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REVIEW

Biomarker Discovery in Wilson's Disease—A Path Toward Improved Diagnosis and Management: A Comprehensive Review

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Abstract

Wilson's disease (WD) is a rare autosomal recessive disorder characterized by defective copper metabolism, which leads to hepatic and neurological damage. The clinical presentation of WD varies significantly, often resulting in delayed diagnosis and an increased risk of irreversible complications. Current diagnostic tools, including biochemical assays, imaging techniques, and genetic testing, lack sufficient specificity and sensitivity, highlighting the need for novel biomarkers for early diagnosis and treatment monitoring. This review explores emerging biomarkers for both hepatic and neurological manifestations of WD, including blood-based molecular markers such as cytokines, proteases, oxidative stress indicators, inflammasomes, and gut microbiota signatures. Recent studies have identified neurofilament light chain (NfL), pentraxin 3 (PTX3), caspase-3/XIAP, and NLRP3 inflammasome activation as promising indicators of neurological impairment. Additionally, markers like soluble CD163 (sCD163) and apoptosis antigen 1 (APO-1) show potential for assessing hepatic dysfunction. Metabolomic and proteomic analyses further suggest distinct molecular profiles associated with different WD subtypes, while microRNA-based biomarkers offer novel insights into disease progression. Identifying and validating these biomarkers could enhance early diagnosis, predict neurological deterioration, and optimize treatment strategies, ultimately improving patient outcomes. Further research is needed to integrate these biomarkers into clinical practice and establish standardized protocols for their use in WD management.

Keywords Wilson's disease · Novel biomarkers · Early diagnosis · Neurological impairment · Treatment monitoring

Abbreviations

AD	Alzheimer disease
APO1	Apoptosis antigen 1
ATP7B	Copper-transporting P-type ATPase
Bcl-2	B cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
CTR1	Copper transporter 1
Cu	Copper
CuONPs	Copper oxide nanoparticles
CREB	CAMP response element-binding protein
FHF	Fulminant hepatic failure
GAS	Global assessment scale
GEC	Galactose elimination capacity
GSH	Glutathione
HILIC-QTOF MS	Hydrophilic interaction liquid chromatography-quadrupole time of flight mass spectrometry
IL-10	Interleukin-10
IL-8	Interleukin-8
IL-23	Interleukin-23
LNC RNA	Long non-coding RNA
MDA	Malondialdehyde
MIRNA	Micro-RNA
MRI	Magnetic resonance imaging

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NfL	Neurofilament light chain
NLRP3	NOD-, LRR-, and Pyrin domain-containing protein 3
PAMPs	Pathogen-associated molecular patterns
PTX3	Pentraxin 3
ROS	Reactive oxygen species
TAC	Total antioxidant capacity
Th	T helper
TrkB	Tropomyosin receptor kinase B
UWDRS	Unified Wilson's Disease Rating Scale
WD	Wilson disease
WHO	World Health Organization
XIAP	X-linked inhibitors of apoptosis protein

Introduction

Wilson disease (WD) is an inherited autosomal recessive disorder of copper metabolism that mainly affects the liver and the brain [1]. The mutation of the gene ATP7B encoding the copper transporter leads to its impaired function and excessive deposition of copper in the tissues [2]. The clinical manifestation of WD is highly variable; the symptoms are nonspecific and might be very subtle. However, the key features include a combination of liver disease and neurological impairment during adolescence or early adulthood. The toxic effects of copper lead to varied forms of liver damage, ranging from mild hepatitis to cirrhosis or acute liver failure. Neurological disturbances may progress over time and include symptoms like tremors, involuntary movements, dystonia, and dysarthria [3]. Some patients remain asymptomatic and are detected by family screening [4].

Due to the broad and nonspecific clinical spectrum of WD, delayed diagnoses remain an issue. The 2022 practice guidance from the American Association for the Study of Liver Diseases emphasizes a multidisciplinary approach to diagnosing and managing Wilson disease [5]. It highlights the importance of integrating genetic testing, biochemical markers, and clinical evaluation for accurate diagnosis. Detailed clinical and neurological examination including the examination of the Kayser Fleischer ring on the ophthalmological slit lamp and the clinical scales like Unified Wilson's Disease Rating Scale (UWDRS), or Leipzig Score, are key elements in the diagnosis and disease severity assessment in both hepatic and neurological manifestation of WD [6]. Biochemical tests, including serum ceruloplasmin levels, serum copper, urinary copper excretion, and liver biopsy, are commonly used. However, other diseases may cause false-positive results. Neuroimaging methods might also be helpful as various changes in the brain, especially in the putamen, are observed, but the abnormalities are nonspecific for WD [7]. Moreover, a better understanding of the molecular defect underlying WD allowed for the introduction of genetic testing, which has greatly improved the diagnostic process [6]. Genetic testing for WD focuses on the *ATP7B* gene, which provides instructions for a protein crucial for copper metabolism. This protein, copper-transporting ATPase 2, helps regulate copper levels in the liver [8]. When mutations occur in the *ATP7B* gene, this protein malfunctions, leading to copper buildup in various organs and causing the symptoms of WD. The testing process involves collecting a blood or saliva sample and extracting DNA. These mutations are compared against existing databases and research to understand their potential effect on the protein's function. Over 800 mutations have been identified in the *ATP7B* gene, ranging from small changes to larger deletions or insertions [9].

Despite its benefits, genetic testing has limitations. It should be analyzed alongside clinical findings, family history, and other diagnostic tests. Current methods may not detect all possible mutations, and cost and accessibility can be barriers.

While many available diagnostic tools exist, the mean time between the first symptoms and the diagnosis usually exceeds 2 years [10]. Furthermore, the delay in diagnosis may worsen the clinical outcome and prognosis and have implications for other family members regarding early screening [11]. Importantly, what differentiates WD from other neurodegenerative diseases is the fact that misdiagnosis and delay in treatment are clinically relevant, as proper diagnosis and therapy can prevent and stop the progression of the disease [12]. Thus, there is a great need for novel, blood-based molecular biomarkers, which would allow for early diagnosis of specific subtypes of WD and early prediction of neurological impairment of patients with hepatic manifestation. This review aims to discuss the diagnostic and prognostic utility of non-genetic and genetic biomarkers of hepatic and neurological subtypes of WD patients.

Article Search Process

Electronic databases Pubmed and Scopus were searched between May 2023 and December 2024, and original studies were reviewed to evaluate the role of novel potential biomarkers in WD. Review articles and meta-analyses were incorporated into this, as well as their secondary references for possible inclusion. Titles and abstracts were screened by two independent operators. The following search syntax was used: "Search ("biomarkers" [MeSH Terms] OR "blood-biomarker" [MeSH Terms] OR "cytokines" [MeSH Terms] OR "metabolomic" [MeSH Terms] OR "oxidative stress" [MeSH Terms] OR "XIAP" [MeSH Terms] OR "proteomic" [MeSH Terms] OR "inflammasome" [MeSH Terms] OR

"caspase" [MeSH Terms] OR "BDNF" [MeSH Terms] AND ("Wilson's Disease" [All Fields] OR "Wilson Disease" [All Fields]). Our search was limited to WD only; we excluded the studies that evaluated other neurodegenerative diseases, such as Parkinson's and Huntington's diseases. The human studies section did not exclude studies based on the ethnicity of study participants (Fig. 1).

Current Knowledge of Treatment Strategies for WD

Treatment options primarily involve chelating agents like penicillamine and zinc therapy, while emerging therapies, including gene therapy, offer promising alternatives. For severe cases, liver transplantation remains a crucial intervention [13].

