umol/L)	8.6±3.6	8.6±3.7	8.8±3.7	8.0±2.2	0.852
Serum Cp (mg/L)	180.9 (158.4, 214.8)	186.1 (163.6, 215.2)	174.2 (162.0, 209.1)	153.3 (146.1, 212.1)	0.200
Sco (IU/L)	0.211 (0.160, 0.322)	0.211 (0.174, 0.345)	0.223±0.104	0.236±0.110	0.524
Basic urinary copper (ug/24h)	170.7 (113.7, 408.3)	155.0 (108.9-479.8)	176.4 (126.1, 429.7)	214.1±117.6	0.826
Urinary copper with DMPS (ug/24h)	2094.6 (1335.4, 3673.1)	1850.3 (1267.5, 4012.5)	2234.3 (1678.4, 3626.2)	1916.6±1015.9	0.399
Complete blood count					
NE ($\times10^9$/L)	2.4 (1.5, 3.2)	2.2 (1.6, 2.9)	2.3±1.1	3.5±1.2	0.059
PLT ($\times10^9$/L)	134.5 (81.3, 233.8)	140.0 (79.0, 268.0)	117.0±52.7	199.4±88.1	0.056
RDW (fL)	45.3±7.0	45.5±7.9	45.7±5.8	42.8±2.7	0.551
ALT (IU/L)	43.0 (24.0, 76.8)	60.0 (32.0, 135.0)	28.0 (21.0, 45.0)	36.3±29.6	0.000

Variables	ALL	Heptic type	H-N† type	Neurologic type	*P*
AST (IU/L)	42.5 (23.8, 71.0)	58.0 (29.0, 84.0)	29.0 (20.0, 39.0)	44.5 (18.0, 70.8)	0.001
γ-GT (IU/L)	50.5 (27.0, 108.8)	66.5 (29.0, 151.5)	40.0 (27.0, 91.5)	37.0±26.6	0.025
AP (IU/L)	118.0 (82.0, 241.5)	146.0 (87.3, 303.5)	113.8±56.7	88.0 (85.5, 116.8)	0.030
CHE (IU/L)	5088.4±2536.6	5206.0 (2170.0, 8229.0)	5357.5 (3164.3, 5900.5)	7032.5 (4101.0, 7622.5)	0.277
Albumin (g/L)	40.2 (32.1, 43.7)	37.8±8.5	38.7±6.5	41.8±3.4	0.394
Total bilirubin (umol/L)	17.0 (11.0, 32.0)	22.3 (9.4, 41.3)	17.3 (13.0, 26.3)	14.1±5.6	0.433
Cr (umol/L)	47.2±18.6	44.3±18.6	46.8 (39.2, 54.1)	57.4±19.6	0.173
CHO (mmol/L)	4.2±1.2	4.1±1.0	3.8 (3.4, 4.4)	4.8±0.7	0.054
PT (s)	13.6 (12.7, 16.7)	14.1 (12.6, 18.9)	13.5 (13.0, 16.4)	12.3±1.0	0.021
INR	1.1 (1.0, 1.4)	1.1 (1.0, 1.5)	1.1 (1.1, 1.4)	1.0±0.1	0.072
Liver cirrhosis (n, %)	57 (66.3%)	30 (58.8%)	27 (100%)	0 (0)	0.000
Died (n, %)	6 (7.0%)	3 (5.9%)	3 (11.1%)	0 (0)	0.495
Child-Pugh scores	5 (5, 9)	5 (5, 9)	5 (5, 7)	5 (5, 5)	0.051
class A (n)	59	31	20	8	0.116
class B (n)	13	8	5	0	
class C (n)	14	12	2	0	

† Hepatic and Neurologic presentations; ‡ From the time of onset to the first hospitalization. The normal distribution parameters are shown as mean±standard deviation, or else median and quantile (25%, 75%).

