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Impact of treatment on blood–brain barrier impairment in Wilson's disease

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ABSTRACT

Introduction. Our study assessed changes in concentrations of serum markers for brain damage and blood–brain barrier (BBB) dysfunction in untreated and treated Wilson's disease (WD) patients, and examined correlations between these changes and neurological impairment.

Objective. These results hold the potential to determine BBB impairment and neurological advancement in WD to develop the most effective treatment for patients with severe neurological deterioration.

Material and methods. The study groups included 171 patients with WD (77 with hepatic and 94 with neurological manifestations), treated either for up to 5 or 15 years, and 88 healthy controls. Serum concentrations of intercellular adhesion molecule 1 (ICAM1), P-selectin, matrix metallopeptidase 9 (MMP9), glial fibrillary acidic protein (GFAP), and S100 calcium-binding protein B (S100B) were measured before and during anti-copper treatment. The Unified Wilson's disease Rating Scale (UWDRS) was used to assess neurological advancement.

Results. ICAM1 concentrations were elevated before and during anti-copper treatment compared to controls ($p < 0.01$), but therapy led to substantial decreases both in patients with hepatic ($p < 0.01$) and in patients with neurological manifestations ($p < 0.05$). P-selectin concentrations remained elevated before and during treatment ($p < 0.05$) regardless of the treatment duration and disease form. MMP9 concentrations before treatment were lower ($p < 0.05$), but reached control levels during treatment. GFAP concentrations were significantly elevated only in untreated patients with neurological symptoms in the longer-treated group compared to controls ($p < 0.05$). A significant reduction during treatment was observed only in the shorter-treated neurological group ($p < 0.05$). No substantial changes were observed in S100B. Only ICAM1 concentrations positively correlated ($r = 0.27$, $p < 0.001$) with the UWDRS.

Conclusions. Our results provide evidence of endothelial activation in WD. However, inconclusive GFAP results, and no increase in S100B, do not allow us to conclude whether the reactive gliosis is not prominent or alternatively whether the BBB is disrupted. Elevated ICAM1 concentrations and their correlation with neurological advancement indicate BBB impairment. A decrease in ICAM1 during treatment suggests that the inflammatory process is reduced, and the BBB partially repaired. Decreased MMP9 concentrations may be the result of active liver fibrosis and higher copper concentrations. Elevated P-selectin concentrations indicate a systemic inflammatory process.

Key words: blood–brain barrier, serum inflammatory markers, UWDRS, neurodegeneration, Wilson's disease

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Introduction

Wilson's disease (WD) is a rare autosomal recessive disorder of the copper metabolism caused by mutations in the copper-transporting P-type adenosine triphosphate (*ATP7B*) gene, resulting in copper overload in hepatocytes with associated liver pathology [1]. Excess copper is also released into the circulation with secondary pathological accumulation in other tissues, particularly the brain, leading to neurological and psychiatric symptoms. In WD, concentrations of total serum copper may be decreased due to low ceruloplasmin formation, but concentrations of toxic non-ceruloplasmin-bound copper (NCC) are elevated.

The mechanisms by which copper crosses the blood–brain barrier (BBB) remain unclear. However, it has been demonstrated that copper transport into the brain is mainly achieved in the form of NCC [2]. Furthermore, copper importer Cu transporter 1 (CTR1), and exporters ATP7A and ATP7B, are essential in ensuring copper-requiring processes and preventing copper accumulation in the brain [3].

In WD patients, elevated NCC concentrations with a concomitant uptake of copper into the brain through CTR1, and an impaired copper re-export into the blood due to an ATP7B defect can result in brain copper accumulation [4]. Intracellular copper accumulation can induce mitochondrial stress, leading to brain cell death [5]. As a result, an inflammatory process may be triggered, which can aggravate the brain damage. This inflammatory process is characterised by endothelial cell activation, cytokines production, oxidative stress induction, the stimulation of microglia, astrocytes, and the further migration of inflammatory cells into the central nervous system (CNS) [6].

The BBB consists of a tightly connected monolayer of brain endothelial cells and pericytes separated by the basement membrane and unsheathed by astrocytic end-feet [7]. Entry of leukocytes from the blood into a tissue is a multi-step process that includes rolling adhesion, firm adhesion, and extravasation. This requires a series of different leukocyte adhesion molecules, including selectins for rolling adhesion, and immunoglobulin family members for firm adhesion [8]. Under normal conditions, the endothelial layer remains at rest and the expression of adhesive molecules, such as intercellular adhesion molecule 1 (ICAM1) [9–15] and P-selectin [16–19], increases under the influence of inflammatory processes. With the increase in expression, adhesive molecules may be shed from the surface of the activated endothelial cells and released into the circulation in soluble form.