Although penicillamine and zinc salts are standard therapy for WD and clinical improvement is observed in most of the cases of WD patients, in the neurological manifestation, treatment can lead to early neurological worsening and exacerbation of the symptoms. Kalita et al. reported that approximately 30.2% of the patients with a neurological form of WD deteriorated after treatment initiation [14]. Typically, the neurological worsening is observed in the first 6 months of the therapy. However, most of the cases are noticed during the first month [15]. Moreover, some of those symptoms are severe and irreversible as half of the patients never improve to the baseline even after drug discontinuation [14].

Neurological worsening is often observed in chelator initiation rather than in zinc salts [16]. It was reported that penicillamine can cause neurological worsening in 10–50% of the patients with neurological subtype of WD at the early stage of administration [17], while only in 3–7% of patients taking zinc salts [18]. On the other hand, other authors reported that the neurological deterioration is observed with similar frequency in both groups, 9.1% for chelating agents and 7.3% for zinc salts, respectively [18].

The mechanism of this pathological process is not fully understood. One hypothesis is that the treatment triggers a rapid mobilization of unbound copper, which leads to cytotoxic effects in neuronal tissue [19]. Nevertheless, the zinc salts mechanism of action involves reduction of intestinal copper absorption, thus does not influence copper balance

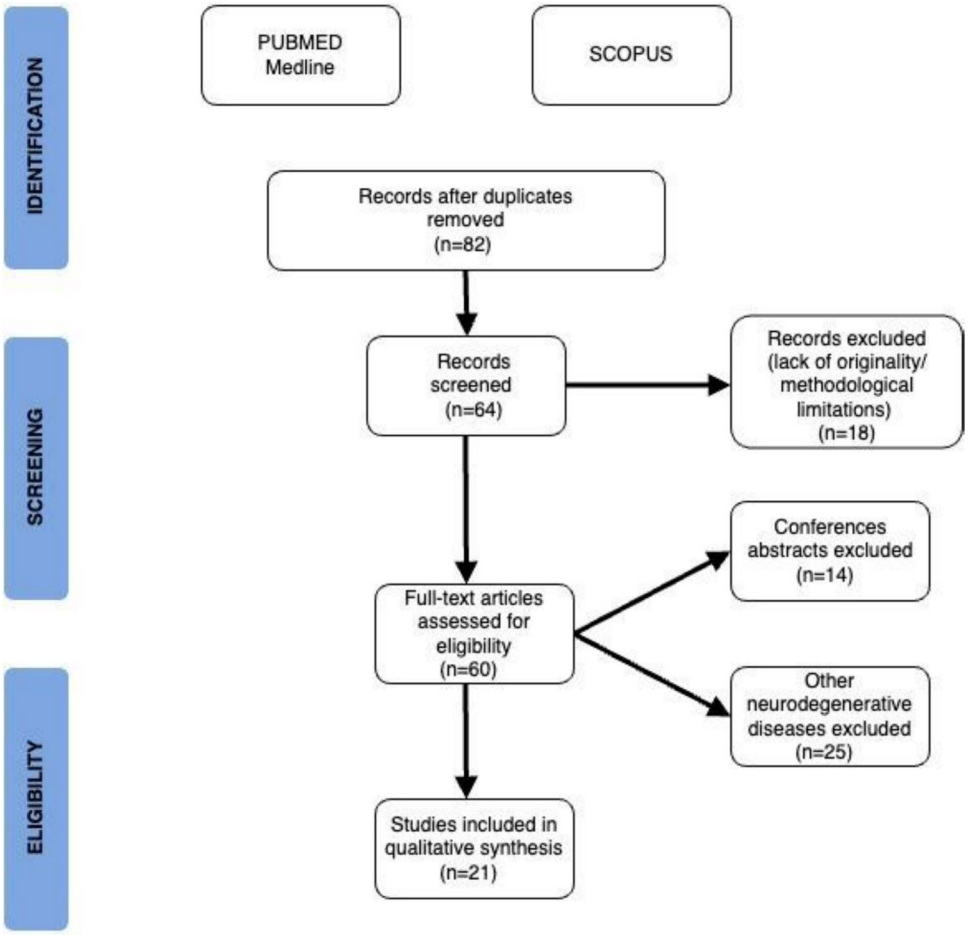

Fig. 1 Article selection flowchart. The figure schematically depicts the article selection process, from the literature search, through the screening, up to the final assessment of eligibility

as rapidly as chelating agents. Thus, the hypothesis needs to be further evaluated as the mechanisms of neurological worsening after zinc salts remain unclear [18].

Some prediction factors of neurological worsening after treatment initiation such as the severity of baseline neurological symptoms or MRI changes of the thalamus and brain stem were reported [20]. There were reports that symptoms such as drooling, leukopenia, thrombocytopenia, splenomegaly, and chronic liver disease are significantly associated with neurological worsening; however, they are not specific for WD [14]. Up to date, there are no blood-based genetic or non-genetic biomarkers that would be able to predict neurological deterioration after treatment initiation. Thus, there is a great need for novel blood-based biomarkers that would allow for the prediction of neurological worsening in those patients, therapy guidance, and the prevention of serious and life-threatening complications.

Another crucial issue is prediction of the course of the disease and its subtype as well as the development of symptoms in asymptomatic individuals. Moreover, new studies should focus on the search for biomarkers that would help prognose disease severity, as well as the reversibility of the neuronal damage and treatment response [21]. Neurologic symptoms in WD are primarily reversible with anti-copper treatment, but most patients have at least minor residual neuropsychiatric impairment, and approximately 20% of patients have unfavorable outcomes with severe disability or death. The prognosis of WD is much better when treatment is started before neurologic symptoms develop. Thus, population screening for WD is well justified but there are no biochemical markers with sufficient sensitivity/specificity and acceptable costs.

Issues Associated with the Neurological Manifestation of WD

Delayed Diagnosis of Neurological Manifestation of WD

The World Health Organization (WHO) estimates that the global prevalence of WD ranges from 1:10,000 to 1:30,000 people worldwide [22]. Neurologic symptoms of WD typically occur between the ages of 20–40 years old; however, the range is extensive [23]. Neurologic symptoms at initial presentation are observed in approximately 18–68% of WD patients [12]. The course of the disease is difficult to predict and the neurological symptoms may either slowly develop over years or rapidly escalate [23]. The most common neurologic symptoms of WD include movement disorders such as tremors, dystonia, parkinsonism, and ataxia, which are frequently associated with dysphagia, dysarthria, and drooling [24]. Those symptoms are nonspecific and are commonly observed in other neurological and neurodegenerative diseases, such as Parkinson's disease and Huntington's disease. While all three conditions can cause movement disorders, some key symptomatic differences can help differentiate WD from these other neurodegenerative diseases. WD patients often show a wider variety of tremors, including postural, intention, and wing-beating tremors [24]. On the other hand, resting tremor is more characteristic of Parkinson's disease, whereas tremor is less prominent in Huntington's disease. Furthermore, dystonia, ataxia, and parkinsonism are characteristic symptoms of WD [24]. In Parkinson's disease, bradykinesia and rigidity are more prominent, whereas, in Huntington's disease, chorea is the hallmark, sometimes accompanied by dystonia [25, 26]. Finally, WD patients often experience cognitive impairment, depression, anxiety, and personality changes [24]. In Parkinson's disease, cognitive changes typically occur later in the disease course [26]. In contrast, significant cognitive decline and psychiatric symptoms, such as depression, anxiety, irritability, and psychosis, are core features of Huntington's disease. While there is no available specific and sensitive blood-based biomarker for differential diagnosis of WD, there is a significant problem of long-term misdiagnosis or unclear diagnosis of WD. Moreover, proper diagnosis of brain changes, even without neurological symptoms, may be critical for the treatment introduction, as it may cause neurological deterioration in stable patients [27]. Moreover, despite the available diagnostic tools, neurological presentation is associated with a significantly longer time from onset of symptoms to diagnosis than hepatic presentation, ranging from 2.5 to 6 years [10]. All of the issues lead to an underestimation of the prevalence of WD. Late diagnosis of neurological impairment results in irreversible complications that significantly impair daily life and are associated with poor prognosis or even death [24]. Moreover, during this time, some of the patients are exposed to invasive and painful procedures and interventions, which could be prevented if properly diagnosed. WD belongs to just a few genetic disorders that can be successfully managed if diagnosed early and correctly treated [28]. Thus, there is a great need for novel, blood-based molecular biomarkers, which would allow for early diagnosis/prediction of neurological impairment and prognosis of WD.

