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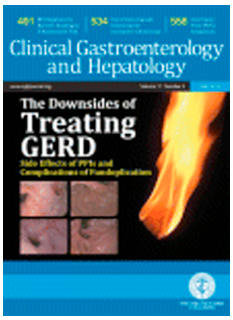

Role for Biochemical Assays and Kayser-Fleischer Rings in Diagnosis of Wilson's Disease

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Title Page

Title: Role for Biochemical Assays and Kayser-Fleischer Rings in Diagnosis of Wilson's Disease

Short Title

Reevaluation of diagnostic indexes

Authors

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Potential Conflicts of Interest

All authors reported no biomedical financial interests or potential conflicts of interest.

Authorship

Study concept and design: Yi Dong, Zhi-Ying Wu; acquisition of data: Yi Dong, Rou-Min Wang, Hao Yu, Guo-Min Yang, Wan-Qing Xu, Juan-Juan Xie; analysis and interpretation of data: Yi Dong; drafting of the manuscript: Yi Dong; statistical analysis: Yi Dong; obtained funding: Yi Dong, Zhi-Ying Wu; administrative, technical, or material support: Yue Zhang, Yu-Chao Chen, Wang Ni, Zhi-Ying Wu; study supervision: Zhi-Ying Wu.

Abstract:

Background & Aims: Wilson's disease is an autosomal recessive disorder that impairs

copper homeostasis and is caused by homozygous or compound heterozygous mutations in *ATP7B*, which encodes a copper-transporting P-type ATPase. Patients have variable clinical manifestations and laboratory test results, resulting in diagnostic dilemmas. We aimed to identify factors associated with symptoms and features of Wilson's disease from a large cohort, over 15 years.

Methods: We collected data from 715 patients (529 with symptoms, 146 without symptoms, and 40 uncategorized) and a genetic confirmation of Wilson's disease (mean age of diagnosis, 18.84 years), recruited from 3 hospitals in China from 2004 through 2019. We analyzed clinical data along with serum levels of ceruloplasmin (available from 636 patients), 24-hr urinary copper excretion (collected from 131 patients), Kayser-Fleisher rings (copper accumulation in eyes, with neurologic data from 355 patients), and magnetic resonance imaging (MRI) abnormalities. Differences among the groups were analyzed using 1-way analysis of variance followed by Tukey's multiple comparison test.

Results: Of the 529 patients with symptoms, 121 had hepatic features, 355 had neurologic features, 28 had osteomuscular features (premature osteoarthritis, skeletal deformities, and pathological bone fractures), and 25 had psychiatric symptoms. Age of onset was significantly younger in patients with hepatic (16.94 ± 1.03 years; P=.0105) or osteomuscular features (13 ± 1.33 years; P=.0001) than patients with neurological features (19.48 ± 0.46 years). Serum levels of ceruloplasmin differed among asymptomatic patients and patients with osteomuscular or neurologic symptoms of Wilson's disease. Serum levels of ceruloplasmin ranged from 18.93 mg/L to approximately 120.00 mg/L (quantiles of 0.025 to approximately 0.975). Fifty-one of 131 patients (39%) had urinary copper excretion levels below 100 µg/24 hr; there was significant variation in levels of urinary copper excretion between patients older than 14 years vs 14 years or younger. Of the 355 patients with neurologic features, 244 patients (69%) had abnormal findings from MRI and Kayser-Fleisher rings; only 1 patient with abnormal findings from brain MRI was negative for Kayser-Fleisher rings.

Conclusions: Serum level of ceruloplasmin, 24-hour urinary copper excretion, and Kayser-Fleisher rings can be used to identify patients who might have Wilson's disease. Patients with serum levels of ceruloplasmin below 120 mg/L and children with urinary copper excretion above 40 µg should undergo genetic testing for Wilson's disease. Patients with movement disorders and brain MRI abnormalities without Kayser-Fleisher rings are not likely to have Wilson's disease.

KEY WORDS: diagnostic factors, disease subtype, phenotype, categorization

Need to Know

Background: Wilson's disease is an autosomal recessive disorder that impairs copper homeostasis. Patients have variable clinical manifestations and laboratory test results, posing challenges to diagnosis.

Findings: Serum level of ceruloplasmin, 24-hour urinary copper excretion, and Kayser-Fleisher rings can be used to identify patients who might have Wilson's disease.

