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Evaluation of salivary ceruloplasmin for the diagnosis of Wilson's disease

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The serum ceruloplasmin assay is the most commonly used test for diagnosing Wilson's disease (WD). Despite the utility of non-invasive tests for diagnosing WD, no such tests have been developed. Therefore, we aimed to identify a safe and non-invasive assay and determine the factors associated with salivary ceruloplasmin. The sample comprised 167 participants: 130 patients with WD (experimental group) and 37 individuals without WD (control group). Salivary ceruloplasmin's diagnostic performance was assessed using a receiver operating characteristic curve analysis, and the diagnostic thresholds were determined. The Mann–Whitney U test for independent samples was used to compare intergroup variability between the control and WD groups. We used Pearson's correlation coefficient to determine intergroup correlations between the blood and salivary ceruloplasmin levels of patients with WD. Salivary ceruloplasmin levels were significantly lower in the WD group than in the control group. Serum and salivary ceruloplasmin levels were positively correlated. The area under the curve for salivary ceruloplasmin was 0.9977. The critical value of salivary ceruloplasmin was 8.885 ng/mL, with a sensitivity of 99.23% and specificity of 100%. Evidently, salivary ceruloplasmin has substantial diagnostic value. Therefore, saliva analysis can be used as a non-invasive alternative to serum analysis for diagnosing WD.

Keywords Serum, Saliva, Ceruloplasmin, Wilson's disease

Wilson's disease (WD) is a hereditary metabolic disease caused by mutations in the ATP7B gene[1]. It is one of the most prevalent genetic disorders affecting the nervous system, and its prevalence varies across regions, with a global prevalence rate of 1–3 per 10,000 individuals[2]. The incidence rate in Europe and the United States is 0.5–3 per 100,000 individuals[3], which is lower than that in Japan, Korea, and Southeast Asia, with the incidence rate in Japan being 1.21 per 10,000[4]. Copper transport in the body requires the action of the ATPase to reach the Golgi apparatus, where it is synthesized into ceruloplasmin (CP) and excreted in bile[5]. Wilson's disease is caused by a mutation in the ATP7B gene, which results in a decreased capacity to transport copper, causing a decrease in CP synthesis and leading to varying degrees of copper accumulation in the body[6]. Its primary clinical manifestations are hepatic and neurologic involvement[7]. According to the diagnostic consensus, decreased serum CP in patients with WD, as evidenced by a serum CP level of < 200,000 ng/mL, is usually considered the major diagnostic threshold for WD[8]. In some patients, owing to copper deposition in the cornea, a positive corneal Kayser–Fleischer (K–F) ring can be observed under a slit lamp or normal light[9]. The K–F ring is one of the clinically significant features of WD. Early detection of WD is essential to reduce disease severity and prevent complications[10]. Currently, the tests used to diagnose WD include serum CP, liver biopsy, and genetic testing[11]. Liver biopsies for copper quantification are limited to patients who meet the clinical and biochemical criteria[12]. Additionally, WD can be diagnosed via the direct sequencing of ATP7B; however, this method is expensive[13]. Serum CP deficiency in patients with WD was first reported in 1952[14]. Currently, the most widely used indicator for the clinical diagnosis of WD is serum CP. A significant number of patients have psychiatric symptoms and poor compliance, and frequent testing may lead to symptoms of anxiety in patients. Some patients have thrombocytopenia, which can lead to bleeding risk after blood sampling.Moreover, these diagnostic tests are invasive, and trauma can exacerbate the neurological symptoms in patients with WD[15]. In addition, blood collection requires trained medical personnel to operate, which limits timely diagnosis for patients with limited

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resources, such as those in rural areas. Determining a non-invasive sample collection method has far-reaching implications for patients and healthcare.

Saliva is a human bodily fluid with a complex and varied composition. Similar to the serum, the saliva contains various amino acids, proteins, hormones, ions, enzymes, electrolytes, and many other substances[16]. It has substantial potential for diagnosing diseases[17]. As a biological sample, saliva is easy to collect, is non-invasive, and helps screen for diseases[18]. According to previous studies, saliva can be used as a diagnostic tool for systemic diseases[19].

The Encephalopathy Center of the First Affiliated Hospital of the Anhui University of Traditional Chinese Medicine (AUTCM), where this research group is located, is a major center for diagnosing and treating WD in China. The Center treats thousands of patients with WD annually. Currently, the internationally available serum CP test is the most commonly used method for diagnosing WD. However, a safer and non-invasive salivary CP test has not yet been developed. The purpose of this study was to investigate whether salivary CP can replace serum CP as a diagnostic tool for WD and to provide a new, safe, and non-invasive method for diagnosing WD in the clinic.

Materials and methods

Study population

This study was approved by the Ethics Committee of the First Affiliated Hospital of Anhui University of Traditional Chinese Medicine (Approval number: 2023AH-56). The participants and/or their legal guardians were provided with information about the study and signed informed consent forms. All methods in this study were carried out in accordance with relevant guidelines and regulations. Research was performed in accordance with the Declaration of Helsinki. A total of 130 patients diagnosed with WD underwent serum CP testing during hospitalization between July 2023 and May 2024 at the First Affiliated Hospital of the AUTCM. In addition, 37 individuals without any clinical symptoms of WD were recruited as the control group.