The pathogenesis of diseases associated with BBB damage may involve metalloproteinases, enzymes involved in the degradation of basement membranes, and extracellular matrix proteins. One of the most widely investigated metalloproteinases is matrix metallopeptidase 9 (MMP9) [20].

WD is neuropathologically characterised by a dominant alteration of astrocytes. Therefore, it has been considered as a primary gliopathy, represented by progressive astrocytic changes, taking the form of generalised proliferation and hypertrophy concomitant with nonspecific degeneration of astrocytes [21].

Activated or damaged astrocytes can release specific substances into the cerebrospinal fluid (CSF) and blood, which can serve as biomarkers of CNS injury and BBB disruption. One of these proteins is glial fibrillary acidic protein (GFAP), an emerging biomarker in brain and spinal cord disorders [22–24].

Another marker of CNS injury is S100 calcium-binding protein B (S100B) [25–30], produced mainly by astrocytes, which is also a marker of early BBB disruption that may precede brain damage. At the same time, massive elevations in S100B are indicators of extensive brain damage [31].

The characteristics of the serum markers for brain damage and blood–brain barrier dysfunction, i.e. ICAM1, P-selectin, MMP9, GFAP and S100B, are summarised in Table 1.

In patients with WD, copper-reducing therapy with D-penicillamine, zinc sulfate, trientine or bis-choline tetrathiomolybdate may lower NCC concentrations with later redistribution from the brain into the blood and subsequent copper excretion [32]. The copper-related toxic effects on the brain and the BBB in neurological WD patients have been demonstrated by an increased albumin ratio (AR) in CSF versus serum which normalises during anti-copper therapy. In addition, an initial worsening of the neurological condition after starting chelator therapy has been linked to the disturbance of BBB function, measured as a transient increase in AR [32].

Thus, brain damage from NCC may start at the BBB, facilitating further unregulated copper entry into the brain [33], and inflammatory processes in the liver and brain may impair BBB function and contribute to CNS damage.

This study aimed to examine changes in concentrations of serum markers for brain damage and BBB dysfunction in untreated and treated WD patients, and assess correlations with the severity of neurological impairment.

Clinical rationale for the study

The aim of this study was to assess changes in serum concentrations of ICAM1, P-selectin, MMP9, GFAP and S100B in untreated and treated WD patients, and examine correlations between these changes and neurological advancement. Our goal was better determining BBB impairment and identifying possible improvements to treatment for WD.

Material and methods

This study was approved by the Committee for Ethics in Human Research at the Institute of Psychiatry and Neurology in Warsaw, Poland. Informed written consent was obtained from each participant. This publication was prepared without any external source of funding. All authors declare that they have no conflict of interest.

Table 1. Characteristics of serum markers for brain damage and blood–brain barrier dysfunction

Serum marker	Type	Location	Function	Clinical significance in neurogenerative and liver diseases
ICAM1	Intercellular adhesion molecule 1, glycoprotein of immunoglobulin family	Expressed constitutively on surface of various cell types, especially endothelial cells	Firm adhesion of leukocytes to endothelium and their transendothelial migration to sites of inflammation [9]	Increased in active multiple sclerosis [10, 11], viral encephalitis [11], acute ischaemic stroke [12], Alzheimer's disease [13], liver diseases and other inflammatory processes [14, 15]
P-selectin	Cell adhesion glycoprotein of selectin family	Stored within platelets and endothelial cells, exposed on surface after inflammatory stimulation	Initial recruitment of leukocytes, efficient leukocyte capturing [16]	Reports in neurodegenerative disorders reveal inconsistencies [17, 18], elevated in liver diseases [19]
MMP9	Matrix metallopeptidase 9	Produced by many cell types, including inflammatory cells	Degradation of basement membranes and extracellular matrix proteins [20]	Higher in WD patients with neurological than in hepatic forms and higher in hepatic presentations than in controls [20]
GFAP	Glial fibrillary acidic protein, intermediate filament	Produced mainly by astrocytes	Involved in structure and function of cell's cytoskeleton [22]	Emerging biomarker in brain and spinal cord disorders, elevated in mild traumatic brain injury, progressive multiple sclerosis [22], higher in WD patients with neurological manifestations [23], but in another study no significant differences between neurological, hepatic and control groups, and no association with severity of neurological impairment [24]
S100B	S100 calcium-binding protein B	Produced mainly by astrocytes	Involved in cell cycle progression, cell differentiation, and cytoskeletal-membrane interactions [25]	Potential parameter of glial activation in brain damage and neurodegeneration [26], studied in Parkinson's disease [27], Alzheimer's disease [28, 29] and Creutzfeldt–Jakob disease [30], but not yet in WD