Current Diagnostic Tools for the Neurological Manifestation of WD

None of the available diagnostic tools, including laboratory tests and imaging techniques, is perfect and specific for WD [7]. Commonly used blood-based biochemical tests such as serum ceruloplasmin and serum copper are unreliable as their levels may also be disturbed in other diseases and modulated by inflammatory processes. Moreover, serum

ceruloplasmin levels may be normal in almost 50% of patients with active WD [7].

The diagnostic process to evaluate neurological disturbances and brain changes in patients with WD includes neurological examination and neuroimaging techniques. Several clinical rating scales were created to assess the severity of neurological disturbances in WD. One of them, the unified Wilson disease rating scale (UWDRS), contains three subscales: consciousness, a historical review of activities of daily living adapted from the Barthel index, and neurological examination. Another scale, the global assessment scale (GAS) for WD, is also commonly used. Both scales require a long duration to complete the assessment. However, it was reported that the shorter version of the UWDRS, called the "minimal UWDRS," involving only the neurological subscale, can be a valuable tool for preclinical screening and is significantly correlated with the UWDRS total score [29].

Another clinical hallmark of WD is the Kayser–Fleischer ring, which occurs only in 50% of patients with the presenting hepatic form but in approximately 95% of patients with the neurological form of WD and can be examined with an ophthalmological examination [7].

Most common neurologic symptoms appear older than hepatic symptoms and are caused by brain tissue damage due to copper accumulation. The pathomechanism of copper toxicity involves various mechanisms such as mitochondrial damage, oxidative stress, or cell membrane damage. Most of the patients with the neurologic subtype of WD have abnormalities in brain magnetic resonance imaging (MRI). Most affected regions involve the putamen, pons, midbrain, and thalamus [30]. Importantly, it was reported that there is a significant correlation between WD diagnosis lag time and specific brain region damage [30].

Importantly, brain tissue damage can be prevented with early diagnosis and early treatment introduction [24]; thus, searching for neurological biomarkers is crucial.

Neurologic symptoms in WD are primarily reversible with anti-copper treatment but most patients have at least minor residual neuropsychiatric impairment and approximately 20% of patients have unfavourable outcomes with severe disability or death. The prognosis of WD is much better when treatment is started before neurologic symptoms develop. Thus, population screening for WD is well justified but there are no biochemical markers with sufficient sensitivity/specificity and acceptable costs. Moreover, a single-test strategy for the diagnosis of WD may lead to false-negative results and thus delay the treatment [11]. The sensitivity and specificity of those tests vary from 50 to 94.4% and from 52.2 to 98.3%, respectively [11]. Thus, there is a great need for novel, blood-based, molecularly characterized biomarkers for the diagnosis of specific subtypes of WD as well as early prediction of neurological impairment of patients with hepatic manifestation.

Novel Biomarkers for Neurological Manifestation

Active Caspase-3 and X-Linked Inhibitors of Apoptosis Protein

Recent evidence suggests that caspase activity, a protease responsible for morphological changes of apoptotic cells, can be a potential apoptosis biomarker in non-alcoholic liver disease [31]. Kalita et al. investigated the role of active caspase-3 and X-linked inhibitors of apoptosis protein (XIAP) in the neurological subtype of WD and their correlation with disease severity as well as pro- and anti-apoptotic markers. Elevated intracellular copper levels induce a conformational modification in XIAP, promoting its proteasomal degradation and attenuating its capacity to inhibit caspase-3 activation. This dysregulation of XIAP function consequently lowers the apoptotic threshold, rendering cells more susceptible to apoptotic stimuli and ultimately leading to increased cell death [32]. The study included 54 patients with WDN and 36 healthy individuals as a control group. First, the level of active caspase-3 and death signals was found to be higher in WDN patients. In contrast, the XIAP and survival signals (glutathione) were lower compared to the control group. Furthermore, active caspase-3 increased in WD patients and was positively correlated with the severity of the disease and death signals (TNF, IL-8, MDA, and free Cu), whereas the XIAP level was positively correlated with survival signals and inversely with death signals. Consequently, this study suggests that XIAP and caspase-3 can be potential apoptosis biomarkers in the neurological form of WD [33] (Fig. 2A).

Pentraxin 3

Pentraxin 3 (PTX3) is an acute-phase protein encoded by the PTX3 gene and plays a pivotal role in innate immunity and inflammation processes [34]. Increased levels of PTX3 were observed in both acute and chronic neurological diseases [35]. As it is suggested that PTX3 plays an important role in neurodegenerative diseases, the role of PTX3 in the neurological form of WD should be also evaluated. The study involved 86 WD patients and 28 healthy individuals. It was revealed that plasma PTX3 levels were significantly higher in patients with neurological WD than in the control group and were correlated with neurological disease severity assessed by the neurological Global Assessment Scale (GAS). Thus, the study shows that PTX3 can be a potential biomarker of disease severity in patients with neurological WD [34].