K-F ring, Kayser-Fleischer ring; Cp, ceruloplasmin; Sco, serum ceruloplasmin oxidase; DMPS, dimercaptopropansulfonate sodium; WBC, white blood cell; PLT, platelet; RDW, red cell distribution width; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GT, γ-glutamyltransferase; AP, alkaline phosphatase; CHE, cholinesterase; Cr, creatine; CHO, cholesterol; PT, prothrombin time; INR, international normalised ratio.

The K-F ring postive rate was the highest in H-N type patients (100%), followed by N type (87.5%) and H type (77.9%) (*P*=0.001). Similarly, liver metabolic indicators of H type, like ALT, AST, γ-glutamyltransferase (γ-GT) and prothrombin time (PT), are significantly higher than those of H-N type and N type (*P*=0.000, 0.001, 0.025, 0.021). Overall, 57 (66.3%) patients complicated with LC, of which 6 (7.0%) patients died due to complications of LC during follow-up, involving liver failure, upper gastrointestinal bleeding, hepatic encephalopathy, infection and so on. Most of the other biochemical results in three groups had no significant differences, for example serum Cp, Sco and Cu, urinary copper, white blood cell and platelet (PLT), TB and creatine (Cr), and so forth (Table 1).

Mutations analysis of *ATP7B* gene

There were 64 patients (H: 43, H-N: 16, N: 5) having ATP7B gene sequencing results. We found 115 mutations (107 missense, 5 frameshift, 3 splicing-site) of 47 sites in 16 exons (Fig. 2), which had 6 homozygous, 43 compound heterozygous, 13 heterozygous mutations and 2 negative. Four patients were found having three variants. The mutation hotspots were p.R778L (exon 8), P992L (exon 13) and p.R919G (exon 12) (Fig. S1, Table S1). The mutation hotspots of the patients whose serum Cp concentrations were always higher than 140mg/L (H: 22, H-N: 5, N: 1, data not show) or fluctuated around 140mg/L (H: 19, H-N: 11, N: 4, data not show) had no obviously different (*P*=0.750, 0.332) (Table S1).

Factors associated with Cp change in WD cohort

Follow-up data of 86 WD patients were grouped according to serum Cp levels (group A1 vs. A2, Fig. 1) in order to identify factors associated with CP changes.

Clinical and Biochemical Findings of group A1 and A2 are shown in Table 2. During follow-up, the serum Cp fluctuated in most patients according to the boundary value ≥140mg/L: from high to low and back or not (n=22, 23. Data not shown), always at a high level (n=32), and only once record (n=12). Linear regression analysis showed that serum copper, SCO and CP, in 352 medical records, have an obvious linear correlation, R^2= 0.769 and 0.775 (Fig. S2). The 24-hour urinary copper which were basic and treated with DMPS, were significantly higher in group A1 (both *P*=0.000), 137.2 (95.8, 213.1) and 1534.7 (1054.1, 2265.3), than in group A2, 112.5 (82.7, 147.9) and 983.7 (523.7, 1618.9). Group A1 significantly differ from group A2 in items like AST (*P*=0.011), γ-GT (*P*=0.000), albumin (ALB) (*P*=0.000), cholesterol (*P*=0.017).

Table 2
Clinical and biochemical findings in 352 hospitalization records of 86 WD patients.

