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ORIGINAL ARTICLE

Decreased serum antioxidant capacity in patients with Wilson disease is associated with neurological symptoms

Radan Bruha · Libor Vitek · Zdenek Marecek · Lenka Pospisilova · Sona Nevsimalova · Pavel Martasek · Jaromir Petrtyl · Petr Urbanek · Alena Jiraskova · Ivana Malikova · Martin Haluzik · Peter Ferenci

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Abstract
Background & Aims Wilson disease (WD) is an inherited disorder of copper disposition caused by an *ATP7B* transporter gene mutation, leading to copper accumulation in predisposed tissues. In addition to a genetic predisposition, other factors are likely to contribute to its clinical manifestation. The aim of the study was to assess whether oxidative stress affects the phenotypic manifestation of WD.
Methods In 56 patients with WD (29 men; 26 with the hepatic form, 22 with the neurologic form, and eight asymptomatic; mean age 38.5±12 years), total serum antioxidant capacity (TAC) and inflammatory parameters (hs-CRP, IL-1β, IL-2, IL-6, IL-10, and TNF-α) were analyzed and related to the clinical manifestation, and mutations of the *ATP7B* gene. The control group for the TAC and inflammatory parameters consisted of 50 age- and gender-matched healthy individuals.
Results WD patients had a significantly lower TAC ($p<0.00001$), lower IL-10 levels ($p=0.039$), as well as both higher IL-1β ($p=0.019$) and IL-6 ($p=0.005$) levels compared to the control subjects. TNF-α, hs-CRP, and IL-2 did not differ from the controls. Patients with the neurological form of WD had a significantly lower TAC than those with the hepatic form ($p<0.001$). In addition, the lower TAC was associated with the severity of the neurological symptoms ($p=0.02$). No relationship between the inflammatory parameters and clinical symptoms was found.
Conclusions Data from our study suggest that the increased oxidative stress contributes significantly to the clinical

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R. Bruha · L. Vitek · J. Petrtyl
First Faculty of Medicine, 4th Department of Internal Medicine,
Charles University in Prague,
Prague, Czech Republic

L. Vitek · A. Jiraskova · I. Malikova
1st Faculty of Medicine, Institute of Clinical Biochemistry and
Laboratory Diagnostics, Charles University in Prague,
Prague, Czech Republic

Z. Marecek · P. Urbanek
Department of Internal Medicine, Central Military Hospital and
1st Faculty of Medicine, Charles University,
Prague, Czech Republic

L. Pospisilova · P. Martasek
1st Faculty of Medicine, Pediatric Department, Laboratory for
Mitochondrial Disorders, Charles University in Prague,
Prague, Czech Republic

S. Nevsimalova
1st Faculty of Medicine, Department of Neurology,
Charles University in Prague,
Prague, Czech Republic

M. Haluzik
1st Faculty of Medicine, 3rd Department of Internal Medicine,
Charles University in Prague,
Prague, Czech Republic

P. Ferenci
Department of Internal Medicine III,
Medical University of Vienna,
Vienna, Austria

R. Bruha (✉)
4th Department of Internal Medicine, General Teaching Hospital,
U Nemocnice 2,
128 08, Prague 2, Czech Republic
e-mail: bruha@cesnet.cz

manifestation of WD; as a lower TAC is associated with the neurological symptoms in WD patients.

Introduction

Wilson disease (WD) is an autosomal recessive inherited disorder of copper metabolism, resulting in the accumulation of copper in the organs and tissues; principally in the liver, brain, cornea, and kidneys (Scheinberg et al. 1981). The disease is caused by a deficiency of a copper-transporting P-type ATPase, encoded by the *ATP7B* gene (Bull et al. 1993, Tanzi et al. 1993), which is located on chromosome 13. More than 500 different mutations have been identified in this gene. The H1069Q mutation seems to be prevalent in Central Europe (Thomas et al. 1995). Copper accumulation results in different symptoms, ranging from acute or chronic liver disease to neuropsychiatric disturbances (Roberts and Schilsky 2008, Sternlieb and Scheinberg 1968, Merle et al. 2007).