Inclusion criteria

(1) Patients who met the diagnostic criteria for WD[20] and received conventional anti-copper therapy; (2) patients who could cooperate during the collection of their saliva and urine; (3) patients who were willing to participate in the saliva and urine collection and signed the informed consent form; and (4) patients with WD diagnosed before July 1, 2023.

Exclusion criteria

(1) Patients with mental disorders, cognitive dysfunction, or severe dysarthria who could not cooperate, and (2) patients who refused to participate for various reasons.

Saliva collection

All participants underwent a clinical oral cavity examination prior to the saliva collection. The participants and/or their legal guardians were provided with information about the study and signed informed consent forms.

ELISA method for serum and salivary ceruloplasmin

The level of human CP in the specimens was determined by the double-antibody sandwich method using an ELISA kit (Wuhan GeneMed Technology Co., Ltd., JYM1187Hu). The absorbance (OD value) was measured at 450 nm using an enzyme labelling instrument (Radiometer, RT-6100), and the concentration of human CP in the samples was calculated using a standard curve.

24-hour urine copper assay method

The 24-hour urine was retained on the third day of each course. We collected the patients' The urine was then thoroughly mixed, and 5 mL of the sample was poured into a dedicated test tube and sent for analysis. The Calculation was performed using Flame Atomic Absorption Spectrophotometry method. The participants and/or their legal guardians were provided with information about the study and signed informed consent forms.

Salivary copper ion assay

The participants were asked to fast for two hours prior to collection, and saliva was collected between 9:00 a.m. and 4:00 p.m. Saliva samples were collected using the spit method. Each participant spit into a calibrated sterile centrifuge tube every 60 s after rinsing their mouths with distilled water until approximately 1 mL of saliva was obtained[21]. The sample was stored in a −80 °C refrigerator for preservation and sent to the laboratory for analysis, followed by the use of a centrifuge for approximately 20 min (2,000–3,000 rpm). Copper ion content was detected using a copper ion detection kit (Nanjing Jiancheng Bioengineering Research Institute, E010-1-1).

Genetic testing

Genetic mutation locus testing was performed in 130 patients using the Sanger sequencing method, assisted by Bekom Medical Ltd. The participants and/or their legal guardians were provided with information about the study and signed informed consent forms.

Statistical analysis

We used SPSS (version 25.0) for the analysis. For descriptive statistics, quantitative data were expressed as mean ± standard deviation, median, and interquartile range (IQR) based on normality or non-normality. The Mann–Whitney U test was used to compare the two groups. A Pearson's correlation analysis was performed to determine the correlation between the participants' blood and salivary CP levels and between their urinary

and salivary copper concentrations. A receiver operating characteristic (ROC) curve analysis was performed to determine the diagnostic value of salivary CP and correctly distinguish patients with WD from healthy controls. The overall performance was determined by the total area under the curve (AUC), and the threshold values for optimal diagnosis were selected based on their sensitivity and specificity. The level of statistical significance was set at $p < 0.05$.

Results

General characteristics of the participants

The WD group included 130 patients with WD and the control group included 37 healthy individuals. The distribution of all participants' statistics is presented in Table 1. The patients were recruited from 21 provinces in China (Fig. 1; Table 2). Among them, the main manifestations were the onset of disease in the form of a hepatic phenotype for 36 patients, a cerebral phenotype for 92 patients, no other symptoms besides copper biochemical abnormalities for 1 patient, and other symptoms for 1 patient.

Significant difference in ceruloplasmin between patients with Wilson's disease and healthy controls

We statistically analyzed the salivary CP levels in the WD and control groups, and the results are shown in Table 3. The mean value of salivary CP in the WD and control groups was 5.9568 ng/ml and 11.4419 ng/ml, respectively. The WD group had significantly lower salivary CP levels than the control group ($p < 0.05$; Fig. 4A).

Optimal cut-off values for saliva and blood ceruloplasmin diagnostics

We conducted salivary CP (Fig. 2A) and serum CP (Fig. 2B) ROC analyses for the WD and control groups. The total AUC for salivary CP was 0.9977 (Table 4). The sensitivity and specificity of various levels of salivary CP were measured. A critical value of 8.885 ng/ml was estimated, as this yielded an optimal trade-off with a sensitivity of 99.23% (95% confidence interval [CI]: 95.77–99.96%) and specificity of 100% (95% CI: 90.59–100.0%; Table 5). One false-negative result was found among the patients, corresponding to a false-negative rate of 0.7%. The patient presented with hepatic failure. The total AUC of serum CP was 0.9997, with 230,000 ng/mL as the optimal threshold, a sensitivity of 100% (95% CI: 97.13–100.0%), and a specificity of 94.59% (95% CI: 82.30–99.04%). Two false positives were found in heterozygous relatives and patients with liver failure. Diagnoses based on saliva are exceptionally sensitive and specific and can reduce the patients' risk of infection and anxiety; therefore, saliva testing has clinical practical utility.