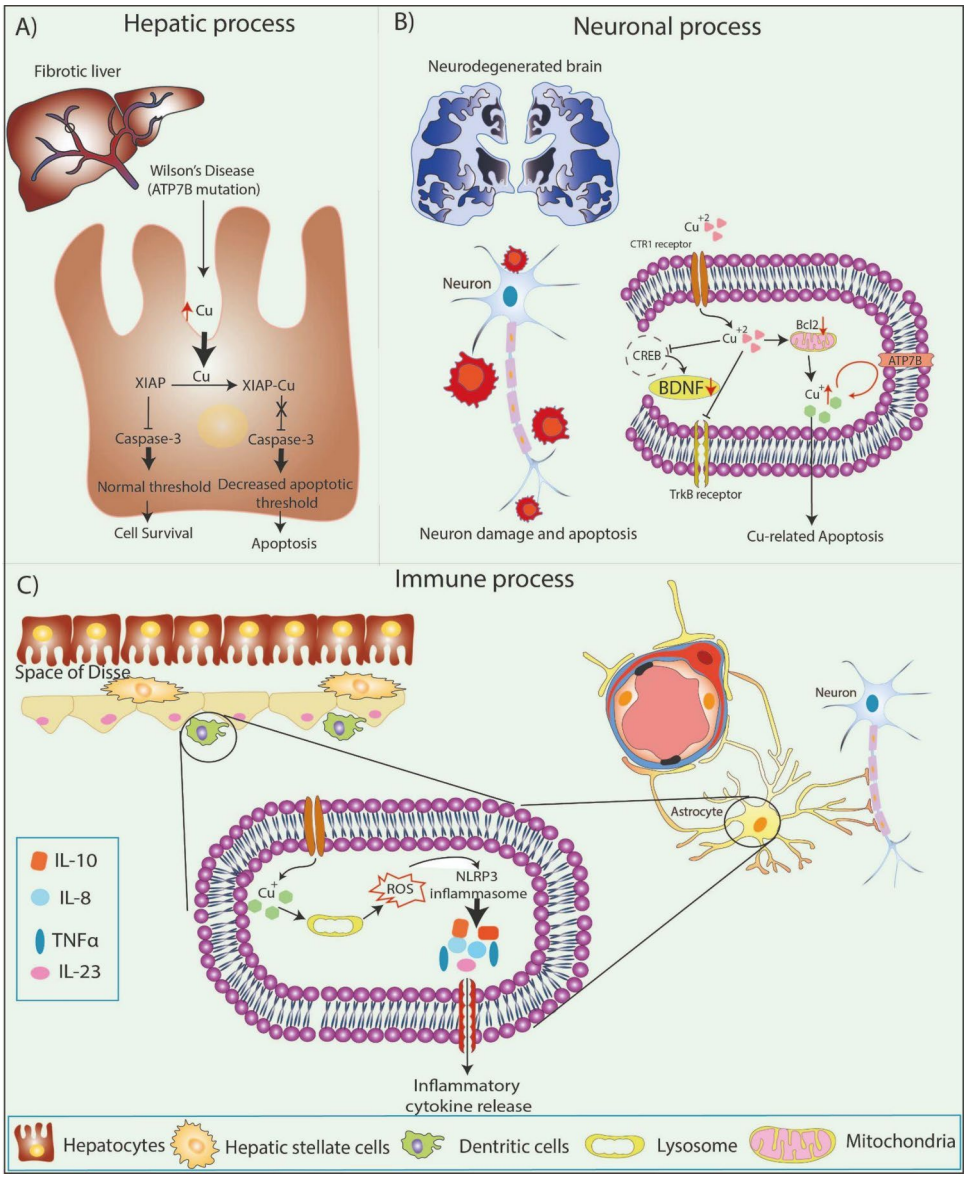

Fig. 2 Pathomechanisms related to copper toxicity involved in neurodegeneration, hepatotoxicity, and immune system damage. **A** Elevated intracellular copper levels induce a conformational modification in XIAP, promoting its proteasomal degradation and attenuating its capacity to inhibit caspase-3 activation. This dysregulation of XIAP function consequently lowers the apoptotic threshold, rendering cells more susceptible to apoptotic stimuli and ultimately leading to increased cell death. **B** Excess Cu leads to reduced CREB activation which in turn, decreases BDNF gene transcription, impairing neuronal survival and plasticity. Cu toxicity leads to TrkB downregulation or desensitization, reducing BDNF-mediated neuroprotection. Bcl-2 level, which is downregulated under Cu-induced stress, promotes mitochondrial outer membrane permeabilization triggering apoptosis. **C** Excess Cu can activate the NLRP3 inflammasome by increasing ROS production and cellular stress. Overactivation of the NLRP3 inflammasome results in the excessive release of proinflammatory interleukins. Abbreviations: BDNF, brain-derived neurotrophic factor; IL-10, interleukin-10; IL-8, interleukin-8; IL-23, interleukin-23; TNFα, TNF receptor-associated factor; XIAP, X-linked inhibitors of apoptosis protein; CTR1, copper transporter 1; CREB, cAMP response element-binding protein; Bcl-2, B cell lymphoma 2; Cu, Copper; TrkB, tropomyosin receptor kinase B; ATP7B, copper-transporting P-type ATPase; ROS, reactive oxygen species; NLRP3, NOD-, LRR-, and Pyrin domain-containing protein 3

Plasma Neurofilament Light Chain

Recently, neurofilament light chain (NfL) appears to be a promising biomarker of neurological and neurodegenerative diseases. Axonal damage and neurodegenerative processes lead to NfL release to cerebrospinal fluid and blood plasma, reflecting neuronal damage [36]. It has already been reported that the levels of NfL in blood plasma and CSF are increased in Alzheimer's disease patients and correlate with disease severity [37]. Moreover, NfL was shown to be a predictive

biomarker of disease onset in patients without symptoms [38]. Besides, plasma NfL concentration was independently associated with multiple sclerosis severity and disease status, whereas it decreased when proper treatment was introduced [39]. Finally, NfL was shown to be a predictive biomarker for post-stroke cognitive impairment [40].

Yang et al. aimed to analyze plasma NfL concentrations in patients with neurological and hepatic manifestations of WD and its correlations with neurological symptoms and brain atrophy. The study involved 75 patients with WD and 27 healthy individuals. It was reported that NfL concentrations were significantly higher in neurological type WD than in hepatic type WD. Moreover, NfL was positively associated with UWDRS scores and UWDRS-N scores. Finally, significant negative associations between NfL and brain grey matter volumes were found. To sum up, the study suggests that NfL may act as a biomarker of the neurological subtype of WD and is associated with disease severity and brain atrophy [41].

Schriebman et al. aimed to identify specific biomarkers to diagnose the neurological form of WD. The study involved 40 patients with WD, who were divided into neurological and hepatic subgroups and 38 healthy individuals. First, plasma NfL concentrations were higher in the neurological patients than in hepatic and control groups. Moreover, analysis of neurological severity showed that NfL was higher in patients with an active neurological form of WD compared to stable patients. Last but not least, the highest NfL concentration was in newly diagnosed neurological patients with paradoxical worsening after starting treatment. Consequently, this study presents that aside from being a damage severity tool, NfL can additionally act as a biomarker to identify which treatment and doses improve neurological recovery and to help avoid paradoxical worsening [42]. Interestingly, as NfL reflects neuronal damage, increased levels of NfL in stable patients may provide information about continuous neuronal damage in treated WD patients. Nevertheless, further studies are needed to evaluate the role of NfL in WD.

NLRP3 Inflammasome

Nucleotide-binding oligomerization domain receptor family, leucine-rich repeat, and pyrin domain-containing protein (NLRP) 3 are one of several intracellular inflammasome sensor proteins that are sensitive to particular danger-associated molecular patterns and pathogen-associated molecular patterns (PAMPs). Consequently, they cause IL-1β expression, an essential neuroinflammatory cytokine, through caspase-1 procession, which itself is activated by inflammasomes [43]. Holbrook et al. underline the importance of NLRP3 inflammasomes in the progression of multiple neurodegenerative diseases like Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and prion disease [44]. Moreover, recent studies show that exposure to copper oxide nanoparticles (CuONPs) results in the activation of the NLRP3 inflammasome, which is facilitated through the induction of lysosomal damage and subsequent release of cathepsin B. Furthermore, after lysosomal deposition, CuONPs released Cu^{2+} due to the acidic environment of lysosomes. Cu^{2+} then induced cellular oxidative stress and further mediated the activation of the NLRP3 inflammasome [45].