The striking variability of the phenotypic manifestations in patients with the same genetic defect is one of the most extraordinary features of WD. Some authors suggest that the presence of a specific genotype (*ATP7B* H1069Q mutation) is associated with the late and neurological presentation of WD (Stapelbroek et al. 2004); while others could find no such correlation (Vrabelova et al. 2005, Nicastro et al. 2009). It is evident, that factors other than mutations in the *ATP7B* gene are likely to be involved in the clinical manifestation (Wright et al. 2009). For instance, certain genetic variants of apolipoprotein E (*APOE*) were shown to have a protective effect in patients with the neurological form of WD (Schiefermeier et al. 2000). Recently, other genes like *murr1* (Weiss et al. 2008), *BIRC4/XIAP* (Weiss et al. 2010) and *Atox 1* (Simon et al. 2008) were suggested as putative modifiers of WD, but their exact role still awaits for elucidation.

The mechanism of tissue damage from copper overload is complex and not fully understood (Gu et al. 2000); Zischka et al. (2011) suggested that liver damage is due to structural and functional mitochondrial impairment induced by excessive mitochondrial copper load. In fact, copper-induced free-radical formation, lipid peroxidation (Videla et al. 2003), and subsequent oxidative damage of hepatocytes' mitochondria are reported to be crucial in WD pathogenesis (Sokol et al. 1994). In fact, decreased levels of different antioxidants and increased oxidative stress (Nagasaka et al. 2009, Ogihara et al. 1995) have been described, using different methods, in patients with the hepatic presentation of WD (Nagasaka et al. 2006). Experiments using HepG2 cells have shown that proteins involved in antioxidant defense are dramatically altered by chronic copper exposure (Jimenez et al. 2002). The idea that antioxidants may protect the isolated hepatocytes from copper toxicity (Sokol et al. 1996), and thus should be explored as potential therapeutic agents in WD, has been articulated (Fryer 2009). Since the susceptibility to oxidative stress may contribute to the severity of both the WD disease symptoms and phenotypic manifestations, the aim of this study was to evaluate serum antioxidant capacity and inflammatory markers in patients with WD, with relationship to the clinical form of the disease.

Patients and methods

The clinical data and laboratory parameters of 56 consecutive patients, with established WD, were evaluated and analyzed at the 4th Department of the Internal Medicine, General University Hospital in Prague. The diagnosis of WD was based on clinical, biochemical, histological, or genetic parameters. All patients met the diagnostic criteria of WD (Ferenci et al. 2003). Blood sampling for TAC and measurement of cytokines was done prospectively between 2008 and 2009. The clinical and laboratory characteristics of the evaluated group are given in Table 1. The patients were classified as having either primarily the hepatic or the neurological form, using the generally accepted criteria (Ferenci 2005). Briefly, the hepatic form was defined as the presence of liver disease and the absence of any neurological symptoms upon a detailed neurological examination at the time of diagnosis. Patients presenting with an acute hepatic disorder with jaundice, in the absence of previous history of liver disease were classified as H1; those patients with any type of chronic liver disease were classified as H2. A diagnosis of liver cirrhosis was based upon liver biopsy. The neuropsychiatric form was defined by the presence of neurological and/or psychiatric symptoms at the time of diagnosis. At the time of presentation, patients with neuropsychiatric symptoms and significant liver disease (i.e., liver cirrhosis) were classified as N1; those without any symptomatic liver disease were classified as N2. The severity of neurologic disability was evaluated using the WD rating scale (WDRS) (Walter et al. 2005). This scaling system includes 5 items: dysarthria, akinesia, ataxia, tremor, and dystonia; each item is rated as follows: 0 - not present, 1 - slight, 2 - moderate, or 3 - severe. All patients with the neuropsychiatric manifestation of WD had undergone a neurological examination, with an estimation based on the WDRS.

Adherence to the treatment was evaluated by monitoring the excretion in the urine of copper or zinc. The response to treatment was evaluated by the improvement of clinical symptoms in the neuropsychiatric patients, or by improvements in the liver tests (or the disappearance of signs of liver failure).