Linear correlation between serum and salivary ceruloplasmin levels

We conducted a correlation analysis to determine whether an association exists between the serum and salivary CP levels of 130 patients in the WD group. Across the cohort, a significant correlation was observed between the blood and salivary CP levels ($r = 0.4456$, $p < 0.0001$). The linear regression analysis of the patients' serum and salivary CP levels showed a linear correlation (Fig. 3A).

Correlation of saliva and blood ceruloplasmin with the Wilson's disease rating scale

The Wilson's Disease Rating Scale (UWDRS) is advantageous for assessing the disease severity. The UWDRS provides a comprehensive assessment of the signs and symptoms of the hepatic, neurological, and psychiatric systems in patients with WD[22]. In our study, Our findings also revealed that the UWDRS was not significantly correlated with blood ($r = 0.067$, $p = 0.4437$; Fig. 3B) or salivary ($r = 0.03052$, $p = 0.7326$; Fig. 3C) CP.

Parameter	Case (*n*)
Patients with WD male/female (*n*)	78/52
Age of patients with WD at onset (years)	
Range	
3.25–51.65	130
Control male/female (*n*)	24/13
Age of control group (years)	
Range	
15.35–63.75	37
Kayser–Fleischer ring of patients with WD	
Positive	96
Negative	34
Form of onset of patients with WD	
Hepatic	36
Neurologic	92
Asymptomatic	1
Others	1

Table 1. Demographics of patients with Wilson's disease (WD) and healthy controls.

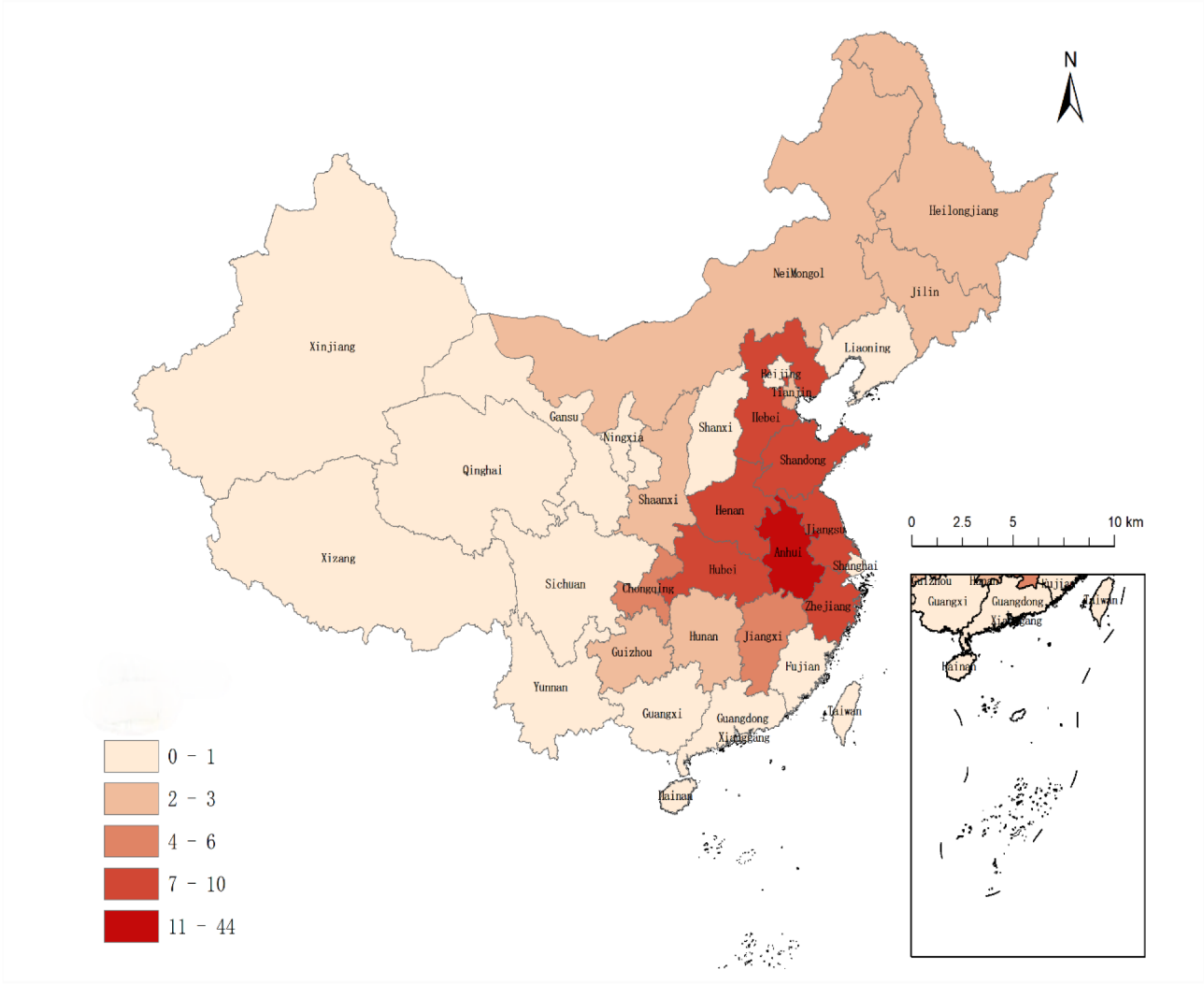

Fig. 1. Patients from 21 provinces in China.