Variables	Group A1	Group A2	*P*†
Records (n)	231	121	
Patients (n)	86	43	
Heptic type	51	23	
H-N type	27	16	
Neurologic type	8	4	
Age	27.0 (18.0, 39.0)	25.0 (16.0, 36.0)	0.362
Serum Cu (umol/L)	8.2 (6.2, 10.2)	3.5±1.6	0.000
Serum Cp (mg/L)	186.2 (166.8, 218.4)	94.2±26.9	0.000
Sco (IU/L)	0.219 (0.173, 0.336)	0.085 (0.058, 0.115)	0.000
Basic Urinary copper (ug/24h)	137.2 (95.8, 213.1)	112.5 (82.7, 147.9)	0.014
Urinary copper with DMPS (ug/24h)	1534.7 (1054.1, 2265.3)	983.7 (523.7, 1618.9)	0.000
Complete blood count			
WBC	5.0 (4.1, 5.9)	5.0±1.5	0.272
PLT ($\times 10^9$/L)	169.0 (111.0, 240.0)	186.1±87.1	0.362
RDW (fL)	42.2 (39.8, 46.4)	43.0 (40.5, 45.7)	0.995
ALT (IU/L)	36.0 (22.0, 55.0)	30.0 (19.0, 46.0)	0.189
AST (IU/L)	31.0 (22.0, 48.0)	26.0 (21.0, 32.5)	0.011
γ-GT (IU/L)	38.0 (23.5, 73.5)	22.0 (17.0, 43.0)	0.000
AP (IU/L)	112.0 (79.0, 198.0)	102.5 (71.8, 184.0)	0.380
CHE (IU/L)	5734.8±2334.1	5880.8±1439.5	0.717
Albumin (g/L)	42.1 (36.5, 45.0)	43.9 (40.7, 47.5)	0.000
Total bilirubin (umol/L)	13.6 (9.4, 23.3)	14.6 (10.5, 19.7)	0.793
Cr (umol/L)	49.7 (40.3, 63.0)	53.0 (42.4, 61.2)	0.728
CHO (mmol/L)	4.3±1.1	4.1±0.9	0.017
PT (s)	12.7 (11.7, 14.2)	12.3 (11.9, 13.6)	0.323
INR	1.1 (1.0, 1.2)	1.0 (1.0, 1.1)	0.228

Variables	Group A1	Group A2	*P*†
Liver cirrhosis	141 (61.0%)	64 (52.9%)	0.133
Child-Pugh scores	5.0 (5.0, 6.0)	5.0 (5.0, 5.0)	0.997
class A (n)	186	115	1.000‡
class B&C (n)	45	6	

† Univariate generalized linear mixed models analysis. ‡ Child-Pugh class B and C were merged compared to class A due to the limit of mixed effect model. The normal distribution parameters are shown as mean±standard deviation, or else median and quantile (25%, 75%).

H-N, hepatic-neurologic. Cp, ceruloplasmin. Sco, serum ceruloplasmin oxidase. DMPS, dimercaptopropansulfonate sodium. WBC, white blood cell. PLT, platelet. RDW, red cell distribution width. ALT, alanine aminotransferase. AST, aspartate aminotransferase. γ-GT, γ-glutamyltransferase. AP, alkaline phosphatase. CHE, cholinesterase. CB, conjugated bilirubin. Cr, creatine. CHO, cholesterol. TG, triglycerides. PT, prothrombin time. INR, international normalised ratio.

Factors associated with WD and develop a simplified model

The characteristics of the training and validation sets of group A and B are listed in Table 3 and Table S2 respectively. In the training set, the serum Cp level was significant lower than control group (*P*=0.002). Most of the liver metabolic indexes of two groups had no significant difference, according to each factor of univariate binary logistic regression analysis, like ALT, AST, γ-GT, cholinesterase (CHE), ALB, TB, PT, INR, Child-Pugh scores and proportion of LC or Child-Pugh classification B&C (*P*=0.075-0.748). While the age (*P*=0.000), AP (*P*=0.040), serum Cr (*P*=0.000) and cholesterol (Cho, *P*=0.001) revealed major divergence between two group. Multivariate binary logistic regression analysis identified 2 variables as independent predictors of WD: age and serum Cr (Table 4) with an AUC of 0.950 (95% CI: 0.907-0.993, *P*=0.000) in the training set and 0.903 (95% CI: 0.837-0.970, *P*=0.000) in the validation set respectively (Total cohort: 0.926, 95% CI: 0.877-0.965, *P*=0.000) (Fig. 2A).