Table 1 Clinical characteristics and laboratory parameters in 56 patients with Wilson disease at our center. All asymptomatic patients were siblings of indexed cases

Parameter	A Neurological-psychiatric form	B Hepatic form	C Asymptomatic patients	*P*-value
Number of patients	22	26	8	
Male/female (no. patients)	7/15	18/8	4/4	AxB: 0.023
Age at testing	45.6±9.2	31.5±8.9	41.6±12.9	AxB: <0.001 BxC: 0.017
H1069Q *ATPB1* gene mutations: homo/compound hetero/no mutation (No. of patients)	9/9/4	10/11/5	2/5/1	AxB: NS
Copper/ dry weight of liver tissue (μg/g)	682.5±303	753.9±305	943.5±594	AxBxC: NS
Ceruloplasmin (g/L)	0.126±0.13	0.105±0.05	0.08±0.05	AxBxC: NS
Cu in urine (μmol/24 h)	7.02±4.9	6.36±4.8	6.22±4.4	AxBxC NS
Diagnostic score according to international criteria (17)	10.73±2.7	8.12±2.5	7.75±2.3	AxB: p=0.001 AxC: NS BxC:NS
Type of presentation (No. of patients)	N1: 16 N2: 6	H1: 3 H2: 23	-	-
Cirrhosis (% of patients)	59	38	25	AxB: NS
Length of follow-up (years)	16.9±12	12.9±7	22.54±11	AxBxC: NS

SD – standard deviation

The control group for TAC and inflammatory parameters consisted of 50 age- and gender-matched healthy individuals recruited from the employees of the General University Hospital in Prague.

The study was approved by the local Ethics Committee, and constructed to conform to the latest ethical guidelines of the Declaration of Helsinki. Informed consent, including consent for a genetic examination, was obtained from all subjects.

Laboratory examination

Biochemical parameters were measured by routine laboratory techniques. A genetic examination was performed in all 56 patients. The serum TAC was determined as the serum peroxyl radical scavenging capacity, using the fluorometrical method, measuring the relative proportion of chain breaking antioxidant consumption present in the serum compared to that of Trolox (a reference and calibration antioxidant compound) (Iuliano et al. 2000). In this test, dipyridamole is used as a highly efficient chain breaking antioxidant, whose consumption is blocked in the presence of endogenous chain breaking antioxidants, such as ascorbic acid, vitamin A or E, or uric acid. The TAC test was validated against the oxygen consumption method (Iuliano et al. 2000).

Highly sensitive CRP (hs-CRP) was measured by immunonephelometry (Behring Nephelometer II), inflammatory cytokines (IL-1β, IL-2, IL-6, IL-10, and TNF-α multiplex kit from Linco Res., USA) by Luminex technology. Up to 1994, assays of the copper content in the liver tissue were performed using the spectrophotometric method (Ojeda et al. 1995); and since then by atomic absorbance spectroscopy technique (Evenson 1988). Ceruloplasmin was measured using immunonephelometric method from sera obtained between 2008 and 2009 in all patients.

Molecular analyses

Mutations in the *ATP7B* gene were analyzed using a combination of polymerase chain reaction (PCR)/restriction fragment length polymorphism (RFLP) analyses and DNA sequencing. Shortly, genomic DNA was extracted from peripheral blood samples, anticoagulated with EDTA, according to a standard protocol. The screening of the prevalent mutation H1069Q in exon 14 of the ATP7B gene was carried out as published elsewhere (Maier-Dobersberger et al. 1997). Four other mutations in *ATP7B* gene- W779X and P768fs in exon 8, P1133fs in exon 15, and Q447fs in exon 3 were screened by PCR/RFLP. Second, the coding sequences of exons 1–21 of the ATP7B gene, with the flanking exon/intron boundaries, were analyzed by DNA sequencing (Bruha et al. 2011). The implementation of genetic testing in the diagnostic process was subsequently introduced, as the different mutations were published.