Correlation of salivary ceruloplasmin with phenotype in patients with Wilson's disease

As patients with WD mainly present with hepatic and cerebral damage, we compared the salivary CP content of patients with the hepatic phenotype as the starting form of the disease ($n = 92$) with that of patients with the cerebral phenotype as the starting form of the disease ($n = 36$). We found that the difference in the salivary CP levels between the hepatic and cerebral phenotypes was not significant ($p = 0.2459$; Fig. 4B). Additionally, the presence or absence of K–F rings in the patients did not differ significantly ($p = 0.3874$) in the salivary CP content (Fig. 4C).

Patients with an age of onset of less than 10 years (4.970 ng/mL, IQR: 4.825–5.305) had lower salivary CP levels, whereas those with an age of onset greater than 30 years had higher salivary CP levels (6.470 ng/mL, IQR: 6.188–7.055; Fig. 4D).

Correlation between salivary copper and 24-hour urinary copper

To understand the correlation between salivary copper and 24-hour urinary copper levels, we collected samples from 10 patients. Each patient received two to four sessions of copper drainage during hospitalization, with each session lasting eight days. The 24-hour urine and saliva samples of the patients were collected on the same day before and during each session. We observed a significant negative correlation between the salivary copper content and 24-hour urinary copper amount ($r = -0.3258$, $p = 0.0491$; Fig. 3D). The amount of urinary copper gradually increased and salivary copper gradually decreased in patients during the copper removal sessions. Most of the copper in the patients' bodies was excreted in urine, and a small portion was excreted in saliva and blood.

Salivary ceruloplasmin and genotype

We analyzed 130 patients with WD using gene sequencing. Based on the sequencing results, 108 patients had 2 pathogenic variants (12 pure and 96 compound heterozygotes). In addition, seven WD cases had three

Region	All ($N = 130$)
AnHui	44 (33.8%)
Tianjin	2 (1.5%)
HeiLongJiang	3 (2.3%)
HuBei	9 (6.9%)
GanSu	1 (0.8%)
HeBei	9 (6.9%)
JiLin	2 (1.5%)
JiangSu	8 (6.2%)
ShanDong	10 (7.7%)
NeiMengGu	3 (2.3%)
HeNan	9 (6.9%)
GuiZhou	2 (1.5%)
ShanXi	2 (1.5%)
FuJian	1 (0.8%)
ZheJiang	10 (7.7%)
ChongQing	4 (3.1%)
JiangXi	6 (4.6%)
GuangDong	1 (0.8%)
LiaoNing	1 (0.8%)
HuNan	2 (1.5%)
ShanXi	1 (0.8%)

Table 2. Patients from 21 provinces in China.

	Groups	*n*	Mean value	(Statistics) Standard deviation	z	*p*
Salivary ceruloplasmin	Control group	37	11.4419	0.69670	8.046	0.0001
	WD group	130	5.9568	1.01844		

Table 3. Salivary ceruloplasmin in the Wilson's disease (WD) group versus the control group.