Based on the relationship of the 2 regression coefficients of the model and ease of use, the following simplified formula (WD index) was derived:

WD index = 0.001 × age [yr] × Cr [umol/L].

Table 3 Baseline characteristics of WD patients in training sets of group A and B.

Variables	Group A	Group B	$P^{\#}$
N	43	49	
Sex (male, %)	25 (58.1%)	38 (77.6%)	0.048
Age	25.3±12.8	50.0 (38.6, 53.5)	0.000
Serum Cp (mg/L)	180.3 (152.4, 215.2)	245.5±75.1	0.002
Complete blood count			
WBC ($\times 10^9$/L)	4.8±2.0	4.2±2.0	0.089
PLT ($\times 10^9$/L)	177.7±104.2	103.0 (54.0, 189.0)	0.026
ALT (IU/L)	45.0 (22.0, 96.0)	86.0 (20.5, 183.0)	0.210
AST (IU/L)	46.0 (22.0, 70.0)	44.0 (27.5, 101.0)	0.606
γ-GT (IU/L)	54.0 (29.0, 99.0)	68.0 (30.0, 167.5)	0.370
AP (IU/L)	112.5 (84.3, 246.0)	92.0 (77.5, 144.0)	0.040
CHE (IU/L)	5372.1±2551.2	4488.0 (3151.5, 6931.5)	0.806
Albumin (g/L)	39.3±8.2	36.3±8.2	0.091
Total bilirubin (umol/L)	16.4 (10.9, 26.3)	17.3 (12.0, 45.1)	0.075
Cr (umol/L)	46.7±18.5	71.1±18.2	0.000
CHO (mmol/L)	4.3±0.9	3.5±1.2	0.001
PT (s)	13.4 (12.3, 16.6)	13.9±2.3	0.237
INR	1.1 (1.0, 1.4)	1.2±0.2	0.748
Liver cirrhosis	26 (60.5%)	25 (51.0%)	0.364
Child-Pugh scores	5 (5, 8)	6 (5, 7)	0.638
class A (n)	31	27	0.094
class B&C (n)	12	22	

#, *P* values of univariate binary logistic regression analysis, depending on whether it is WD or not. The normal distribution parameters are shown as mean± standard deviation, or else median and quantile (25%, 75%).

Cp, ceruloplasmin; WBC, white blood cell; PLT, platelet; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GT, γ-glutamyltransferase; AP, alkaline phosphatase; CHE, cholinesterase; Cr, creatine; CHO, cholesterol; PT, prothrombin time; INR, international normalised ratio.

The performance of binary logistic regression analysis.

ıtory ɔr	*β*	*SE*	*t*	*OR*	Lower 95% Limit of *OR*	Upper 95% Limit of *OR*	Significance (*P*)
t	13.523	3.081	19.270	746553.8			0.000
ars)	-0.189	0.044	18.368	0.828	0.760	0.903	0.000
e)	-0.109	0.030	13.627	0.897	0.846	0.950	0.000

dard error; OR, odds ratio.

The values of this index were from 0.1 to 8.6. The univariate binary logistic regression analysis using above WD index as predictive factor had a AUC of 0.950 (95% CI: 0.909-0.990, P=0.000) in the training set, 0.900 (95% CI: 0.833-0.968, *P*=0.000) in validation set (Total cohort: 0.923, 95% CI: 0.883-0.962, *P*=0.000) (Fig. 2B), which are almost identical to the original model. According to the AUC of WD index,

two cuttoff values were chosen to identify WD patients (Table 5). Using cutoff values of 1.9 and 2.5, 92.3% of patients in the training set with a WD index >2.5 would not be WD patients, whereas a WD index ≤ 1.9 would have a specificity of 95.9% and a PPV of 94.4% for WD (Table 5). Their performance in validation set is slightly lower. In the total cohort, the PPV for WD patients with cutoff value of ≤ 1.9 achieves 88.2%, with a specificity of 90.8%. On the other hand, sensitivity is only 77.9%.