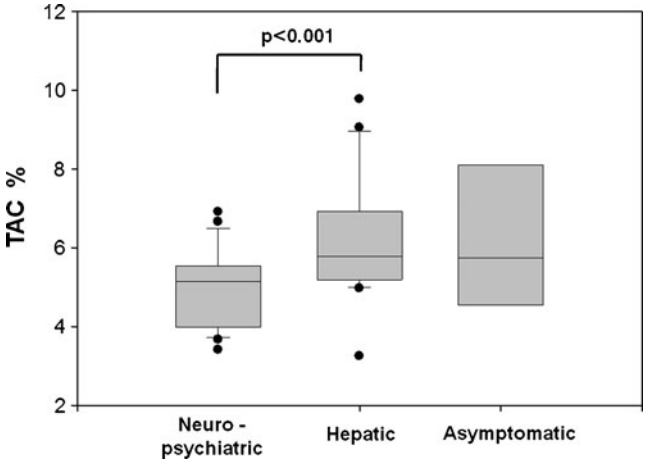

Fig. 1 Total antioxidant capacity in different groups of patients with WD

Statistical analysis

The results are expressed as the mean ± SD and/or median (IQ range) when data were not distributed normally. The t-test and the Mann–Whitney Rank Sum Test were used for data comparison. An ANOVA test was used to compare TAC in the neurological, hepatic, and asymptomatic forms of WD and the clinical parameters. The correlation between genetic mutations and the WD phenotype was evaluated with the Fisher's exact test. A stepwise discriminant analysis was performed to assess the variables affecting WD manifestation. The values were adjusted for age, when required. The level of significance for the entire study was set at $P<0.05$. All of the statistics were computed using BDMP Statistical Software version PC90 (Cork Technology Park, Ireland), and Statistica CZ 08.

Table 2 The influence of selected laboratory and clinical variables on the neuropsychiatric manifestation of WD

Variable	Odds ratio	95% CI	P-value
TAC	4.272	1.41-12.96	0.01
Cu in liver	1.001	0.99-1.00	0.61
Length of treatment	1.025	0.92-1.14	0.641
Cirrhosis	0.222	0.02-2.2	0.196
H1069Q homo	1.541	0.19-12.72	0.688
Gender	5.552	0.77-40.1	0.089

CI: confidence interval

Results

Fifty-six patients with confirmed WD (29 men and 27 women, with a mean age of 38.5±11.6, range 16–63 years old) were enrolled in the study. Patients were followed up for 15.5±10 years (1–41 years) at the time of evaluation. The type of WD manifestation, age at its appearance, and basic diagnostic parameters are given in Table 1. The presence of the neurological form of WD was more frequently associated with female gender (p=0.023, Table 1). The *ATP7B* H1069Q mutation was proven to be the most frequent molecular defect, with a prevalence rate of 55%. No relationship between the genetic mutations and the clinical manifestation of WD was found in the group of patients examined.

Total antioxidant capacity

Patients with WD had a significantly lower TAC than did the controls (median; IQ range: 5.50; 4.90 - 6.55 vs. 6.51;

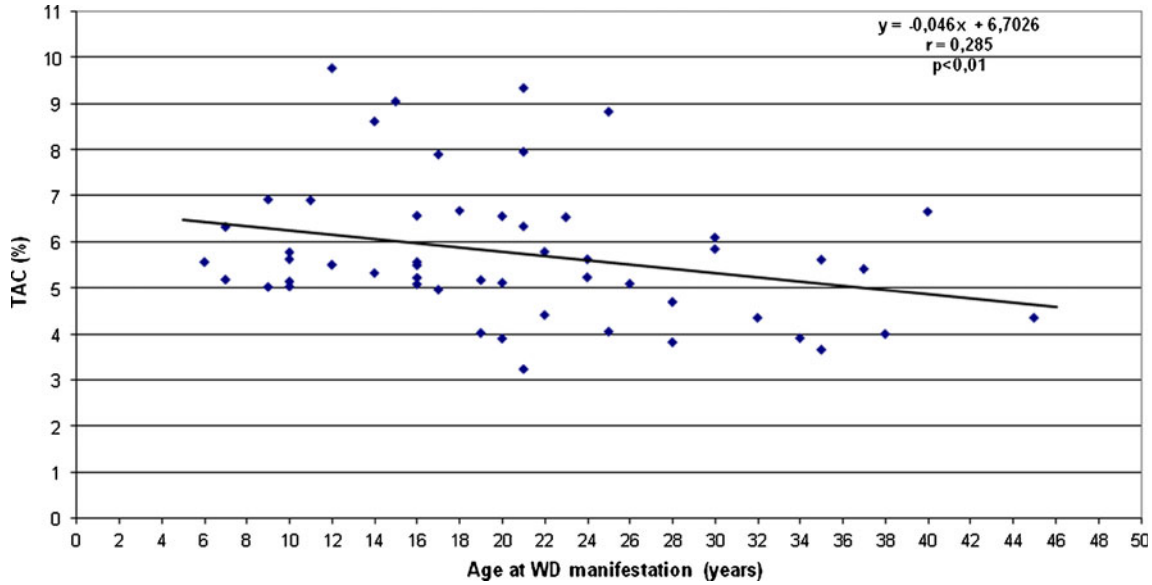

Fig. 2 Relationship between total antioxidant capacity and age of the onset of Wilson disease