Implications for patient care: Patients with serum levels of ceruloplasmin below 120 mg/L and children with urinary copper excretion above 40 µg should undergo genetic testing for Wilson's disease. Patients with movement disorders and brain MRI abnormalities without Kayser-Fleisher rings are not likely to have Wilson's disease.

Introduction

Wilson's disease (WD) is an autosomal recessive disorder that impairs copper homeostasis in the human body. It is caused by homozygous or compound heterozygous mutations within *ATP7B*, encoding a copper-transporting P-type ATPase. Defective ATP7B function influences the biliary excretion of copper and the synthesis of serum ceruloplasmin, with pathological copper accumulations in several organs and low serum ceruloplasmin levels[1, 2]. The worldwide prevalence

of WD is estimated to be one per 30~100,000 [3-5]. In Asia, there seems to be a higher prevalence of WD, with estimates as high as 1 in 7561 in Korea [6]. Epidemiological studies of WD in China are rare, except one that reported a prevalence of one per 40,000 people in Hanshan County [7].

There is considerable variation in phenotypic characteristics of WD, and the knowledge of various disease manifestations, including hepatic, neurological, psychiatric, ophthalmological and other organ involvements is important for clinicians. The spectrum of liver disease varies from increased serum aminotransferase levels to hepatic steatosis to acute liver failure to cirrhosis[8, 9]. The neurological expressions range from mild disturbance to severe disability [10, 11]. There have been a few population-based studies on the clinical outcomes and genetic traits of WD[12, 13]. We first reported that p.R778L within *ATP7B* was the most common mutation and had a deleterious impact on Chinese WD patients [14], and we further summarized the genotypic data in a large WD cohort [15]. Although phenotypic and genotypic characterizations were further analyzed in large-scale Chinese WD patients, most patients were diagnosed only via rapid multiplex PCR-MassArray screening, with the possibility of *ATP7B* heterozygous mutation carriers being misdiagnosed as WD patients[16].

WD is one of a few genetic disorders that could be successfully managed with early diagnosis and standardized treatment[11, 17]. The clinical guidelines emphasizes the importance of timely diagnosis. Abnormal serum ceruloplasmin and 24-hour urinary copper excretion are used for WD diagnosis before genetic determination[4, 18]. However, decreased ceruloplasmin levels and increased 24-hour urinary copper excretion levels within diagnostic limits could be absent in WD patients, and they are increasingly recognized to have low predictive values.[4]

Moreover, these two diagnostic parameters mostly originated from studies conducted 20 years ago. Therefore, there is an unmet need for updating and explaining these diagnostic parameters because not all patients were able to undergo genetic WD diagnosis. We sought to update these diagnostic parameters to facilitate more suitable management for the affected individuals.

Among various clinical expressions, Kayser-Fleischer (K-F) rings are regarded as the hallmark of WD. Pathological copper accumulations in the eyes lead to these ophthalmological features that occur in almost 100% of neurological WD patients [19]. Some studies have focused on the correlation between K-F rings and clinical severity and therapy among neurological WD patients [20-22]. Similar to K-F rings, characteristic imaging abnormalities in the common basal ganglia, thalamus and brainstem also indicate copper deposition in the corresponding brain areas of WD patients. However, there is a great lack of studies exploring the correlation between K-F rings and brain magnetic resonance imaging (MRI) in neurological WD. Therefore, as the second part of our study, we further investigated the frequency and relevance of these imaging abnormalities in Chinese WD patients.

Materials and methods

Patients

In this multicenter observational cohort study, subjects were recruited from the Departments of Neurology at three participating centers of Zhejiang University, Fudan University and Fujian Medical University from January 2004 to February 2019. Patients were clinically diagnosed according to the Leipzig Score[8] and included in the study when they were confirmed to carry *ATP7B* pathogenic variants in two different alleles[15, 23]. Variants were accessed for pathogenicity

based on the American College of Medical Genetics (ACMG) standards and guidelines [24]. This study was approved by the ethics committees of the local hospitals. After obtaining informed consent from the affected individuals or legal guardians, clinical and genetic records were evaluated and analyzed.