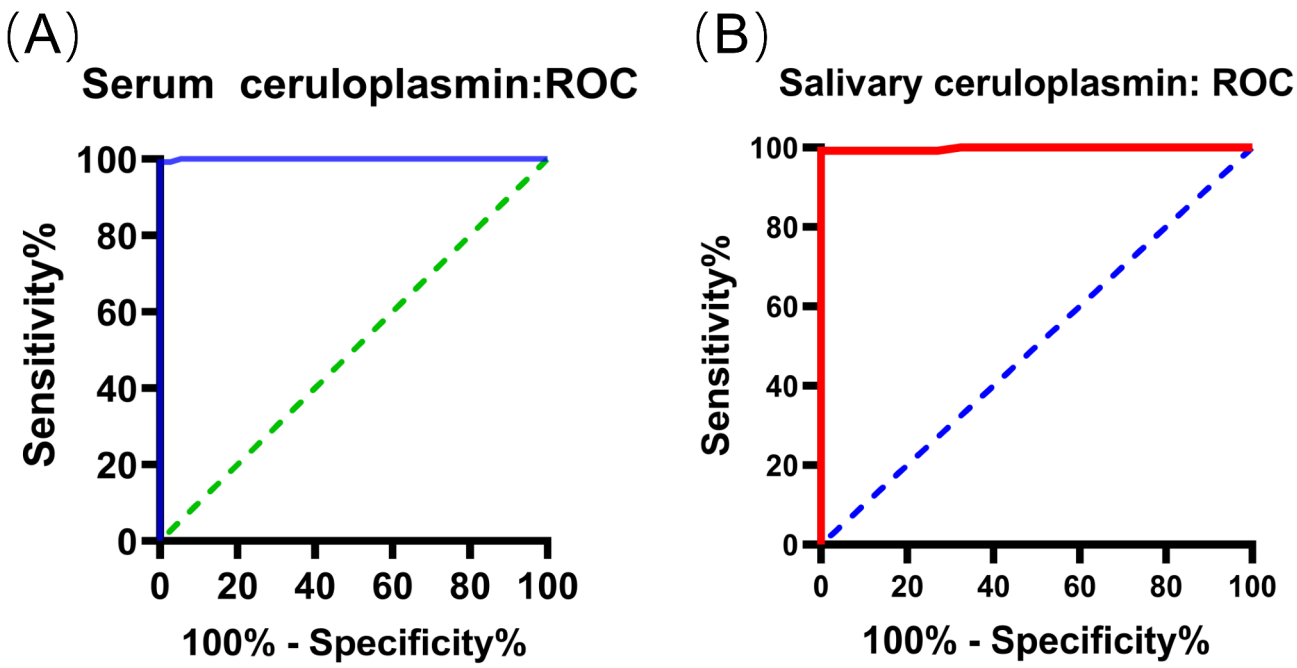

Fig. 2. (**A**) Receiver operating characteristic curve of serum ceruloplasmin. (**B**) Receiver operating characteristic curve of salivary ceruloplasmin.

	Area	Standard error	*p*	95% confidence interval	
				Upper bound	Lower bound
Salivary ceruloplasmin	0.9977	0.002420	< 0.001	0.9930	1.000
Serum ceruloplasmin	0.9996	0.0004656	< 0.0001	0.9988	1.000

Table 4. Area under the receiver operating characteristic curve for ceruloplasmin.

Salivary ceruloplasmin (ng/ml)	Sensitivity (%)	Specificity (%)
< 7.175	96.15	100.0
< 7.275	96.92	100.0
< 7.360	97.69	100.0
< 7.420	98.46	100.0
< 8.885	99.23	100.0
< 10.39	99.23	97.30
< 10.51	99.23	94.59
< 10.59	99.23	91.89
< 10.63	99.23	86.49

Table 5. Sensitivity and specificity of salivary ceruloplasmin diagnosis.

heterozygous pathogenic variants. Eight patients with WD had only one heterozygous variant. We identified seven patients without any potentially pathogenic variants. Among the five genotypes, patients who were heterozygous had the lowest levels of salivary CP (4.95 ng/mL, IQR: 4.82–5.22), and the highest salivary CP levels were found in those without any mutation (7.24 ng/mL, IQR: 6.89–7.41; Fig. 5A).

Among the heterozygous patients, salivary CP levels were lower in patients with the R778L/R778L mutation (4.865 ng/mL, IQR: 4.745–4.968) than in those with other heterozygous genotypes (5.305 ng/mL, IQR: 5.030–5.423; Fig. 5B). Among the heterozygous patients, salivary CP levels were higher in patients with the PAV/PAV gene (5.860 ng/mL, IQR: 5.300–6.490) than in those with the PTV/PAV gene (5.430 ng/mL, IQR: 4.970–6.015; Fig. 5C). Among patients with PAV/PAV, salivary CP levels were lower in patients with the R778L/P992L genotype (5.175 ng/mL, IQR: 5.043–5.330) than in those with other genotypes (6.060 ng/mL, IQR: 5.350–6.520; Fig. 5D).

Discussion

Currently, WD is mostly diagnosed based on its clinical presentation and blood tests. Blood CP is the most commonly used test to diagnose WD[23]. Blood testing is invasive. Saliva also becomes a copper excretion pathway when the copper levels in the body increase. Ceruloplasmin is a biomolecule found in the saliva[24], making saliva testing possible.

An objective of this study was to determine whether saliva could be used for diagnosing WD. Our findings showed that salivary CP levels were significantly higher in the control group than in the WD group. To determine whether a correlation existed between salivary and serum CP in patients with WD, we performed a correlation analysis, which showed a linear correlation between salivary and serum CP levels in the WD group. No significant correlation of the UWDRS score was observed with salivary or serum CP levels in patients with WD.

The diagnostic value of saliva testing must be compared with that of blood testing to determine whether saliva testing can be used as a new diagnostic method for WD. The diagnostic value of this assay depends on how accurately it distinguishes between patients with and without WD. The sensitivity and specificity of the ROC curve are indicators used to determine diagnostic accuracy, and the ROC analysis was used to determine the diagnostic potential of the saliva assay. The diagnostic accuracy was estimated from the AUC. Our study showed that the AUC of CP protein was 0.9977, indicating that salivary CP levels can effectively differentiate patients with WD. We used the ROC analysis to determine the critical level of salivary CP for the diagnosis of WD, with a critical value of 8.885 ng/mL, sensitivity of 99.23%, and specificity of 100%. This indicated that the individuals with a salivary CP level > 8.885 ng/mL were more likely to not have WD and those with a salivary CP level below 8.885 ng/mL were more likely to have WD. These findings suggested that the saliva can be used as a biosample for CP assays in patients with WD.

In terms of the clinical phenotype, no significant difference in salivary CP levels was found between the liver and brain subtypes. In addition, there were no significant differences in the CP levels in the saliva of patients with WD with or without K–F rings. Patients with a lower age of onset had less salivary CP and those with a higher age of onset had higher salivary CP than those with other ages of onset.