Dong et al. focused on the role of inhibiting the activation of NLRP3 inflammasome due to the copper excess in the neurological progression of WD in a murine model. Since copper is crucial for inflammasome activation, it was deduced that its accumulation starts with NLRP3 inflammasome-mediated neuroinflammation in the corpus striatum, hippocampus, cortex, and cerebellum and is associated with activation of microglia. Firstly, it was assessed whether the inflammatory cytokine levels were comparable in the sera of WD patients and healthy controls [43]. The findings indicated significantly augmented concentrations of IL-1β, IL-18, IL-6, and TNF-α in the serum of patients with WD, as well as in the cerebral tissue of the murine subjects, in conjunction with marked elevations of caspase-1, ASC, and IL-1β within the brains of these animals. Moreover, heightened levels of NLRP3 were observed within the corpus striatum and hippocampus of the mice, in contrast to their wild-type counterparts. Moreover, as initially predicted, it was found that silencing NLRP3 expression prevents copper-related neuroinflammation by inhibiting IL-1β and IL-18 production, additionally resulting in improved cognitive functions and the reversal of behavioral deficits. Therefore, the study came across the therapeutic value of NLRP3 inflammasome activation inhibition in the CNS complications of WD, which not only aids in its pharmacological potential but also prognostic value through the neuropathological mechanisms of injury [43] (Fig. 2B–C).

The Role of Plasma Inflammatory Cytokines in the Neurological Manifestation of WD

Increased levels of Cu in WD are highly toxic and lead to reduced antioxidants, increased oxidative stress, cytokines, and excitotoxic injury [46]. It has already been shown that dietary Cu levels may modulate circulating inflammatory cytokines and neuroinflammation in animal models [47, 48]. Additionally, another study reported that high levels of Cu were associated with inflammatory responses in mice livers, modulated via NF-κB and MAPK signaling pathways with high expression of various proinflammatory cytokines [49]. Nevertheless, the knowledge about the role of cytokines in WD and their correlation with the phenotype and therapeutic status is very limited [49].

Wu et al. aimed to investigate the effect of inflammatory cytokines on WD manifestation. The study involved 99 patients with WD divided into three subgroups: neurological, hepatic, or both, neurological and hepatic manifestations, and 32 healthy individuals as a control group. It was shown that plasma levels of T helper (Th) 1 cells (IL2, TNF-α, and TNF-β), Th 2 cells (IL-5, IL-10, and IL-13), and Th 17 (IL-23) were higher in patients with hepatic WD than in the control groups. In the neurological manifestation, higher plasma Th 1 cells (IL-2, TNF-α, and TNF-β), Th 2 cells (IL-13), Th 3 (TGF-β1), and Th 17 (IL-23) levels were found compared with control groups. Furthermore, Th 1 cells (TNF-α and TNF-β) and Th 17 (TGF-β1, IL-23) levels were significantly higher in hepatic and neurological patients compared to the control group [50].

Oxidative Stress

Recently, there have been two forms of copper increase in WD patients' blood, including copper-bound and free states. In the bound state, copper is linked to ceruloplasmin, while in the free state, it can loosely bind to small molecules such as albumins [46]. Ogihara et al. already reported that the loosely bound level is elevated in the serum of WD patients, however, the total serum copper concentration is usually low [51]. Importantly, in healthy individuals, the serum-free copper is approximately 10–15% of the total body copper, while in WD patients, it is highly increased and can cross the brain-blood barrier, resulting in oxidative neuronal tissue damage [52, 53].

Kalita et al. aimed to evaluate the role of oxidative stress markers, cytokines, and glutamate in the neurological form of WD. The study involved 29 neurologic WD patients and 9 asymptomatic siblings. It was found that the level of glutathione (GSH) and total antioxidant capacity (TAC) was significantly lower in neurologic WD patients, whereas the malondialdehyde (MDA), IL-6, TNFα, and glutamate levels were significantly increased when compared to asymptomatic siblings. Similar results were found in patients who obtained treatment either with penicillamine or zinc compared to the treatment-naive asymptomatic group. No statistical differences were found in the IL-8 and IL-10 between the WD patients and asymptomatic siblings [46].

Brain-Derived Neurotrophic Factor

Our team has intensively studied the role of brain-derived neurotrophic factor (BDNF) in the context of neurodegenerative diseases. Recent studies show that Cu impairs CREB/BDNF signaling in mice hippocampal. Thus, it can primarily affect neurodegenerative processes [54]. To the best of our knowledge, the first article on this topic was published by the experts who are co-authors of this review paper. The objective of this study was to assess how often the BDNF Val66Met and C-270 T polymorphisms occur in patients with WD compared to healthy individuals, as well as to analyze the impact of these polymorphisms on the clinical features of WD [55]. It was shown that BDNF polymorphism was more common among patients with WD than in healthy individuals, showing that they have an increased susceptibility to WD. On the other hand, no correlation between BDNF polymorphism and disease subtype or age of onset was found. Further studies on this topic are definitely needed (Fig. 2B).

Novel Biomarkers Regardless of WD Manifestations

Altered Composition of Gut Microflora in WD

Gut microbiota is a heritable composition of various microorganisms in the human gastrointestinal system, which is determined by multiple genetic and environmental factors [56]. In recent years, the role of human gut microbiota in the pathophysiology and development of multiple diseases was investigated. Current evidence suggests a correlation between intestinal flora polymorphism and genetic, neurodegenerative, or metabolic disorders [57]. Moreover, multiple studies suggest that gut microbiota and microbiota-derived metabolites may act as biomarkers for various neurological diseases and cognitive impairment [58, 59]. Thus, the identification of differentially abundant metabolites in the serum of WD patients may be potentially used as novel biomarkers for the disease.

Geng et al. aimed to investigate the difference between gut microbiota composition in patients with WD and healthy individuals [60]. The study involved 22 WD patients and 22 healthy individuals whose fecal samples were analyzed with 16S rRNA sequencing. It was reported that there were significant differences at the phylum level in microbial composition in the two groups and that the gut microflora of WD patients was significantly less diverse compared to the control group. The abundance of Bacteroidetes was significantly lower in WD patients compared to the control group, whereas the abundance of Firmicutes, Proteobacteria, and Fusobacteria was significantly higher.

A similar study was conducted by Cai et al., who aimed to investigate the diversity and composition of the gut microbiome in patients with WD [56]. The study involved 14 WD patients and 16 healthy individuals whose fecal samples were analyzed with 16S rRNA sequencing. It was found that WD patients had in general significantly less diverse and impoverished composition of gut microbiome than the control group. Some of the species, including Gemellaceae, Pseudomonadaceae, and Spirochaetaceae, were markedly

elevated, whereas others, like Actinobacteria, Firmicutes, and Verrucomicrobia, were significantly decreased compared to the control group. Moreover, it was revealed that the microbiome of WD patients involved fewer bacteria responsible for the host immune and the pathways of transcription factors and ABC-type transporters than healthy individuals.

Consequently, the studies show that dysbiosis in WD patients may have an impact on the disease progress as each of the altered bacteria has a specific function, and lack or excess of some species may disrupt the immune, neurological, and cognitive systems, physiological homeostasis, and promote proinflammatory effect [56]. Thus, the gut microbiome may serve as a non-invasive diagnosis tool for WD (Table 1).

Metabolomics Profiles of Patients with WD

Nowadays, more and more researchers focus on metabolomic profiling, whose main aim is to measure multiple small molecule metabolites in biological specimens, including body fluids and tissues using either nuclear magnetic resonance or mass-spectrometry. Similar studies are performed in WD patients in order to find particular diagnostic biomarkers for hepatic, neurological, and asymptomatic manifestations of the disease.