Phenotype evaluation

All patients underwent a standardized clinical review, physical examinations and biochemical tests. Detailed clinical and laboratory data were prospectively collected. Age at onset and symptoms at presentation are regarded as important phenotypic markers of WD[18]. Age at onset was defined as the age when the initial symptom developed due to WD. At the baseline visit, initial syndromes were distinguished as hepatic, neurological, psychiatric, osteomuscular and asymptomatic subtypes, and renal and hematological involvement [8, 19]. Specifically, abnormal liver function at routine testing or accidental finding of K-F rings was classified as asymptomatic. Osteomuscular subtype mainly included premature osteoarthritis, skeletal deformity and pathological bone fractures. Serum ceruloplasmin was measured enzymatically by its copper-dependent oxidase activity and 24-hour urinary copper was collected avoiding contamination. A slit-lamp examination by an experienced ophthalmologist was required to detect corneal Kayser-Fleischer. Structural brain abnormalities, including focal high-intensity lesions and atrophy, were investigated using brain MRI scans.

Statistical analysis

Quantitative data were expressed as the mean ± the SD or median (interquartile range) based on normality (age at onset, serum ceruloplasmin and 24-hour urinary copper excretion). For the data meeting the normal distribution and homogeneity of variance, differences between two groups were analyzed by Student's *t* test,

and differences among three or more groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. For non-normally distributed data or unequal variance parameters, we used the Mann-Whitney U test to compare the difference between two groups and the Kruskal-Wallis test and post hoc test to compare differences among three or more groups. Categorical data (phenotypic classification, the presence or absence of K-F rings, the abnormality or normality of brain MRI) are depicted as numbers of patients (n) and percentages (%). Pearson chi-squared, continuity correction, or Fisher's exact test was applied to assess the distribution of categorical variables among groups. Statistical significance was indicated at $p< 0.05$. All statistical analyses were performed using SPSS (version 26.0) and Prism (version 8.0) statistical software.

Results

Mutation spectrum

A definitive diagnosis of WD was made in 715 unrelated patients based on Leipzig criteria[8] and *ATP7B* mutation screening, and mutation analysis of 696 patients had been described previously[15, 23]. Specifically, the current finding revealed in the sequence of p.N1270S and p.G943D was slightly different from that previously reported[15].

Clinical profiles

The sample size of the summarized clinical and genetic data could be different among some items (**Table 1).** The mean age at onset was 17.0 years (range 1~59), while the mean age at diagnosis was 18.8 years (range 1~62). Among 715 unrelated WD patients, 529 patients were symptomatic, whereas 146 were asymptomatic. The remaining 40 patients were uncategorized because of a

combination of clinical presentations. Of 529 symptomatic patients, 121 were categorized as hepatic subtype, 355 as neurological, 28 as osteomuscular, and 25 as psychiatric. Together, the age at onset of patients with hepatic (16.9 ± 1.0, p=0.0105) or osteomuscular subtypes (13.0 ± 1.3, p=0.0001) were significantly younger than those with neurological subtypes (19.4 ± 0.45). There was no significant difference in age at onset between patients with neurological and psychiatric subtypes (p=0.5257) or between those with hepatic and osteomuscular subtypes (p=0.0788) (**Figure 1A**).

Serum ceruloplasmin

Serum ceruloplasmin levels were collected among 636 unrelated WD patients, ranging from 6 to 170 mg/L (**Table 1**). After confirming a non-Gaussian distribution, the reference range of serum ceruloplasmin level was determined to be 18.93~120.00 mg/L according to a quantile of 0.025~0.975. Specifically, among 636 patients, the initial symptoms were hepatic in 117, neurological in 325, psychiatric in 22, osteomuscular in 28, and asymptomatic in 137, while 7 patients were uncategorized. The median serum ceruloplasmin level was significantly higher in asymptomatic WD patients [30.00 mg/L (range, 20.50~70.00)] than in neurological WD patients [27.00 mg/L (range 20.00~41.00, p=0.003)] and osteomuscular WD patients [23.00 (range 20.00~39.75, p=0.048)] (**Figure 1B**). The comparisons among other groups were not statistically significant. Moreover, no significant difference was detected in ceruloplasmin levels between WD patients with K-F rings (28.70(20.00~42.00) mg/L) and those without K-F rings (30.00(20.00~69.00) mg/L, z=-1.920, p=0.055) (**Figure 2A**).

Urinary copper excretion

The measurement of 24-hour urine copper excretions may help in WD diagnosis.