Urinary copper reflects the amount of copper in circulation, and 24-hour copper levels are elevated in patients with WD[25]. Tests that indicate patients' response to clinical treatments include urinary copper testing[9]. Previous studies have shown a decrease in fecal copper excretion and an increase in urinary copper excretion following a copper excretion treatment. A decrease in fecal copper was significantly correlated with a decrease in biliary excretion[26]. The use of copper-chelating agents, such as penicillamine, led to an increase in copper in

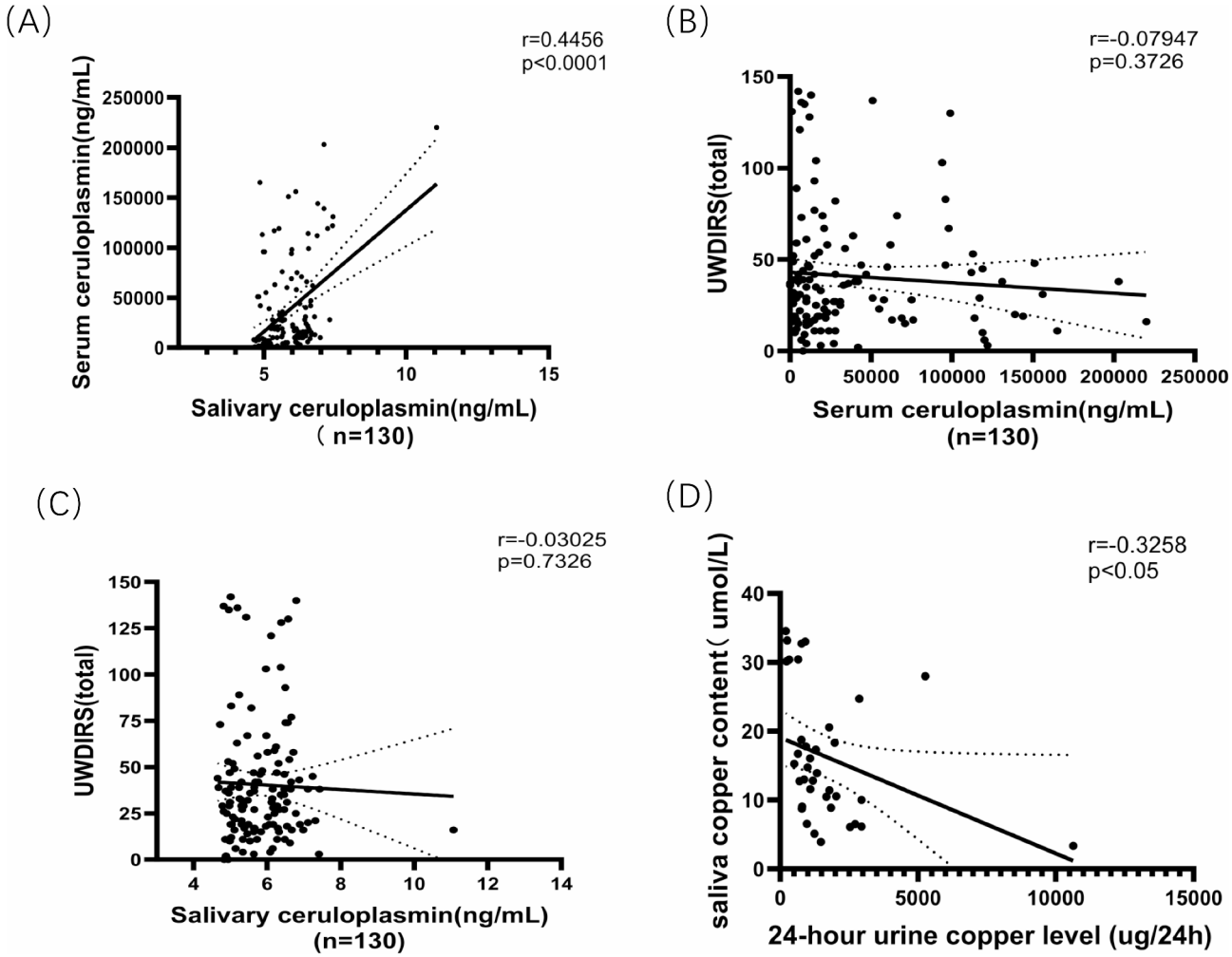

Fig. 3. (**A**) Correlation curve of salivary ceruloplasmin with serum ceruloplasmin in 130 patients. (**B**) Correlation curve of serum ceruloplasmin level with total score of UWDRS. (**C**) Correlation curve of salivary ceruloplasmin levels with total UWDRS score; UWDRS, Joint Wilson's Disease Rating Scale. (**D**) Correlation curve between salivary copper concentration and 24-hour urinary copper.

the urine and a decrease in copper in the blood[27]. Our results showed a significant negative correlation between patients' 24-hour urinary copper and salivary copper levels. As more copper was excreted from the body in the urine, the copper in the saliva gradually decreased. After the copper excretion treatment, the copper content in the saliva decreased. This may be due to most of the copper in the patient's body being excreted from the urine after treatment. Moreover, after a period of time, the copper in the body decreased, with both urinary and salivary copper gradually decreasing.