Table 5
Accuracy of the Simplified Model in Predicting WD Patients.

Group (N)	Cutoff value	Sensitivity	Specificity	PPV	NPV
Total (184)	≤ 1.9	77.9%	90.8%	88.2%	82.4%
	≤ 2.5	90.7%	72.4%	74.3%	89.9%
Training set (92)	≤ 1.9	79.1%	95.9%	94.4%	83.9%
	≤ 2.5	93.0%	73.5%	75.5%	92.3%
Validation set (92)	≤ 1.9	76.7%	85.7%	82.5%	80.8%
	≤ 2.5	88.4%	71.4%	73.1%	87.5%

AUC, area under the receiver operating characteristic curves; PPV, positive predictive value; NPV, negative predictive value.

Discussion

This is a retrospective cohort study with a very long follow-up time (9.8 years). The longitudinal study showed that the increased Cp in WD patients is related to the deterioration of liver function indexes and the rise of urinary copper excretion (Table 2). Then we conducted a case-control study and build a WD index using two easily available markers: age and Cr to identify WD from hepatopathy patients whose serum Cp are nearly normal.

This study found that, in WD patients, the rise of serum Cp concentration mainly happened when they presented liver function injury or complicated with LC, like the abnormal change of AST, γ-GT, ALB, cholesterol and internal copper load (Table 1&2). It is consistent with previous research which the serum Cp concentration may be normal in hepatic WD with active liver disease[12–14], and could go down to low values after copper chelator treatment.[15] By analyzing liver histology of 27 WD patients whose serum Cp levels varied from 210 to 269 mg/L, Peter Ferenci thought it correlated with liver histologic activity ($r=0.47$, $p<0.05$).[15] We found the similar Cp change trend after treatment. However, after terminating the treatment, the serum Cp would rise again. Maybe, it can be explained with that Cp is mainly produced by liver and an acute phase response protein to infection and inflammatory agents,[7,8] liver injury caused by copper burden will push the Cp to rise. This is likely why the “normal” Cp is more common in WD patients with obvious hepatic damage than in N type WD patients (Table 1&2).[15]

Cp is the main copper binding glycoprotein in blood plasma, transporting 40–70% of total serum copper.[8] The serum Cp tested via immunological method includes the biologically inactive apo-Cp and biologically active holo-Cp binding with copper.[18] Sco represents the level of biologically active holo-Cp and has greater diagnostic value than Cp concentration determined by immunological technique.[18,19] So we can easily understand that, in longitudinal study, serum Cu and Sco had significant linear correlation with serum Cp (R^2=0.769 and 0.775, both P=0.000, Fig. S2).

The mutations distribution and allele frequency in ATP7B gene from 64 WD paitents in our study shows the information that the hotspots are p.R778L (exon 8), p.P992L (exon 13), and p.R919G (exon 12) (Fig. S1) which are in accordence with Chinese prevailing hotspot mutations in WD patients.[20] There was no difference in mutation hotspots between the two groups patients whose serum Cp was always higher than 140mg/L or fluctuated (P=0.332) (Table S1). These data did not show that the mutations of ATP7B gene were associated with the change of Cp. The same findings have been found in the North American, Russian, and Swedish samples whose the most common WD mutation (p.H1069E) had no a significant correlation with the ceruloplasmin activity.[21]