Here, 24-hour urinary copper was collected from 131 untreated WD patients. According to the previous guidelines[4, 18], a 24-h urinary copper excretion level above 100 μg is taken as indicative of WD, while greater than 40 μg could be indicative of childhood WD. In our study, urinary copper concentrations were abnormal (>100 μg/24 h) in 80 out of 131 (61%) patients, of whom 43 patients were aged >14 y. Urinary copper excretion levels > 40 μg/24 h were found in 57 out of 67 patients aged ≤14 y and in 61 out of 64 patients >14 y. The proportion of patients whose urinary copper excretion levels were ≤40 μg was much larger in the younger group (age≤14 y) than in the older group (age>14 y) (p=0.001). Furthermore, we analyzed urinary copper excretion variation between patients who were positive and negative for K-F rings. The 24-hour urinary copper excretion level was significantly higher in WD patients with K-F rings (160.30 μg, range 85.93~367.34) than in those without K-F rings (104.78 μg, rnage 57.33~187.75 μg, z=-2.601, p=0.009) (**Figure 2B**). Of 80 patients having positive K-F rings and urinary cooper recordings simultaneously, 8 were asymptomatic, 52 neurological, 14 hepatic, 3 psychiatric, and 3 osteomuscular. Whereas, of 44 patients with negative K-F rings and urinary cooper recordings simultaneously, 37 were asymptomatic, 2 neurological, 2 hepatic, 2 psychiatric, and 1 osteomuscular. However, age variation was not found between the normal and abnormal urinary copper excretion subgroups (p=0.6558).

Correlation between the presence of K-F rings and brain MRI abnormalities

Among 355 WD patients with neurological involvement, 329 were positive for K-F rings. Of the remaining 26 patients, 8 were negative for K-F rings, and 18 refused ophthalmological examination when visiting the clinic. Additionally, of 355 neurological WD patients, 277 underwent brain MRI. The characteristic lesions of

widespread and focal T2 hyperintense signal regions were detected in 245 WD patients, including in the basal nuclei, thalamus, brainstem activating system, cerebellum, and white matter, and even extensive brain atrophy, whereas 32 WD patients had normal MRI. The remaining 78 patients did not undergo brain MRI examination.

K-F rings were detected in 97.6% (329/337) of patients with neurological symptoms, whereas 88.4% (245/277) of neurological WD patients manifested brain MRI abnormalities. Furthermore, we analyzed the correlation between the presence of K-F rings and MRI abnormalities among patients with neurological involvement. We found that 244 patients had simultaneous manifestations of abnormal MRI findings and K-F rings, and only one patient with abnormal brain MRI findings was simultaneously negative for the presence of K-F rings. Among 32 patients with normal MRI findings, 20 were positive for K-F rings, and 2 were negative. The remaining 10 had no record of K-F ring findings (**Figure 3**).

Discussion

This study represents the largest cohort of genetically diagnosed WD patients to date. We studied phenotypic characteristics and diagnostic parameters in 715 unrelated WD patients. Overall, men and women seemed to be equally affected in our study. The proportion of patients with psychiatric onset (n=25, 3.5%) was relatively low in our study compared with other reports, with a rate of 4~10%[25-27]. Additionally, 28 patients initially presented with osteomuscular involvement, of whom 8 were reported previously [28]. This slightly higher proportion allowed us to identify the rare subtype and to avoid unnecessary surgery and irreversible damage.

The serum ceruloplasmin level is generally lower than 200 mg/L in WD

patients[4], although a small number of WD patients may manifest normal levels[29, 30], which are thought to be the consequence of hepatic inflammation. Even so, most studies showed that 90~100% of patients had decreased ceruloplasmin[31-33]. Our results showed that all patients had ceruloplasmin concentrations lower than 200 mg/L, confirming the high sensitivity for the detection of WD. These data demonstrated that serum ceruloplasmin could help to detect WD patients. Furthermore, the 97.5% confidence interval for ceruloplasmin level in our data showed that if suspected WD patients had ceruloplasmin levels <120 mg/L, this would be strongly indicative of a diagnosis of WD.