The analysis of the relationship between genotypic factors showed that salivary CP levels were lower in pure heterozygous patients than in heterozygous patients and in patients with one, three, and no variants. Among the heterozygous patients, salivary CP levels were lower in patients with the PTV/PAV gene than in those with PAV/PAV. Furthermore, patients with the R778L/R919G genotype had higher salivary CP levels than those with other normal variations. These differences are consistent with those of blood CP[28].

Therefore, our study demonstrated that salivary CP can be used as an early diagnostic tool to determine the optimal cutoff value for diagnosing patients with WD. Moreover, we examined the relationship between salivary CP levels and ATP7B mutations as well as the reduction in salivary copper levels after treatment. This study offers valuable insights for the diagnosis and counseling of patients with WD by providing a non-invasive test for early clinical diagnostic screening.

Conclusion

We found that the serum CP levels of patients with WD were positively correlated with their salivary levels. Salivary CP had high sensitivity and specificity as determined by the ROC analysis, with high accuracy. Saliva collection is a non-invasive method that is easy to perform, reduces the risk of bleeding associated with blood collection, and does not damage patients with WD, facilitating early diagnosis. The most crucial finding of this study is that CP levels in patients with WD can be estimated using saliva as a non-invasive diagnostic tool.

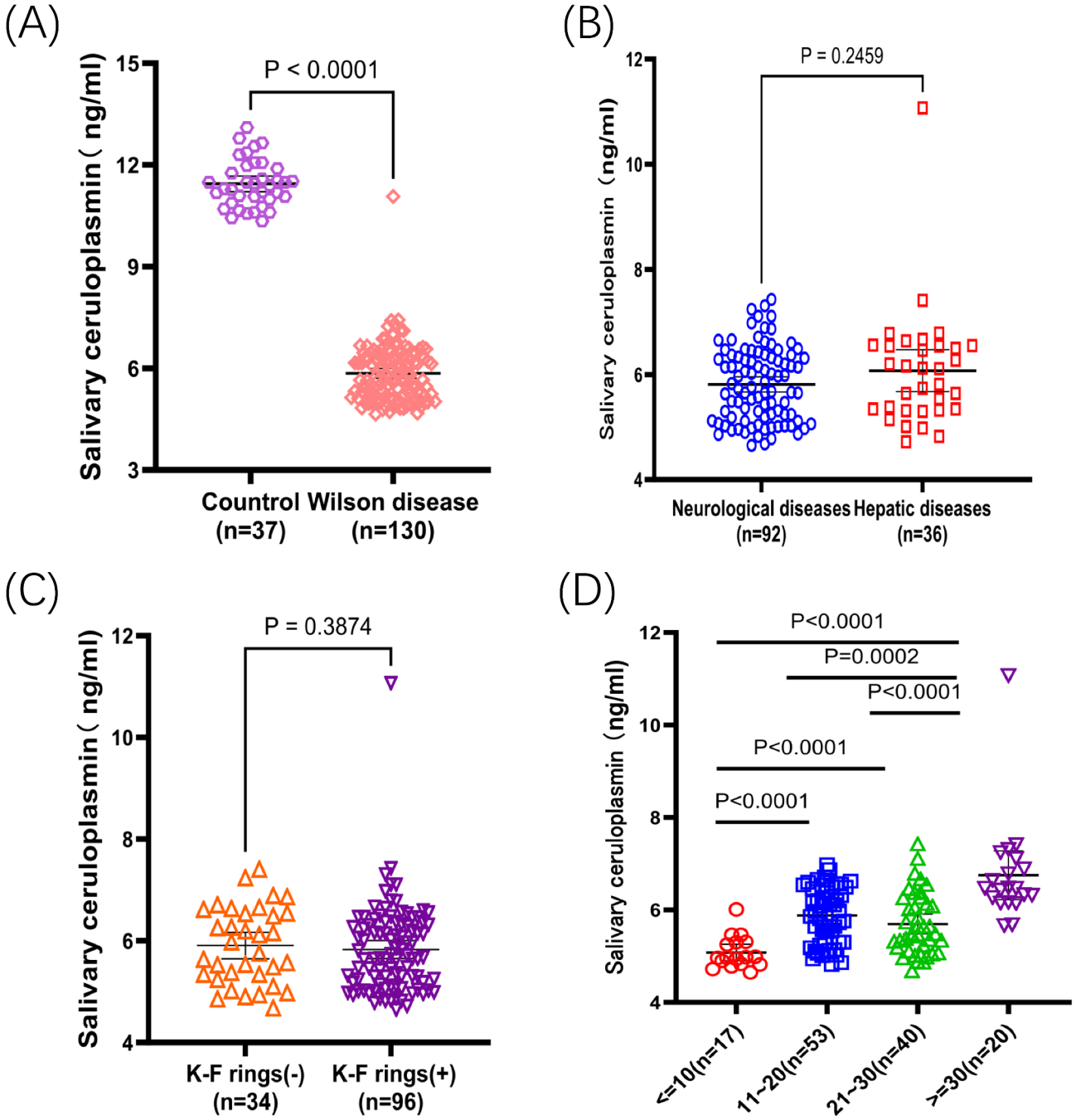

Fig. 4. (**A**) Differences in salivary ceruloplasmin levels between patients with Wilson's Disease and healthy controls. (**B**) Differences in salivary ceruloplasmin levels between cerebral and hepatic types. (**C**) Differences in salivary ceruloplasmin levels between Kayser–Fleischer ring (+) and (−). (**D**) Differences in salivary ceruloplasmin levels by age of onset.