Serum CP and K-F ring are the most recognized and easily remembered tests for WD diagnosis. Unfortunately, they always are concealed and puzzling in hepatic type WD patients[4–6]. European guidelines and Leipzig scoring system[6] have provided a perfect diagnostic pathway for WD diagnosis, but they are based on parameters that are not readily available in ordinary clinical centers, like serum and urinary copper, ATP7B gene or liver biopsy which are also expensive, time-consuming. Nevertheless, no studies have addressed a diagnosis index based on the usual indicators to differentiate WD patients with “normal” serum Cp. We developed a simple formula to recognise these WD paitents. The parameters (age and serum Cr) adopted in WD index are different from those used to identify WD from ALF patients in some other studies, like AST/ALT and AP/TB.[13,16,17,22] Firstly, the majority of WD presents between ages 5 and 35, and liver disease may precede neurologic manifestations by as much as 10 years.[6] In addition, the onset age of other causes liver diseases is generally later, from 30 to 80 years old, for instance hepatitis B, steatosis heptitis and autoimmune hepatitis and so forth.[23,24] Hence, age is a crucial parameter for differentiating WD from other liver diseases. Secondly, supplementing the diet with Cr, one of another parameter in WD index, can relieve liver damage rely on inhibiting liver inflammation, oxidative stress and cellular senescence.[25] Copper ions are an oxidant and may cause obvious increase of oxidative stress and creatine consuption in WD patients whose body have excessive copper deposits, in our study, which may be the reason why the WD patients’ serum Cr is lower than that of other liver diseases. But whether this is a ubiquitous phenomenon in all WD patients remains unclear.

This simplified model (WD index) has a great AUC value, which is almost consistent with the original model (Fig. 2). No diagnostic model is perfect. Our model can only be used for preliminary screening, not definitive diagnosis. So we selected two cutoff values, one with very high PPV and specificity, and the other with very high NPV and sensitivity (Table 5). It means that when you address WD index $\leq$ 1.9 to diagnose WD, 88.2% are correct. Unfortunately, 22.1% WD patients will be missed, due to 77.9% of

sensitivity. If we want to increase the sensitivity, 2.5 may be a better choice. It has a sensitivity of 90.7% and a NPV of 89.9%, which shows that 89.9% of excluded patients really are non-WD patients (Table 5). All in all, 1.9 and 2.5 used as the cutoff values for diagnosing and excluding WD both have great predictive power. Nevertheless, we still need to be careful with WD index from 1.9 to 2.5.

Limitations of our study include that this is a single center and retrospective study which may have bias and decrease the power of our results; the enrolled WD patients are relatively fewer due to it is a rare disease and fewer patients had elevated serum Cp. In addition, because it is a retrospective research we can not get all patients' ATP7B gene, actually, the clinical phenotypes, K-F ring and increased copper excretion were sufficient for diagnosis of WD. At last, the pathological mechanism of reduced serum Cr in WD patients is still unclear.

Conclusions

In summary, in WD patients, their increased serum Cp concentration mostly were pseudo and finally went back to a reasonable level corresponding to their liver improvement treated with copper chelator, and was not significantly related to ATP7B gene mutations. We have established a novel simple index, WD-index, which have good performance in identifying suspicious WD patients from undefined patients whose seurm Cp ≥140mg/L. We hope additional studies in patients who come from multi-center could further verify WD index.

Abbreviations

ALB, albumin; ALF, acute liver failure; ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; AUC, area under the receiver operating characteristic curves; CB, conjugated bilirubin; Cp, ceruloplasmin; Cr, creatine; DMPS, dimercaptopropansulfonate sodium; γ-GT, γ-glutamyltransferase; GLMM, generalized linear mixed effect models; HBV, hepatitis virus B; INR, International Normalised Ratio; K-F ring, Kayser-Fleischer ring; LC, liver cirrhosis; NASH, nonalcoholic steatosis heptitis; NPV, negative predictive value; PLT, platelet; PT, prothrombin time; PPV, positive predictive value; Sco, serum ceruloplasmin oxidase; TB, total bilirubin; WD, Wilson's Disease.

Declarations

Ethics approval and consent to participate

This article does not contain any studies with human or animal subjects. This manuscript does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

Consent for publication

Not applicable

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing of interests

All authors declare that they have no conflict of interest.

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Author' contributions

L C and W-B H prepared material, collected and analysed data, wrote and reviewed manuscript. N W, Z-Q L, C-S W, L-Y P and X-L X collected and analysed data, reviewed manuscript. R-M Y and Y-Z H guarded resources and coordinated collaboration. B R designed and supervised research and interpreted results.