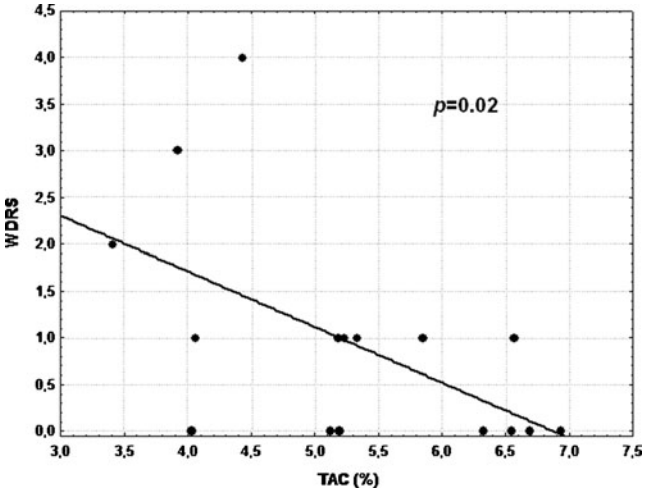

Fig. 3 Relationship between total antioxidant capacity (TAC) and WDRS

5.51 - 8.15, p<0.00001). The TAC value was unrelated to the patients' age. Patients with neurological symptoms had their TAC significantly lower than those with the hepatic form (median; IQ range: 5.14; 4–5.5 vs. 5.78; 5.3 - 6.9, p< 0.001) (Fig. 1). The TAC correlated negatively with the age at disease onset (Fig. 2). No relationships between TAC and either the type or length of treatment, or the presence of cirrhosis, was observed. No relationship between TAC and serum total bilirubin concentration (p=0.33), serum albumin concentration (p=0.39) or INR value (p=0.93) was observed in patients with hepatic form of WD. The TAC values did not differ between patients with neurological presentation irrespective of the presence (median; IQ range 5.2; 4.2-5.6) or the absence (median; IQ range 4.5; 3.8-5.3) of symptomatic liver disease (i.e., N1 or N2 type of presentation; p=0.71).

The multivariate step-by-step regression analysis, comparing the TAC with multiple clinical parameters (Cu content in the liver tissue, presence of liver cirrhosis, presence of H1069Q homozygocity, gender), revealed that the TAC value was the only significant factor for discriminating the presence of either the hepatic or neurological form of WD (Table 2).

In addition, a negative correlation between the severity of neuropsychiatric symptoms and TAC was observed in this group of WD patients (r=0.559, p=0.02, Fig. 3).

Inflammatory cytokines

Patients with WD had significantly higher concentrations of proinflammatory cytokines (IL-1β, IL-6), and lower levels of anti-inflammatory cytokine IL-10 (Table 3 and 4).

The concentrations of IL-2, hs-CRP, and TNF-α did not differ between patients with WD and the controls. No relationship was found between the concentration of inflammatory cytokines and the form of WD presentation. The multivariate step-by-step regression analysis, evaluating the effect of biochemical and pro-inflammatory markers, proved that the TAC was the only significant factor differentiating between the neurological and hepatic forms of WD (Table 5).