WD — Wilson's disease

Study population

The study was performed in the Second Department of Neurology, Institute of Psychiatry and Neurology, Warsaw, Poland. Patients were diagnosed with WD based on a combination of clinical examination, abnormal copper results, the presence of a Kayser-Fleischer ring, typical abnormalities seen by brain magnetic resonance imaging (MRI), and genetic testing results. The form of the disease was determined based on the results of a clinical examination and additional tests (basic laboratory liver tests, ultrasound examination of the liver and brain MRI). Patients classified as hepatic manifestations did not present abnormalities in neurological assessment or brain MRI [34]. Patients were treated with either D-penicillamine or zinc sulfate in standard doses. The Unified Wilson's disease Rating Scale (UWDRS) was used to assess the neurological status advancement, including part II (disability, based on the Barthel Scale) and part III (detailed neurological examination) [35]. Patients with abnormalities in neurological assessment or brain MRI were scored according to the sum of parts II and III of the UWDRS.

The control group consisted of healthy volunteers with similar sex and age distributions and no history of liver disease, neurological or mental disease, chronic inflammatory disease, or infectious disease.

Blood collection

Blood was collected twice from the patients in the experimental group, before and during anti-copper treatment for periods of up to 5 or 15 years, and once from the patients in the control group. After collection, the venous whole blood samples (10 mL) were incubated at room temperature for c.30 minutes to form a clot. Then the blood was centrifuged for 10 minutes at 3,000 rpm at 4°C. After centrifugation, the obtained supernatant was decanted. Serum was pooled successively and stored at –80°C.

ICAM1, P-selectin, MMP9, GFAP and S100B measurements

ICAM1, P-selectin, MMP9, GFAP and S100B serum concentrations were measured with sandwich-type enzyme-linked immunosorbent assays in accordance with the manufacturers' instructions (R&D Systems, Minneapolis, MN, USA; ELK Biotechnology, Wuhan, China). Absorbance at 450 nm was measured with Multiskan Go (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

Shapiro-Wilk test was used to estimate the normality of the studied groups for statistical analyses. Normal distribution

Table 2. Characteristics of Wilson's disease (WD) patients and controls

					WD form						
					Hepatic n = 77				Neurological n = 94		
		Controls (C) n = 88	All WD patients n = 171	p	5-year treated n = 47	p	15-year treated n = 30	p	5-year treated n = 53	p	15-year treated n = 41	p
Age	before	34 (30–43)	29 (22–38)	**< 0.001**	25 (21–31)	**< 0.001**	23.5 (19–41)	**< 0.001**	31 (24–38)	**0.0043**	33 (24–42)	0.18
	during		33 (25–43)	0.070	26 (23–33)	**< 0.001**	33 (26–47)	0.47	32 (27–39)	0.069	44 (33–50)	**0.015**
Sex (female)		46 (52%)	94 (55%)	0.68	29 (62%)	0.29	21 (70%)	0.091	24 (45%)	0.42	20 (49%)	0.71
Treatment												
D-penicillamine		–	72 (42%)	–	21 (45%)	–	10 (33%)	–	27 (51%)	–	14 (34%)	–
Zinc sulfate		–	99 (58%)	–	26 (55%)	–	20 (67%)	–	26 (49%)	–	27 (66%)	–

Results are shown as medians (interquartile range) or numbers (percentages). Statistically significant values are given in bold and $p < 0.05$ was considered a statistically significant difference; p-values refer to comparison of preceding group to controls

was not observed, and therefore results were presented as medians and interquartile ranges (IQR). Nonparametric tests such as Mann-Whitney U and Wilcoxon for matched pairs were used to compare groups. Correlation analysis was performed with the Spearman correlation test. All results for categorical variables were presented as numbers and percentages. Categorical data was analysed with the Chi-square test. Significance was assumed at $p < 0.05$. Statistica 13.3 software was used for data analysis.