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Figures

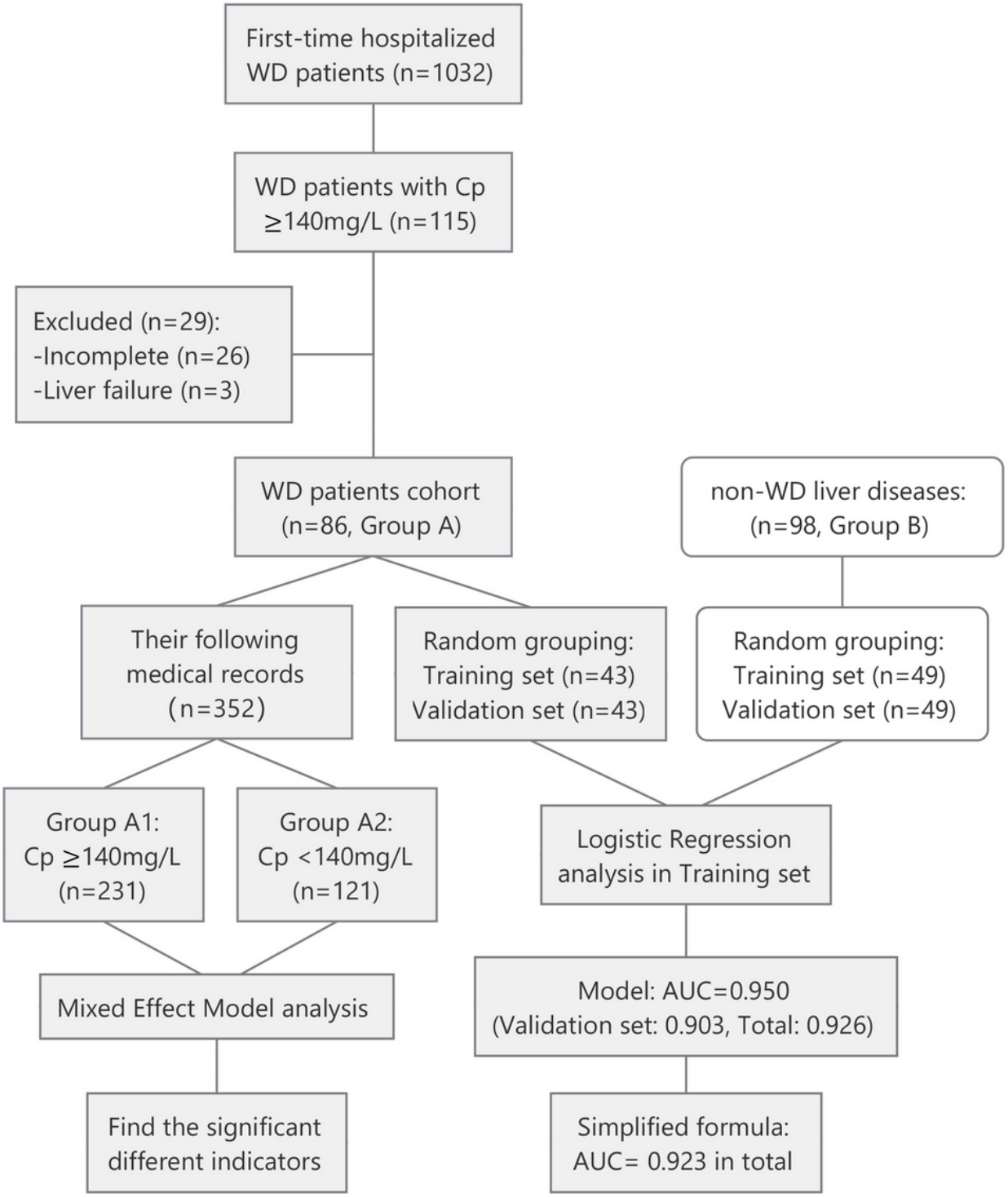

Figure 1

The Flow Chart of this Study.