Sarode et al. analyzed serum metabolomic profiles of WD patients and healthy individuals to find differentially abundant metabolites, which could be potentially used as diagnostic biomarkers [61]. The serum metabolome was evaluated by hydrophilic interaction liquid chromatography-quadrupole time of flight mass spectrometry (HILIC-QTOF MS) in 26 hepatic, 25 neurologic, and 10 preclinical subtypes of WD and 15 healthy subjects. Researchers found 99 different metabolites in the serum of WD patients compared to the control group, which were characterized as amino acids associated with the tricarboxylic acid cycle and choline metabolism as well as metabolites related to the gut microbiota. Moreover, it was revealed that patients with hepatic manifestation had 67 different metabolites when compared to the control group, whereas patients with neurological subtypes had 57 different metabolites, respectively. Additionally, when symptomatic and asymptomatic patients with WD were compared to the control group, 77 and 36 significant metabolites were detected, respectively. Furthermore, they aimed to identify the metabolic pathways associated with the most significant abundant metabolites. It was found that the aminoacyl-tRNA biosynthesis pathway was associated with the highest number of identified metabolites. Collectively, the study shows that patients with WD have unique metabolomic profiles and, more importantly, metabolite profile comparisons could distinguish between different clinical manifestations of WD [61].

Mazi et al. aimed to investigate the role of choline, methionine, aromatic amino acids, and phospholipids in the serum of WD patients. The HILIC-QTOF MS was used to analyze the serum of patients with hepatic, neurological, and asymptomatic manifestations. The study shows that choline is significantly increased in patients with WD, regardless of manifestation. Moreover, upregulation of methionine was observed in patients with WD, especially in hepatic manifestation, whereas specific amino acids such as phenylalanine, tyrosine, and histidine were related to neurological manifestation. The study shows that specific metabolomic profiles in patients with WD can contribute to distinguishing between hepatic and neurological manifestations [62] (Fig. 3).

Proteomic Analysis in WD Patients

Proteomic techniques allow for a cost-efficient and accurate high-throughput screening for differentially expressed proteins. Tested samples can be obtained to a desired degree of invasiveness (urine, blood, tissue biopsy) facilitating the search for biomarkers in disease burdened cohorts.

Park et al. identified three differentially expressed serum proteins, namely C3, FB, and a2MG, in asymptomatic WD patients. C3 and FB are involved in complement activation pathways, while a2MG is a universal protease inhibitor and cytokine transporter. Reduced expression of C3, FB, and a2MG in asymptomatic WD patients may indicate reduced complement activation and blunted acute-phase response due to hepatic copper toxicity. These proteins can be candidates for novel diagnostic biomarkers for WD, particularly in the asymptomatic stage [63].

Wang et al. analyzed the proteome profiles of plasma in WD patients. The study showed that fibrinogen was significantly upregulated in WD patients compared to healthy individuals, which can be associated with inflammatory response and coagulation cascade [64]. On the other hand, Cabras et al. evaluated the proteome/peptidome of the saliva of WD patients. It was reported that WD patients had significantly higher levels of S100A9 and S100A8 proteoforms in the saliva, which are associated with oxidative stress and inflammation [65].

Lee et al. conducted a comprehensive analysis of the hepatic proteome in rats exhibiting a mutation in the human ATP7B homolog, which serves as a murine prototype for WD, and identified modifications indicative of the age-associated dynamics of copper-induced oxidative damage and pro-apoptotic mechanisms. Initial phase alterations demonstrated mitochondrial impairment, characterized by a reduction in the expression of mitochondrial matrix proteins. The metabolic pathways associated with carbohydrates were significantly disrupted. The expression levels of malate dehydrogenase 1, which decreased, and annexin A5, which increased, were modified in a manner

Table 1 Potential biomarkers in WD and their mechanism of action

No	Authors	Biomarker	Mechanism	Human/animal/in vitro/ex vivo/in silico	Material
1	Kalita et al. 2016 [33]	Active caspase-3 and X-linked inhibitors of apoptosis protein (XIAP)	Apoptosis	Human	Blood from WD patients
2	Wang et al. 2016 [34]	Pentraxin 3 (PTX)	Neuroinflammation	Human	Blood from WD patients
3	Yang et al. 2022 [41]	Neurofilament light chain (NfL)	Neuroinflammation	Human	Blood from WD patients
4	Schrimban et al. 2021 [42]	Neurofilament light chain (NfL)	Neuroinflammation	Human	Blood from WD patients
5	Dong et al. 2021 [43]	NLR family pyrin domain containing 3 inflammasome (NLRP3)	Neuroinflammation	Human/ex vivo/in vitro	Blood from WD patients/primary microglia and neuron from mice
6	Wu et al. 2019 [50]	IL-2, TNF-α, TNF-β, IL-5, IL-10, IL-13, IL-23	Inflammation	Human	Blood from WD patients
7	Kalita et al. 2014 [46]	Glutathione, total antioxidant capacity, MDA, IL6, IL8, IL-10, TNFα	Oxidative stress	Human	Blood from WD patients
8	Geng et al. 2018 [60]	Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria, Tenericutes	Intestinal flora polymorphisms	Human	Fecal samples from WD patients
9	Cai et al. 2020 [56]	Gemellaceae, Pseudomonadaceae Spirochaetaceae, Actinobacteria, Firmicutes, Verrucomicrobia, Bacteroidetes, Proteobacteria, Cyanobacteria Fusobacteria	Intestinal flora polymorphisms	Human	Fecal samples from WD patients
10	Mazi et al. 2019 [62]	Choline, methionine, ornithine, proline, phenylalanine, tyrosine, histidine	Metabolomic profiling	Human/ex vivo	Blood from WD patients/liver tissue from mice
11	Park et al. 2009 [63]	Component C3, complement factor B, and alpha-2 macroglobulin	Proteome analysis	Human	Blood from WD patients
12	Wang et al. 2019 [64]	Fibrinogen	Proteome analysis	Human	Blood from WD patients
13	Lee et al. 2011 [66]	Malate dehydrogenase 1, annexin A5, transferrin, *S*-adenosylhomocysteine hydrolase, and sulfite oxidase 1	Proteome analysis	Animal/ex vivo	Serum and liver tissue from rats
14	Cabras et al. 2015 [65]	S100A9 and S100A8 proteoforms	Proteome analysis	Human	Saliva from WD patients
15	Sarode et al. 2019 [61]	Hepatic manifestation had 67 different metabolites; the neurological subtype had 57 different metabolites	Metabolomics	Human	Blood from WD patients
16	Gholizadeh et al. 2020 [68]	Hsa-miR-1275, hsa-miR-21, hsa-miR-222	Copper homeostasis	Ex vivo	Liver tissue samples from WD patients
17	Siaj et al. 2012 [67]	miR-122	-	In vitro	Hepatocytes derived from rats and then hepatocytes culture was set Blood from rats
18	Zhang et al. 2021 [69]	2564 significantly upregulated and 1052 downregulated lncRNAs, and 1576 upregulated and 297 downregulated mRNAs	Apoptosis, drug metabolism-cytochrome P450 pathway, PPAR signalling pathway, Notch signalling pathway, and MAPK signalling pathway	In silico/ex vivo	Liver tissue from mice
19	Glavind et al. 2020 [71]	sCD163	Inflammation, fibrosis	Human	Blood from WD patients

Table 1 (continued)

No	Authors	Biomarker	Mechanism	Human/animal/in vitro/ex vivo/in silico	Material
20	Björklund et al. 2018 [70]	sCD163	Inflammation	Human	Blood and urine from WD patients
21	Strand et al. 1998 [75]	CD95	Apoptosis	Ex vivo/in vitro	Human hepatoma cells

contingent upon the progression of age-related disease. Notably, in aged rats, the expression of S-adenosylhomocysteine hydrolase was significantly downregulated. S-Adenosylhomocysteine acts as a competitive inhibitor of all adenosylmethionine-dependent methyltransferases, which play critical roles in the biosynthesis and metabolism of monoamine neurotransmitters. This alteration may be implicated in the onset of neurological changes and necessitates further investigation [66].