Results

Patient characteristics

Detailed data is set out in Table 2. Of the 181 patients with WD, 10 were lost to follow-up. Of the 171, 100 were treated for up to 5 years (47 with hepatic and 53 with neurological forms) and 71 were treated for up to 15 years (30 with hepatic and 41 with neurological forms). The WD group consisted of 94 women (55%) and 77 men (45%), with a median age of 29 years (IQR, 22–38 years). Regarding treatment, 72 patients (42%) received D-penicillamine and 99 (58%) were treated with zinc sulfate. The group of 88 healthy controls comprised 46 women (52%) and 42 men (48%), with a median age of 34 years (IQR, 30–43 years).

ICAM1, P-selectin, MMP9, GFAP and S100B serum concentrations

Detailed data is set out in Table 3. ICAM1 serum concentrations were significantly elevated before anti-copper treatment in patients with hepatic or neurological forms compared to the control group ($p < 0.001$). Anti-copper therapy led to a substantial decrease both with hepatic ($p < 0.01$) and neurological manifestations ($p < 0.05$) compared to before treatment. In the 15-year treated group in patients with hepatic symptoms, ICAM1 concentrations were not significantly different from the control group. In patients with neurological forms, ICAM1 concentrations remained significantly elevated after 15 years compared to controls ($p < 0.01$).

P-selectin serum concentrations remained elevated before and during treatment compared to the control group ($p < 0.05$) and there were no significant differences between patients with hepatic and neurological manifestations. These values did not decrease regardless of the treatment duration or the disease form.

MMP9 serum concentrations before treatment were lower than in the control group ($p < 0.05$) but reached the level of the controls during the treatment. There were no significant differences in MMP9 concentrations between patients with hepatic and neurological symptoms.

There were no significant differences in GFAP in patients with the hepatic form compared to controls. GFAP serum concentrations were significantly elevated only in untreated patients with neurological symptoms in the longer-treated group compared to controls ($p < 0.05$). There was a significant reduction during treatment only in the shorter-treated group ($p < 0.05$).

No substantial changes were observed in S100B serum concentrations in patients with either form of WD compared to the control group or during treatment.

There were no significant differences in serum concentrations of the tested markers in patients treated with D-penicillamine or zinc sulfate (data not shown).

Table 3. Changes in concentrations of serum markers (ng/ml) for brain damage and blood–brain barrier dysfunction in patients with hepatic and neurological forms, treated for up to 5 or 15 years

		ICAM1	P-selectin	MMP9	GFAP	S100B
Controls (C) n = 88		180 (140–290)	130 (62–180)	560 (210–790)	0.3 (0.0–1.3)	0.78 (0.72–0.88)
Hepatic form, n = 77						
5-year treated group, n = 47	0 y	350 (270–490)	200 (120–330)	290 (170–500)	1.0 (0.0–4.1)	0.83 (0.72–1.0)
	5 y	270 (210–370)	170 (93–310)	470 (340–730)	0.5 (0.0–2.4)	0.77 (0.73–0.92)
	p-value 0 y *vs.* C	**< 0.001**	**0.0012**	**0.0074**	0.052	0.12
	p-value 5 y *vs.* C	**< 0.001**	**0.034**	0.99	0.22	0.55
	p-value 0 y *vs.* 5 y	**0.0016**	0.43	**< 0.001**	0.051	0.061
15-year treated group, n = 30	0 y	340 (250–450)	270 (120–410)	370 (150–750)	0.8 (0.0–3.5)	0.85 (0.74–0.95)
	15 y	220 (170–300)	250 (110–470)	510 (320–760)	0.0 (0.0–2.5)	0.82 (0.75–0.91)
	p-value 0 y *vs.* C	**< 0.001**	**0.0010**	0.41	0.17	0.17
	p-value 15 y *vs.* C	0.16	< 0.001	0.87	1.0	0.17
	p-value 0 y *vs.* 15 y	**0.0011**	0.057	0.29	0.17	0.20
Neurological form, n = 94						
5-year treated group, n = 53	0 y	350 (250–490)	160 (63–250)	320 (140–620)	0.1 (0.0–3.5)	0.82 (0.73–0.97)
	5 y	290 (190–400)	180 (97–300)	420 (230–810)	0.0 (0.0–1.5)	0.84 (0.73–1.0)
	p-value 0 y *vs.* C	**< 0.001**	0.12	**0.021**	0.58	0.23
	p-value 5 y *vs.* C	**0.0020**	**0.0055**	0.87	0.27	0.20
	p-value 0 y *vs.* 5 y	0.027	0.24	**0.016**	**0.014**	0.59
15-year treated group, n = 41	0 y	330 (260–430)	180 (120–300)	320 (210–550)	1.9 (0.0–4.7)	0.86 (0.73–0.96)
	15 y	260 (210–360)	220 (140–410)	550 (340–710)	1.3 (0.0–4.3)	0.80 (0.73–0.97)
	p-value 0 y *vs.* C	**< 0.001**	**0.010**	0.085	**0.021**	0.082
	p-value 15 y *vs.* C	**0.0027**	**< 0.001**	0.81	0.057	0.23
	p-value 0 y *vs.* 15 y	**0.0061**	0.15	**0.0018**	0.68	0.12