Discussion

The clinical manifestation of WD is highly variable, ranging from hepatic symptoms to neurological deterioration, and presenting from early childhood to late middle age. Obviously, factors other than WD gene mutations modifying the phenotypic presentation might contribute. For example, the increased expression of Golgi membrane protein GP73 in the hepatocytes has been found to be a feature of the hepatic form of WD, unrelated to copper overload (Wright et al. 2009). Additionally, certain genetic variants of APOE were shown to have some role in WD pathogenesis (Schiefermeier et al. 2000). Simon et al. (2008) analyzed the metallochaperone antioxidant-1 (*Atox1*) gene sequence in Wilson disease patients, but they found no significant differences between WD patients stratified according to *Atox1*gene variants. The role of *Murr1/COMMD1* in the biliary excretion pathway of copper downstream of Golgi-localized *ATP7B* has been studied by Weiss et al. (2008). The authors concluded that the *Murr1/COMMD1* protein might have a function in the

Table 3 Serum concentrations of inflammatory cytokines and serum total antioxidant capacity in all patients with WD and controls

Parameter	WD all patients (n=56)	Controls (n=50)	*P*-value WD x controls
TAC (%)	5.50 (4.9-6.6)	6.51 (5.5-8.2)	<0.00001
hs-CRP	0.5 (0.2-1.3)	0.4 (0.2-1)	0.329
TNFα (pg/ml)	3.93 (2.8-5.9)	3.96 (2.8-5.3)	0.669
IL-1β (pg/ml)	0.19 (0.1-0.4)	0.13 (0.1-0.2)	0.019
IL-2 (pg/ml)	0.76 (0.3-1.4)	0.53 (0.3-1)	0.360
IL-6 (pg/ml)	1.19 (0.6-2.3)	0.71 (0.3-1.4)	0.005
IL-10 (pg/ml)	9.26 (5.6-13.7)	6.89 (4.3-10)	0.039

Data are expressed as median with IQ range

Table 4 TAC and serum concentrations of inflammatory cytokines in patients with different forms of WD

Parameter	WD hepatic form (n=26)	WD neurologic form (n=22)	WD asymptomatic (n=8)	*P*-value Hepatic x neurological
TAC (%)	5.78 (5.3-6.9)	5.14 (4–5.5)	5.75 (4.7-7.6)	<0.001
hs-CRP	0.5 (0.2-0.8)	0.45 (0.2-1.3)	1.5 (0.9-3.6)	0.959
TNFα (pg/ml)	4.11 (3.0-5.9)	3.92 (2.6-5.3)	4.17 (2.9-6.5)	0.878
IL-1β (pg/ml)	0.23 (0.1-0.4)	0.13 (0.1-0.3)	0.24 (0.2-0.6)	0.156
IL-2 (pg/ml)	0.94 (0.4-1.5)	0.53 (0.2-1.1)	0.9 (0.5-2.3)	0.136
IL-6 (pg/ml)	1.21 (0.6-1.7)	1.17 (0.5-3.3)	1.05 (0.7-3.8)	0.918
IL-10 (pg/ml)	10.11 (5.7-13.3)	7.53 (5.1-14.6)	9.16 (6.3-14.5)	0.877

Data are expressed as median with IQ range

later steps of the copper excretion pathway with no involvement in the copper-mediated ATP7B translocation. The same authors studied the role of *BIRC4/XIAP* polymorphisms in individuals with copper overload (Weiss et al. 2010). In the cohort of 98 patients with WD, the two patients with variants leading to amino acid exchanges in the BIRC4/XIAP protein showed a remarkably early disease onset.

Oxidative stress is believed to play an important role in the pathophysiology of WD (Videla et al. 2003) and the ability of the human body to deal with increased oxidative stress may be one of the factors contributing to the WD disease symptoms' severity and the phenotypic presentation. One potential etiologic factor leading to tissue damage in WD is the oxidation of low-density lipoproteins (LDL) (Ferns et al. 1997). LDL susceptibility toward oxidation depends on the presence of low molecular weight antioxidants, such as vitamin E (α-tocopherol). Von Herbay et al. (1994) described that patients with WD have low vitamin E levels in plasma compared to controls presumably due to enhanced formation of reactive oxygen species. Unfortunately, only 12 patients with WD were included in this study and no data about the type of WD presentation were provided. Similar results were published in the group of 45 patients with WD (Rodo et al. 2000); however, in that study, there was also no reporting of data concerning the type of WD. The WD patients in this study had significantly lower plasma levels of vitamin E compared to controls; but no change in LDL susceptibility toward lipid peroxidation was found; thus, raising the question of whether other antioxidants play a role in the LDL defense against oxidation. In the study by Sinha et al. (2005) on 34 WD patients, most of which with a neurological phenotype, low vitamin E was found compared to the controls. However, the differentiation between the patients with neurological or hepatic symptoms was not given, and the disease description raises some questions, as the mean age of the disease's presentation, of the mostly neurological patients in this study, was only 16 years. In our study, for the first time, TAC in patients was compared with different types of WD presentation (i.e., neurological, hepatic, or symptomatic). As expected, we found a lower TAC in our patients with WD, most probably due to oxidative stress induced by accumulated copper (Videla et al. 2003). This is consistent with decreased levels of different antioxidants (Sinha et al. 2005), and increased oxidative stress (Nagasaka et al. 2009, Ogihara et al. 1995) previously reported. However, in none of these studies were differentiations performed between the hepatic or neurological forms of WD. Surprisingly, we found a strong relationship between low TAC to a neurologic manifestation of WD; suggesting that the serum antioxidant capacity is associated with the phenotypic manifestation of WD. The question remains to be answered, whether higher oxidative stress or a diminished susceptibility to deal with increased oxidative stress, accounts for this observation.