Biomarkers Based on Non-coding RNA

There is a wide range of studies showing the role of microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) as potential biomarkers in hepatic and neurological diseases. Numerous studies reported that miRNAs may serve as diagnostic and prognostic biomarkers in different types of stroke or neuroinflammatory diseases. Nevertheless, there is minimal knowledge about the role of non-coding RNAs in WD.

Siaj et al. aimed to analyze the role of miR-122 in the rat model of WD at various disease time points. The sample included rats with fulminant hepatitis induced by a high-copper diet as a WD model. It was shown that the level of miR-122 was highly elevated in the WD rat model. Moreover, it was shown that the elevated miR-122 level was detected 2 weeks before biochemical liver parameters. Finally, cell therapy with hepatocytes transplantation showed a rapid miR-122 level decrease. Consequently, this study shows that miR-122 can act as an early biomarker of liver damage in WD and can be helpful in treatment effectiveness monitoring [67].

Gholizadeh et al. conducted a large study to identify differentially expressed miRNAs and their target genes in patients with six various liver diseases. The study involved 125 tissue samples from patients' livers obtained during the transplantation procedure. Two key miRNAs were identified among nine liver samples from Wilson Disease patients, which were involved in gene regulation in the disease. Hsa-miR-1275 significantly decreased whereas hsa-miR-21 and hsa-miR-222 significantly increased compared to healthy controls. Notably, it was shown that hsa-miR-222 plays an important role in copper hemostasis and, thus, its role should be further analyzed in WD patients [68].

Zhang et al. aimed to analyze the lncRNA-mRNA regulation network in the WD rat model using in silico analysis. A total of 2564 significantly upregulated and 1052 downregulated lncRNAs, and 1576 upregulated and 297 downregulated mRNAs, were identified. Further analysis showed that these genes were associated with various processes such as apoptosis or drug metabolism-cytochrome P450 pathway [69]. This study shows the importance of further gene analysis to better understand the path mechanism underlying WD.

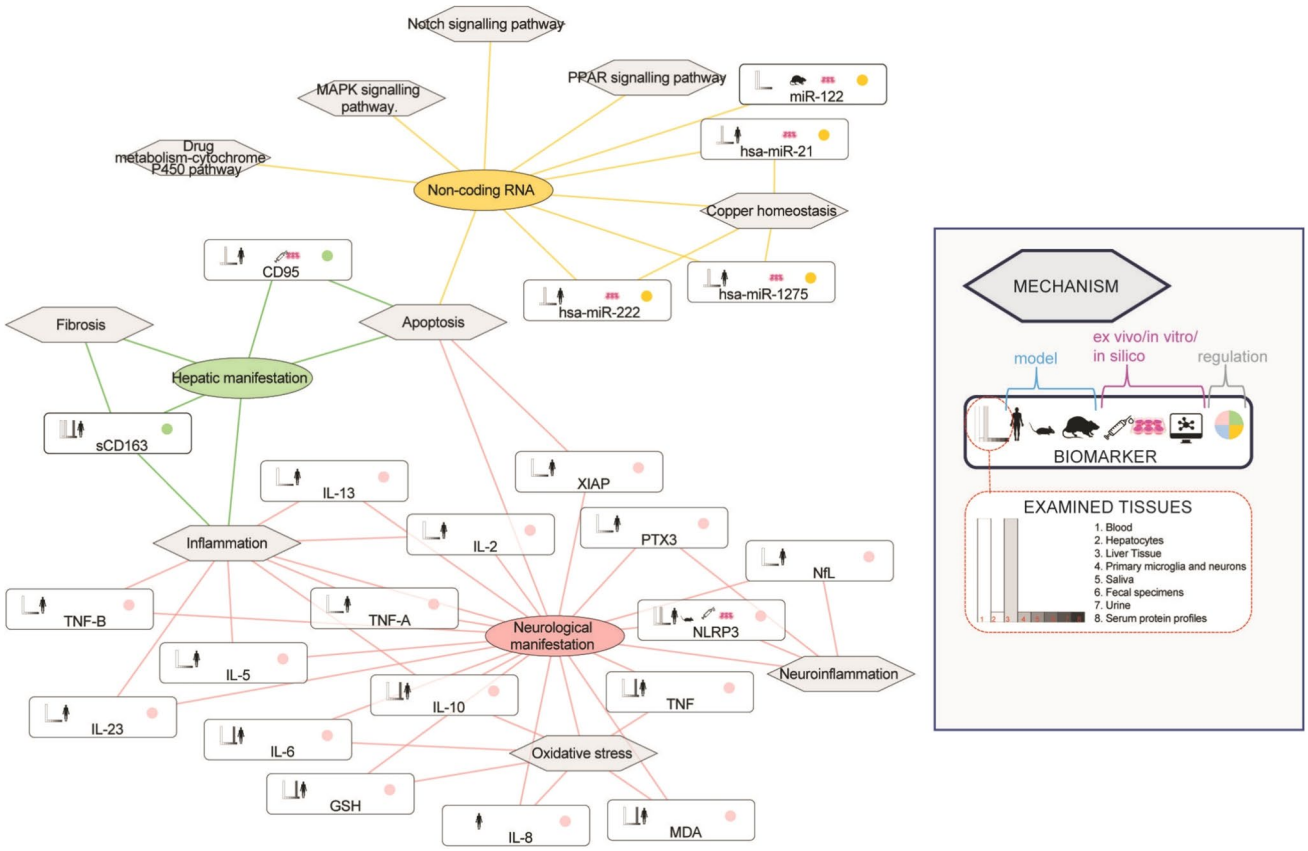

Fig. 3 Networks presenting the specific biomarkers and non-coding RNA associated with specific manifestation of WD, either hepatic or neurological

Novel Biomarkers for the Hepatic Manifestation of WD

Macrophage Activation Marker sCD163

Hepatic macrophages (Kupffer cells) play pivotal roles in many liver diseases and are associated with liver function, and the severity of the disease as well as liver fibrosis [70]. Importantly, the activation of Kupffer cells can be assessed by the macrophage activation marker soluble sCD163, and metabolic liver function by the galactose elimination capacity (GEC) as well as fibrosis by transient elastography [70].

Bjórklund et al. aimed to investigate the effect of Kupffer cells activation on liver functioning in WD patients [70]. The study involved 29 patients with stable forms of WD, who obtained various combinations of treatment and 19 healthy individuals as a control group. To assess the activation of Kupffer cells, sCD163, GEC, and transient elastography were measured. First, it was reported that the median of sCD163 was high in the normal range of the control group, with 24% of patients above the upper limit. Moreover, high sCD163 values significantly correlated with TNF-α, IL-6, IL-8, and low GEC values. Besides, the median GEC of WD patients' was 1.98 mmol/min, which corresponds to 73% of their expected metabolic liver function. Importantly, there was no correlation between the sCD163 and liver fibrosis. Consequently, the study suggests that patients with stable WD presented various degrees of Kupffer cell activation, which may be involved in the loss of metabolic liver function. Thus, sCD163 may be a potential novel biomarker of liver function in WD patients [70].