Results are shown as medians (interquartile range). Statistically significant values are given in bold and $p < 0.05$ was considered a statistically significant difference; GFAP — glial fibrillary acidic protein; ICAM1 — intercellular adhesion molecule 1; MMP9 — matrix metallopeptidase 9; S100B — S100 calcium-binding protein B

Correlations between serum concentrations of brain damage, BBB dysfunction markers, and severity of neurological impairment

The serum concentration of ICAM1 positively correlated ($r = 0.27$, $p < 0.001$) with the advancement of the neurological status assessed according to the sum of parts II and III of the UWDRS (Fig. 1). In contrast, the concentration of MMP9 showed a negative correlation ($r = -0.25$, $p < 0.01$) with the advancement of the neurological status (Fig. 2). The concentrations of the other markers tested did not show significant correlations with the severity of the neurological status.

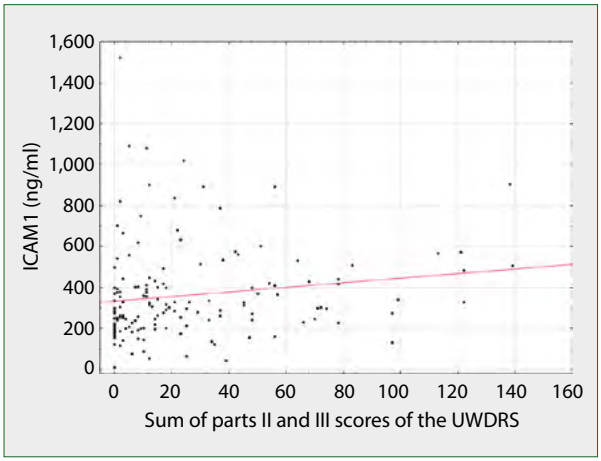

Figure 1. Positive correlation between serum concentration of ICAM1 and advancement of neurological status assessed according to UWDRS parts II and III ($r = 0.27$, $p < 0.001$)

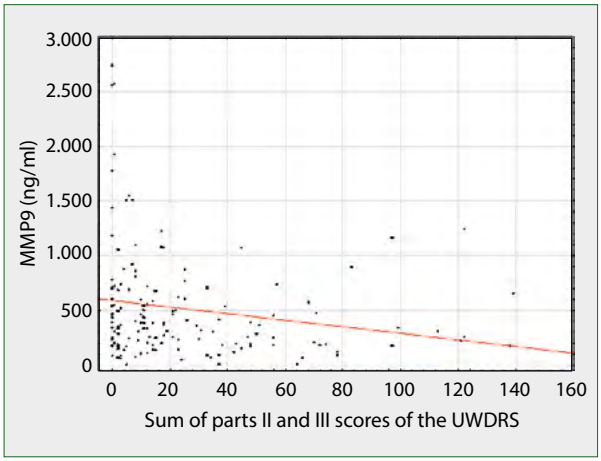

Figure 2. Negative correlation between serum concentration of MMP9 and advancement of neurological status assessed according to UWDRS parts II and III ($r = -0.25$, $p < 0.01$)

Correlations between serum concentrations of brain damage, BBB dysfunction markers, and age

MMP9 concentrations were positively correlated ($r = 0.3$, $p < 0.01$) with age in the control group, but this effect was not observed in the WD group (data not shown). Concentrations of the other BBB dysfunction markers tested showed no correlation with age in the control or WD groups.

Discussion

These results provide evidence of endothelial activation in WD patients, probably due to a toxic copper effect. The most promising result concerns ICAM1. Elevated concentrations of endothelial activation markers have previously been observed not only in neurological disorders but also in chronic liver diseases and other inflammatory processes [14, 15, 19]. Therefore, increased serum values may not necessarily indicate damage to the BBB, but rather a systemic inflammatory process due to liver disease.