On the other hand, copper is also able to induce an antioxidant response as this element is a catalytic center for antioxidant enzymes such as CuZn-dependent SOD, thereby increasing the expression and activity of these enzymes upon acute conditions (Sies 1993). Up to now, little is known about the role of copper and ATP7B in the central nervous system. In some brain areas, such as in the pineal gland, ATP7B is expressed and functionally active; but no reports are available for humans (Borjigin et al. 1999).

Table 5 The role of different inflammatory parameters and TAC on the neuropsychiatric manifestation of WD: multivariate analysis (logistic regression)

Variable	OR	95% CI	P-value
TAC	2.326	1.13-4.78	0.02
IL-2	0.688	0.32-1.49	0.34
IL-6	1.01	0.91-1.13	0.83
IL-10	0.979	0.89-1.07	0.64
IL-β	8.288	0.09-725.2	0.35
hs-CRP	0.942	0.73-1.22	0.65
TNFα	0.975	0.73-1.31	0.867

OR: odds ratio; CI: confidence interval

However, increasing evidence supports an important role for heavy metals in neurobiology, and copper is a major source of free radicals in the brain. It is generally accepted, that an increased systemic copper load contributes to neuronal pathogenetic processes by increasing the production of reactive oxygen species and promoting pro-inflammatory changes, including neurodegenerative pathologies such as Parkinson's, Alzheimer, prion diseases, or amyotrophic lateral sclerosis (Spisni et al. 2009). The results of our clinical examination clearly suggest that the higher the WDRS, the lower the total serum antioxidant capacity; supporting the role of oxidative stress in the pathogenesis of neurological disturbances in WD. This is consistent with our findings demonstrating that decreased TAC was even linked to sleep disturbances in patients with WD (Nevsimalova et al. 2011).

As far as the methodology of our study, several issues should be addressed. First, it might be argued that acute changes in patients with active liver disease may affect the redox status. However, we measured the TAC in a cohort of stabilized patients, treated for a long period (more than 16 years for the neurologic form, and more than 12 years for the hepatic form), where the influence of acute liver disease was minimal. None of our patients had signs of acute liver injury. Second, patients with neurological involvement are older and have had a longer period of treatment and follow-up compared to patients with the hepatic form, which might account for the variable degree of TAC. However, in a multivariate analysis, TAC was not dependent on treatment modality, the length of observation, or the age of our patients. It seems that TAC independently correlates with the clinical presentation. This fact also explains the negative association between TAC and the age of disease onset, as the neurological form manifests later than the hepatic form.

Less information is available concerning the inflammatory markers of patients with WD. The deep association of oxidative stress with inflammation and fibrosis of liver tissue have been shown in various liver diseases, especially non-alcoholic fatty liver (Angulo 2002), but this fact has not been well documented in WD. Nevertheless, we found substantial pro-inflammatory profiles in our WD patients. This fact can reflect the activated inflammatory reaction induced by copper overload (Britton 1996).

In conclusion, data from our study suggest that increased oxidative stress contributes significantly to the clinical presentations of WD, with a particular role in the neurological manifestation of WD. Further, well-designed, and controlled studies are needed to clarify the role of dietary antioxidants in the treatment of WD.

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