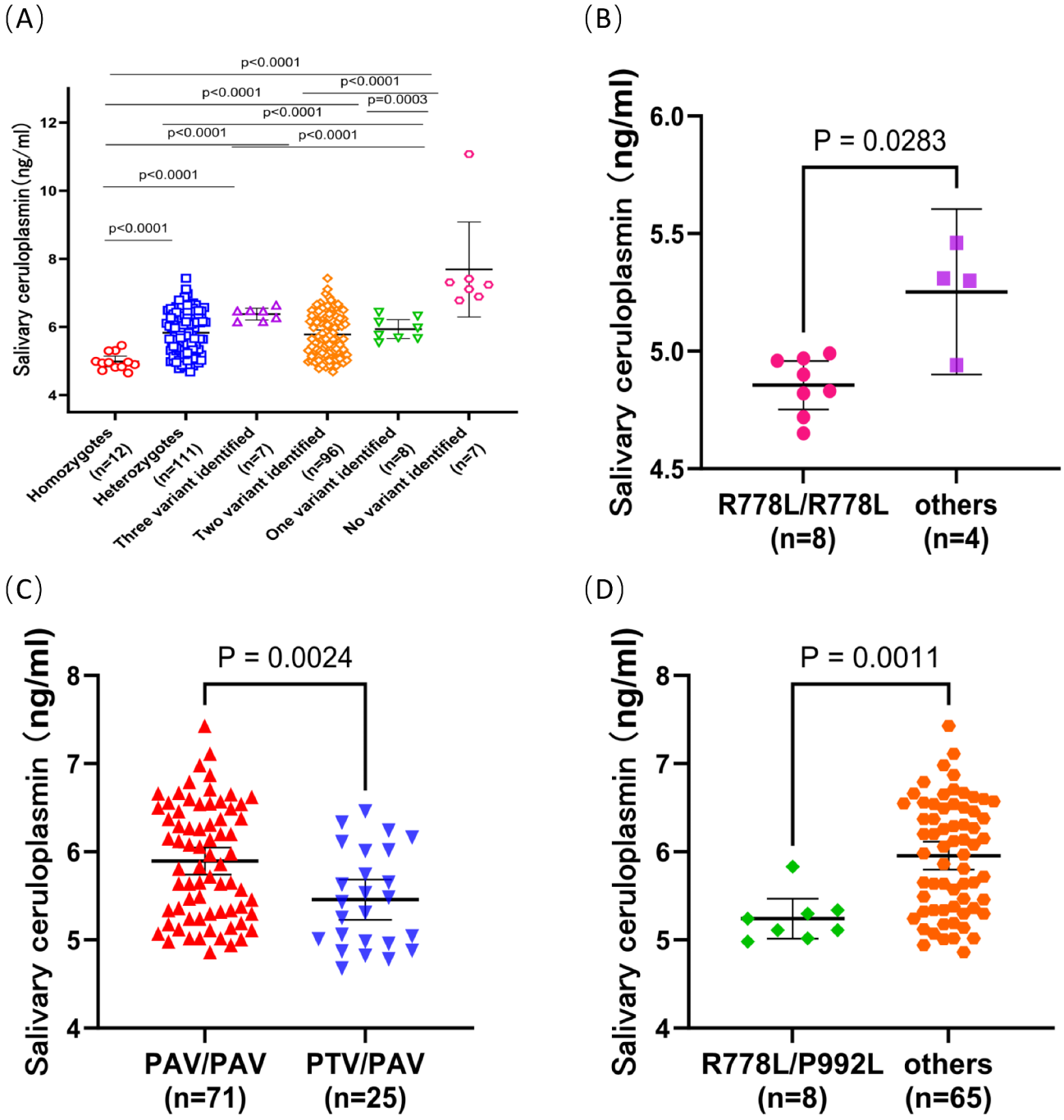

Fig. 5. (**A**) Effect of common ATP7B genotypes on serum ceruloplasmin levels of patients with Wilson's Disease (WD). (**B**) Effect of ATP7B gene heterozygotes on salivary ceruloplasmin levels in patients with WD. (**C**) Effect of ATP7B gene heterozygotes on salivary ceruloplasmin levels in patients with WD. (**D**) Effect of ATP7B gene PAV/PAV on salivary ceruloplasmin levels in patients with WD.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Author contributions

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Declarations

Competing interests

The authors declare no competing interests.

Ethics

This study was approved by the Ethics Committee of the First Affiliated Hospital of Anhui University of Traditional Chinese Medicine (Approval number: 2023AH-56).

Additional information

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