The 24-hour urinary copper level, another important diagnostic parameter in untreated WD patients, represents the amount of non-ceruloplasmin-bound copper in the circulation. In general, the conventional limit taken as a diagnostic of WD is more than 100 μg/24 h[4, 18]. This diagnostic index among WD patients in the present study showed relatively low sensitivity compared with serum ceruloplasmin. Approximately 39% of WD patients were found to have less than 100 μg of urinary copper excretion in 24 h at presentation, which is different from other reports of about 16-23% patients having less than 100μg/24 h urinary copper[30 34, 35]. The proportion of patients with 24-hour urinary copper excretion ≤ 40 μg was significantly different between the >14 age group and the ≤14 age group. This finding indirectly showed that in the case of children, 24-hour urinary copper excretion> 40 μg could be indicative of WD. Moreover, urine copper excretion levels seemed to be related to the presence of K-F rings but unrelated to age. It was reported that K-F rings only exist in about 44-62% of patients with hepatic WD, and even disappear in children with liver disease[4]. In the current study, 65% of patients with positive K-F rings and urinary recordings were neurological, while

84.1% of patients with negative K-F rings and urinary recordings were asymptomatic. Based on phenotype variation, it could serve as reasonable explanation why urine copper excretion seems to be related to K-F rings. In short, the combination of a low serum ceruloplasmin (<120 mg/L) level and a high 24-hour urinary copper excretion (>40 μg) level could help to diagnose WD patients.

When K-F rings are absent, serum ceruloplasmin and 24-hour urinary copper excretion might be unreliable since they are susceptible to variation with liver disease, kidney disease, and even age[4, 18]. Among diverse clinical expressions of WD, K-F rings have been regarded as the key diagnostic trait and seem to be closely correlated to neurological WD, despite being relatively less common in hepatic and asymptomatic disease[22, 36]. Previous studies demonstrated that K-F rings were present in 90.4~100% of patients with neurological and psychiatric WD [37, 38]. Our study shared similarities with a high frequency of 97.6% and highlighted the possibility of invariable presence in patients with neurological symptoms. Furthermore, we found that there are K-F rings paralleling neuroradiological alterations highlighted by brain MRI, and the former seemed to be a prerequisite for abnormal MRI alterations in WD patients. The clinical coexistence emerging from the analysis of our study showed that if a neurological WD patient had MRI pathological findings with the absence of K-F rings, the clinical WD diagnosis for the patient should be highly prudent, and other inherited metabolic disorders should be considered. Patients with aceruloplasminemia overlapped with WD in some aspects, including decreased serum ceruloplasmin and brain MRI abnormalities[39, 40]. Therefore, for the establishment of WD diagnosis, the co-occurrence of MRI abnormalities and K-F rings seems to be more meaningful.

In conclusion, the importance of available and rapid testing to confirm the diagnosis of WD could be underestimated. Serum ceruloplasmin levels (<120 mg/L) and 24-h urinary copper excretion levels (generally>100 μg, even>40 μg in childhood) could strongly be indicative of WD. However, we must realize that no single laboratory test allows a certain diagnosis of WD. The ceruloplasmin level could be correlated with disease phenotype. This indirectly suggested that neurological manifestations in WD could be due to either copper accumulation or ceruloplasmin function abnormalities. Ceruloplasmin seems to be more sensitive for screening for WD than 24-h urinary copper excretion. Serum ceruloplasmin, 24-hour urinary copper excretion and K-F rings equivalently reflect disease status. Most importantly, WD diagnosis should be highly doubted when patients present with both movement disorders and brain MRI abnormalities but not K-F rings.

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Figure legends

Figure 1. A: The comparisons of age at symptomatic onset among 5 different phenotypes. *p<0.05, ***p<0.0005. **B**: Comparisons of serum ceruloplasmin among WD patients with hepatic, neurological, osteomuscular, and psychiatric symptoms and asymptomatic patients. *p<0.05, **p<0.005.

Figure 2. A: The comparisons of serum ceruloplasmin levels between patients who were positive and negative for the presence of K-F rings. **B**: The comparisons of 24-hour urinary copper excretion levels between patients who were positive and negative for the presence of K-F rings. *p<0.05.

Figure 3. The presence and absence of K-F rings and cranial MRI abnormalities among patients with neurologic WD.