However, the positive correlation between serum ICAM1 concentrations and the severity of the neurological status found in our study suggests that the BBB in WD patients is impaired. Thus, ICAM1 may become a potential biomarker of neurological impairment severity. A decrease in ICAM1 concentrations during treatment suggests that the inflammatory process is reduced and the BBB partially repaired. This is also confirmed by ICAM1 returning to control concentrations only in long-treated patients with hepatic symptoms. It might be helpful to determine the concentrations of markers such as ICAM1 in the CSF, but this is not done routinely, and we did not collect CSF samples during our study. It would be interesting to correlate concentrations of these markers with AR in CSF versus serum to minimise the effect of systemic inflammatory response and to confirm the BBB interruption.

In our study, P-selectin concentrations remained elevated with treatment, which may indicate an ongoing inflammatory process in WD patients. P-selectin concentrations did not allow a distinction to be made between hepatic and neurological manifestations and to assess the severity of CNS damage.

Unexpectedly, MMP9 serum concentrations before treatment were lower in WD patients than in the control group, but reached the level of the controls during the treatment. This is inconsistent with previous results [20], in which serum MMP9 concentrations were higher in patients with neurological WD than in patients with hepatic WD, which in turn were higher than in the control group. Nevertheless, our study examined patients regarding their treatment duration, and included larger WD and control groups. The positive correlation between MMP9 concentrations and age could impact upon the results of patients studied after several years, but this correlation was observed only in the control group, and not in the WD group. However, the process of liver fibrosis may be essential. It has been shown that fibrotic matrix stiffness downregulates MMP9 expression and secretion in hepatic stellate cells, thus promoting fibrosis perpetuation [36]. Increased copper concentrations may also downregulate MMP9 expression, as has been demonstrated in rat livers [37]. Thus, untreated patients with higher copper concentrations and liver fibrosis should have lower MMP9 concentrations. Also, MMP9 concentrations increase after anti-copper treatment, which improves liver and brain function and reduces the inflammatory process [38]. Therefore, MMP9 is not a suitable marker for assessing BBB disruption in WD.

Elevated serum GFAP concentrations in untreated WD patients with neurological manifestations can serve as a biomarker for different subtypes of WD. This was previously reported [23], although not confirmed in another study [24]. In our study, GFAP concentrations were significantly elevated

only in untreated patients with neurological symptoms in the longer-treated group compared to controls. This may indicate astrocytic damage in WD patients with neurological manifestation. No substantial changes were observed with serum S100B in our study. Therefore, we cannot definitely determine whether the reactive gliosis is not prominent, or if the BBB is disrupted in WD.

Our study investigated commonly used markers of brain damage and BBB impairment, broadly reviewed in various neurological disorders. Unfortunately, most of them are unspecific for BBB vasculature and depend on their peripheral production. Therefore, it is essential to investigate other promising indicators of BBB disruption, including vascular endothelial cadherin, claudin-5, ocludine, vascular endothelial growth factor, as well as anti-aquaporin 1 antibodies, which have been studied in primary BBB permeability diseases such as neuromyelitis optica spectrum disorders and multiple sclerosis [39].

Further studies involving other inflammatory molecules and brain-specific proteins in serum, but also in CSF, are necessary to get a fuller picture of BBB involvement in WD. It may also be valuable to investigate the concentrations of markers at additional timepoints. Moreover, adherence to therapy is crucial, which should have been considered in this study.

Confirmation of BBB damage in WD patients with severe neurological deterioration may prompt the consideration of temporary immunosuppressive therapy to silence the inflammatory response and rebuild the BBB to reduce further CNS damage, as proposed in some neurological diseases such as refractory status epilepticus [40]. In addition, it might be beneficial to investigate the correlations between BBB dysfunction markers and copper metabolism parameters to determine optimal anti-copper treatment.

Conclusions and clinical implications

Elevated serum concentrations of ICAM1, and their correlation with the advancement of neurological status, suggest that the BBB in WD patients is impaired, especially in patients with neurological symptoms. Furthermore, these results hold the potential to assess neurological impairment and indicate the role of endothelial dysfunction in this process. However, unclear GFAP results and no increase in S100B do not allow us to conclude whether the reactive gliosis is not prominent, or alternatively if the BBB is disrupted in WD. In addition to copper toxicity, impaired immune functions might influence neurological advancement. Therefore, characterising inflammatory molecules and their relationship to neurological deterioration warrants further investigations to determine the most effective treatment for patients with WD.

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