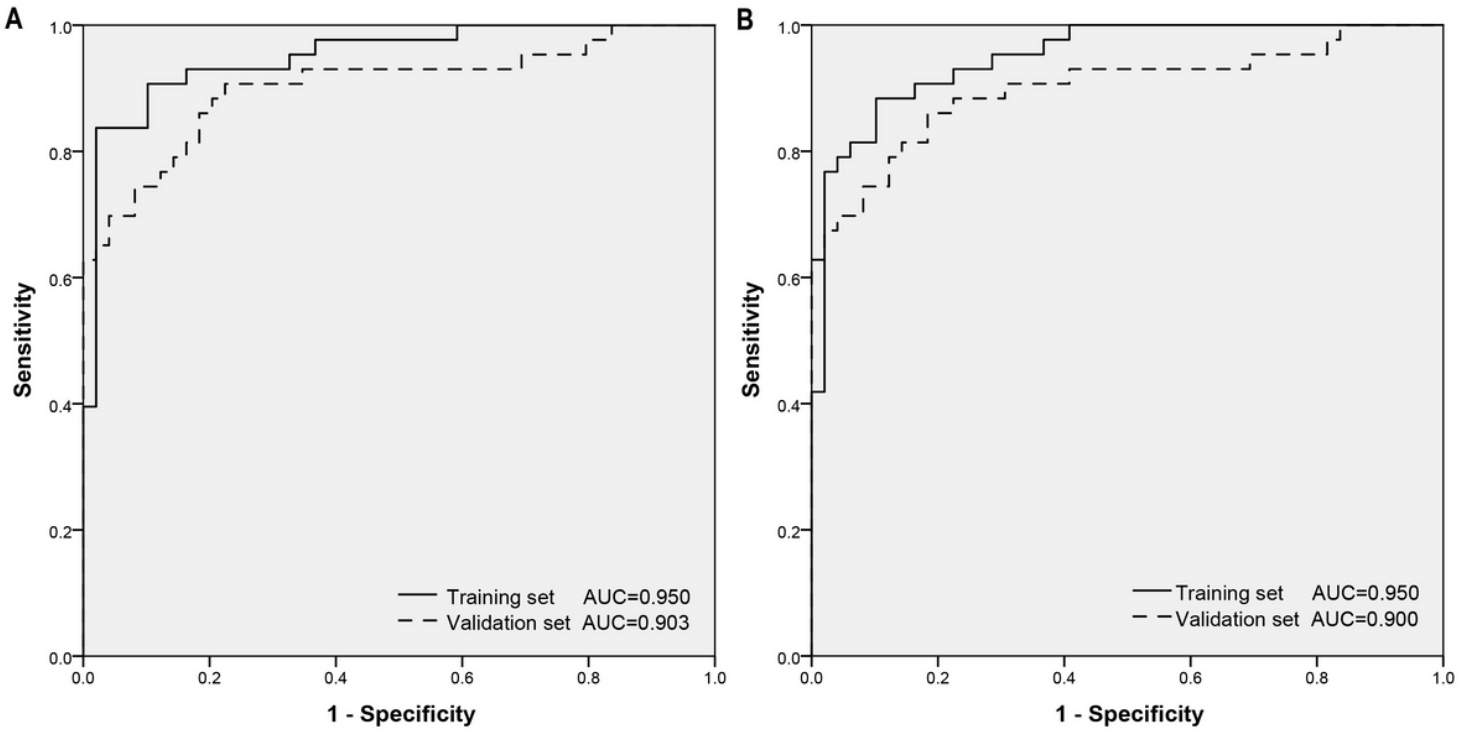

Figure 2

ROC plot in training and validation set for differentiating WD from Non-WD paitents. a Original logistic regression model. b Simplified formula.

Supplementary Files

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