Table 1. The epidemiological and clinical overview of 715 WD patients

			Case (n)
Gender (M/F)			404/311
Age at onset (years)	range	1~59	669
	Mean ± SD	17.03±26.95	
Age at diagnosis(years)	range	1~62	666
	Mean ± SD	18.84±29.61	
Onset form	Asymptomatic		146
	Hepatic		121
	Neurologic		355
	Osseomuscular		28
	Psychiatric		25
Ceruloplasmin (mg/L)	range	6~170	636
	Median	47	
	Quantile(0.025-0.975)	18.93~120	
Copper in urine (μg/24h)	range	10.6~1900	131
	Median	150.2	

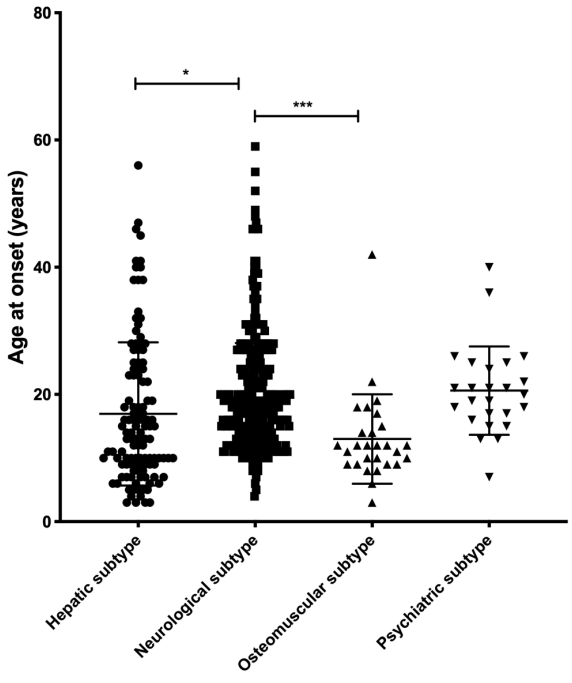

Figure 1A

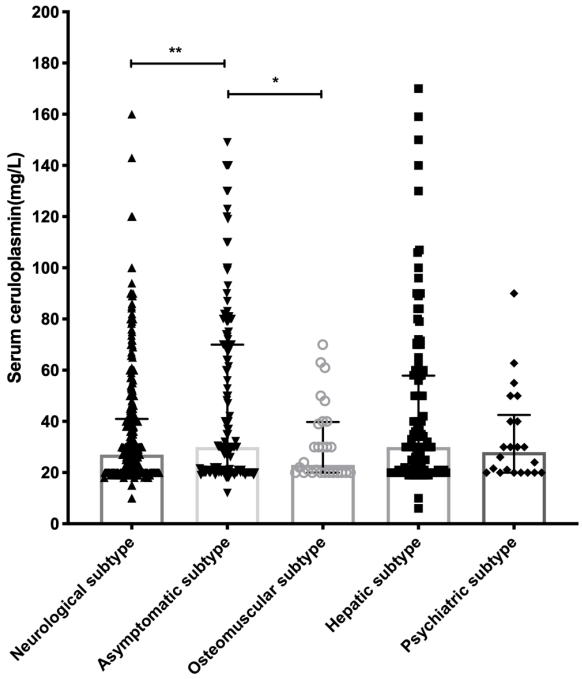

Figure 1B

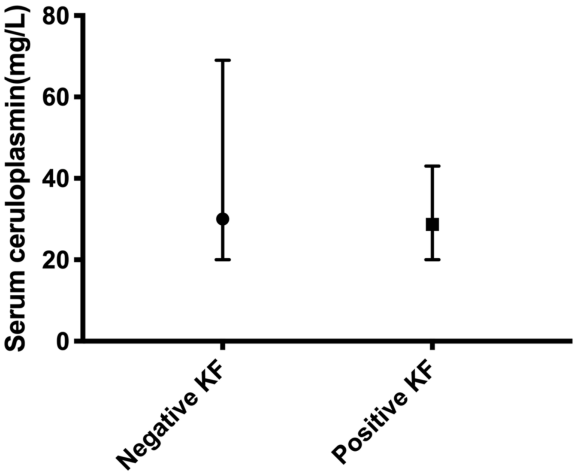

Figure 2A

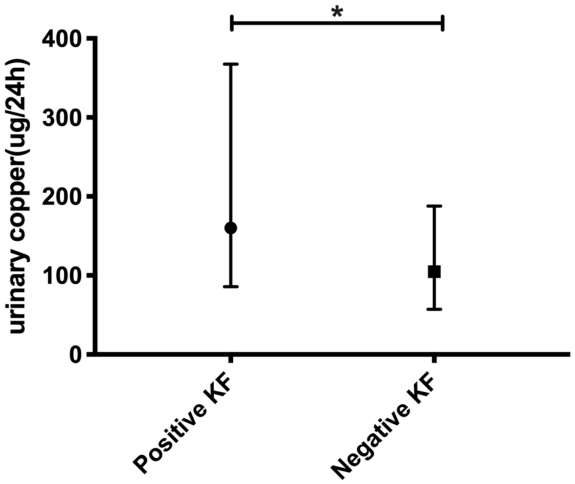

Figure 2B

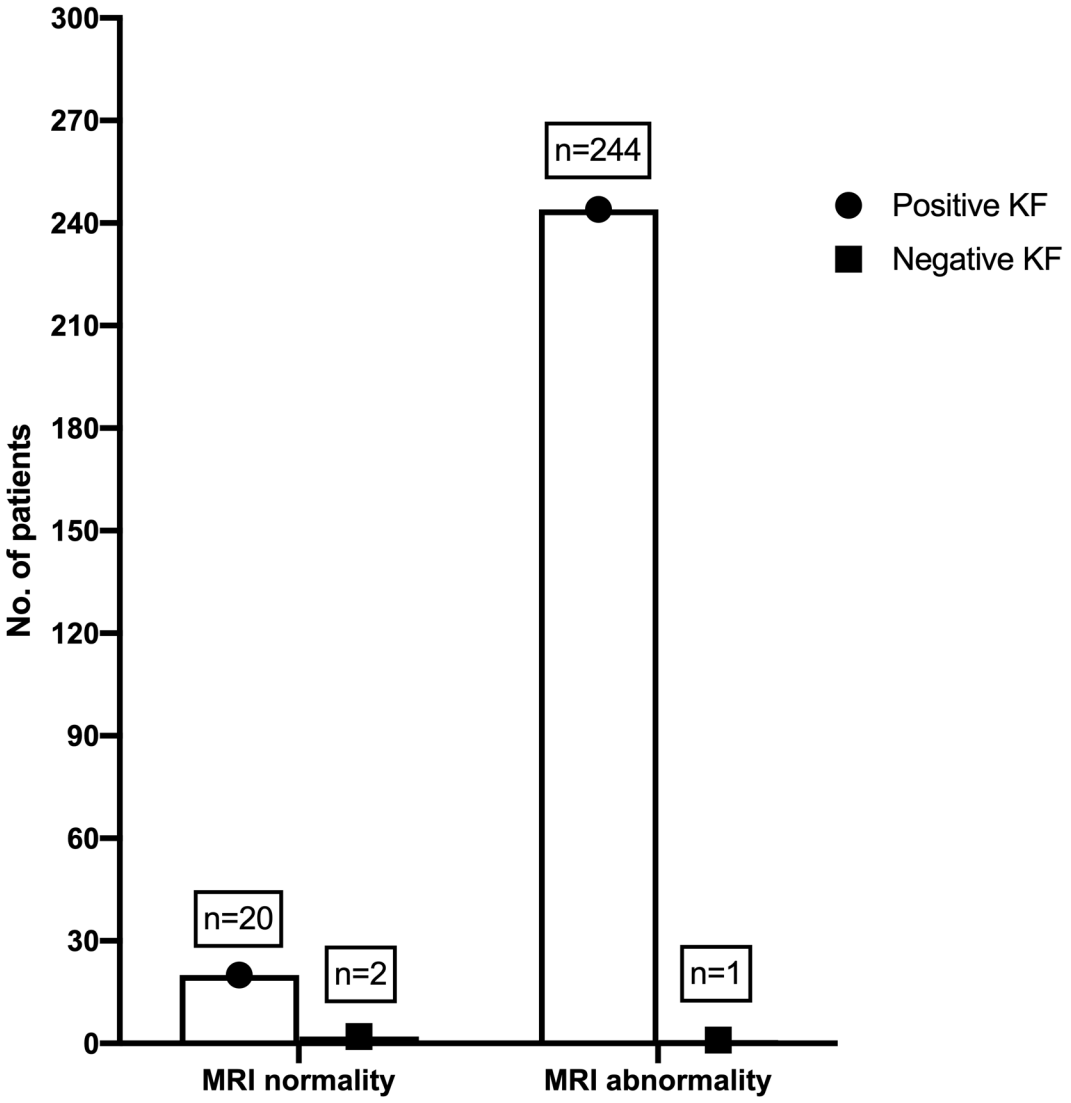
300
270
240
210
180
150
120
90
60
30
0
No. of patients
n=244
n=20
n=2
n=1
Positive KF
Negative KF
MRI normality
MRI abnormality

Figure 3