Similarly, the role of the macrophage activation marker soluble sCD163 was examined in the hepatic manifestation of WD patients. The study involved 28 patients with fulminant WD, 147 patients with chronic WD, and 19 healthy individuals. It was shown that the median sCD163 was tenfold higher in the fulminant group compared to the control group. Importantly, the median sCD163 level was also increased in patients with chronic WD; however, the result was significantly lower than in patients with fulminant WD. Moreover, sCD163 was elevated in patients with cirrhosis compared to patients without cirrhosis and was associated with biochemical markers of liver injury, including ALT, AST, GGT, INR, and albumins [71].

Apoptosis Antigen 1 (APO-1)

Apoptosis is a key factor in the pathogenesis and outcome of many liver diseases, mainly due to resultant inflammation leading to fibrosis [71, 72]. A better understanding of signaling pathways mediating hepatocytes apoptosis may provide a potential tool to assess the severity of the disease and its implications [73]. Nevertheless, the knowledge about the role of apoptosis and its mediators in WD is very limited.

One of the potential biomarkers of apoptosis in WD is the cell surface receptor CD95 (APO-1/Fas), which transduces death signaling in various cells upon stimulation by the Fas ligand or Fas antibodies [74]. Strand et al. aimed to examine the role of CD95 in WD patients with fulminant hepatic failure (FHF) [75]. The study involved an in vitro model of copper overload and an in vivo model of 4 WD patients with FHF as well as normal livers as a control. The morphological analysis of the WD livers revealed large areas of cell death and increased CD95 expression on hepatocytes compared to the control group. Importantly, to confirm whether the enhanced apoptosis was associated with dysregulated copper metabolism, the in vitro study was conducted. It was reported that copper overload induces apoptosis and increases CD95 expression by p53 regulation. Last but not least, reduced apoptosis and reduced induction of CD95 mRNA was observed with the additional treatment of N-acetylcysteine, which is known to have beneficial effects for patients with FHF. To sum up, the study suggests that increased CD95 expression, which is associated with enhanced apoptosis, may be a potential biomarker of FHF in WD [75] (Fig. 4).

Implementation of the Potential Biomarkers in Clinical Practice

To our best knowledge and based on the revision of ClinicalTrials.gov, there is only one clinical trial that evaluates the correlation between the sCD163 biomarker and liver fibrosis in WD (NCT02702765) [70]. The potential significance of emerging biomarkers is extensively examined in other neurodegenerative disorders. For instance, the involvement of BDNF in the pathogenic mechanisms of Huntington's disease (NCT04012411) [76], as well as its prospective utility as a treatment biomarker (NCT00095355) [77], is under rigorous investigation. Parkinson's disease also represents an intensively researched area, with numerous clinical trials attempting to integrate novel biomarkers into standard clinical practice. Among the potential biomarkers studied in Parkinson's disease for diagnosis and treatment is NLRP3 (NCT06822517, NCT06556173, NCT04338997, NCT03918616) [78, 79]. Moreover, BDNF is intensively studied in Parkinson's disease as a marker of treatment efficacy, cognitive exercise, and various physical therapies (NCT03860649, NCT02741765, NCT06580977) [80]. Despite this extensive research, none of the discussed biomarkers are widely used in clinical practice. Further investigation into potential blood-based biomarkers for WD is necessary. This research may ultimately improve diagnosis and prognosis and lead to the implementation of these biomarkers into routine clinical practice.

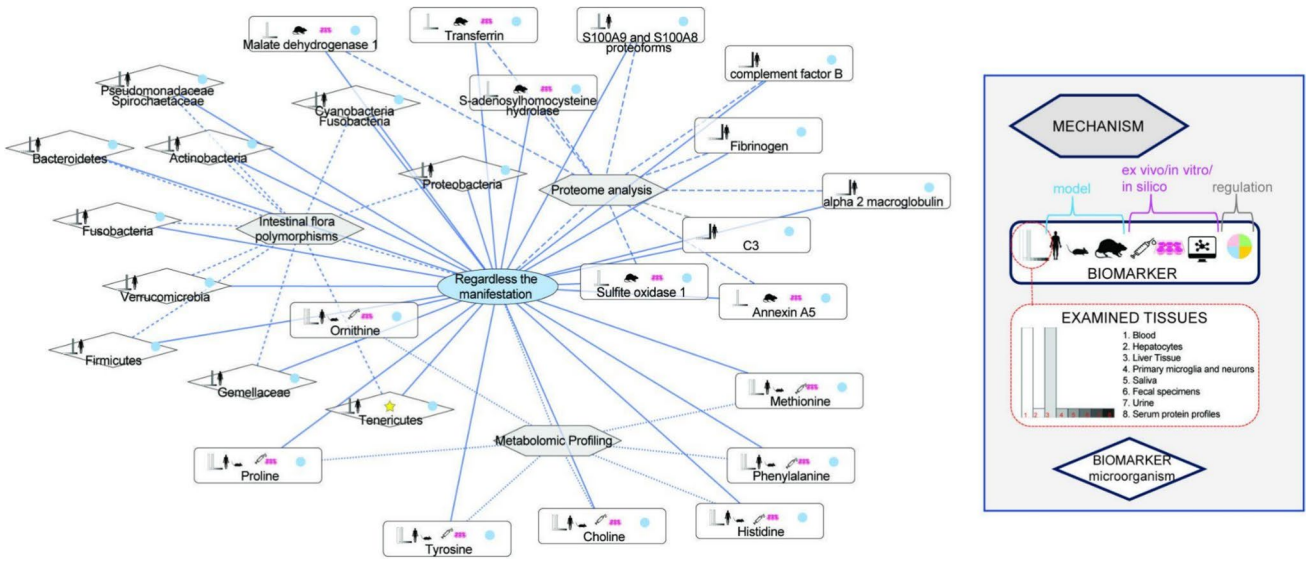

Fig. 4 Networks presenting the specific biomarkers associated with WD regardless of the manifestation

Conclusion

WD is a complex genetic characterized disease by a wide range of hepatic and neurological manifestations, requiring early and accurate diagnosis to prevent complications. Despite advancements in genetic and biochemical diagnostic tools, current methods remain insufficient in differentiating the subtypes of the disease, predicting progression, and assessing drug-response. This review highlights promising novel biomarkers that have emerged as potential tools for improving WD diagnosis and management. Blood-based markers such as active caspase-3, XIAP, PTX3, NfL, and NLRP3 inflammasome activation demonstrate strong potential for identifying neurological impairment. In hepatic manifestations, sCD163 and APO-1 can be promising markers of liver dysfunction and fibrosis. Additionally, metabolomic, proteomic, and gut microbiota analyses provide valuable insights into the molecular and systemic changes associated with WD. Yet, the detailed mechanism of action of the described molecules on processes such as apoptosis, fibrosis, and neuroinflammation has not been fully explained, and more studies should be conducted to evaluate their role. In vitro and animal model studies showed the importance of these markers. However, further validation through large-scale clinical studies is required before they can be integrated into clinical practice. Future research should focus on standardizing biomarker assessment protocols and exploring their predictive value in treatment response. Incorporating these emerging biomarkers into clinical workflows, we may enhance early detection and personalized treatment strategies and, therefore, improve patient outcomes in WD.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethics Approval and Consent to Participate Not applicable.

Patient Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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