Advancing Newborn Screening in Washington State: A Novel Multiplexed LC-MS/MS Proteomic Assay for Wilson Disease and Inborn Errors of Immunity

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Newborn Screening (NBS) is considered one of the most successful public health programs in identifying infants with treatable disorders for early intervention with favorable outcomes. Unfortunately, for many genetic disorders there are no specific metabolic biomarkers nor any analytical methods suitable for population screening even where highly effective pre-emptive treatments are available. Most causative mutations in genetic disorders result in reduction or absence of their proteins, therefore, direct measurements of the peptide as a surrogate marker for these proteins using multiplexed proteomic methods from dried blood spots (DBS) can be highly diagnostic and utilized in population screening.

Direct measurement of signature peptides in DBS has been shown to be a sensitive and specific proteomic screening method for the multiplex detection of patients with Wilson Disease and three life-threatening inborn errors of immunity, X-linked agammaglobulinemia, Wiskott-Aldrich syndrome, and Adenosine Deaminase deficiency. Each of these disorders results in severe negative sequelae if undetected but are treatable if diagnosed early in life. Analysis of signature peptides found statistically significant reduction or absence of peptide levels in affected patients compared to control groups in each case.

A novel proteomic-based IVD (*Invitro* diagnostics) kit for NBS has been manufactured to identify these four conditions in a single-run multiplex assay from DBS with inject-to-inject time at < 3 minutes using LC-MS/MS. For the FDA De Novo Class II application, the screening performance of the kit was determined in a prospective clinical study of routine newborn samples and previously confirmed positive patient samples. In this validation study, a total of 3,294 newborns and 49 genetically confirmed positive samples were tested at three sites. No presumptive positive cases were detected, and all confirmed 49 positive cases were screen positive. Analytical performance including stability, day to day reproducibility, and inter-operator variation was all acceptable at three sites. A pilot study is also underway in conjunction with the WA State public health NBS laboratory. To date, more than 22,000 newborn samples have been screened, and no true presumptive positive cases have been found with only a few false positive cases detected. These ongoing studies support both the feasibility of newborn screening for these conditions and the use of multiplexed proteomic analysis as an effective methodology for pediatric population screening.

Ann. N.Y. Acad. Sci. ISSN 0077-8923

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Issue: *Human Disorders of Copper Metabolism II*

Population screening for Wilson's disease

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Wilson's disease is an autosomal recessive disorder of copper transport caused by mutations in the gene encoding an ATPase, *ATP7B*. Early detection of Wilson's disease is critical because effective medical treatments such as chelating agents and zinc salts are available, which can prevent lifelong neurological disabilities and/or cirrhosis. It is unfortunate that most patients are brought to our attention after they have developed serious complications such as brain damage or cirrhosis, despite the availability of effective treatments. The diagnosis is usually made through copper measurement in the liver tissue, followed by confirmation with genetic testing of the *ATP7B* gene. Currently, there are no effective biomarkers or methods suitable for newborn screening for Wilson's disease. Ceruloplasmin has been tested for pediatric and newborn screening with limited outcome. Recently, liquid chromatography–multiple reaction monitoring–mass spectrometry (LC-MRM-MS) has emerged as a robust technology that may enable multiplex quantification of signature proteotypic peptides with low abundance. The application of this technology may help facilitate the research on Wilson's disease for protein expression, biomarker study, diagnosis, and, hopefully, screening.

Keywords: Wilson's disease; newborn screening; population screening; ceruloplasmin; ATP7B; LC-MS/MS; LC-MRM-MS

Wilson's disease has evolved from a diagnosable to a treatable disease over the 100 years since the first cases were reported in 1912 by S.A.K. Wilson.[1] The disease is progressive and ultimately fatal if untreated and is probably one of the most frequent causes of chronic liver disease in children. Although the prevalence varies across the population, it was estimated to be 1 in 30,000 with a carrier frequency of 1 in 90.[2] Over 500 mutations have been reported, along with >100 polymorphisms, in the *ATP7B* gene in the Human Genome Mutation Database (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=ATP7B). The most common mutation in Europe and North America is p.H1069Q, while p.Arg778L is the most common in Far Eastern Asian populations.

Clinical and laboratory ascertainments for Wilson's disease are often very challenging, as clinical presentations of Wilson's disease show considerable variations and may be even much broader than we know. Guidelines for the diagnosis of Wilson's disease have been approved;[3] however, a small number of patients still cannot be diagnosed with current tests. Despite recent improved awareness and advanced DNA testing, a substantial number of patients still present with delayed diagnosis, which is unfortunate when we have effective treatments available that include copper chelators (trientine, penicillamine) to promote copper excretion from the body and zinc salts to inhibit the copper absorption from the gastrointestinal epithelium. Early diagnosis is critical for better prognosis. Patients with neurological symptoms may experience worsening with the treatment, and half of those patients may develop permanent neurological damage and will never return to the baseline of function.[4] Hepatic presentations range from acute and chronic hepatitis to cirrhosis and acute liver failure. Hepatocellular carcinoma has become an important recent issue for patients with Wilson's disease, as current treatment has improved life expectancy.

Considering all these factors, population screening for Wilson's disease has been extensively discussed, with strong needs; however, no screenable,

doi: 10.1111/nyas.12423

Table 1. Results of population screening trials for Wilson's disease

Year	Study	No. of children	Age range	Positive cases	Method/sample
1993–1995	Yamaguchi *et al.*[7]	126,810	Newborns	0	ELISA, DBS
1977–1996	Yamaguchi *et al.*[7]	24,165	Late infancy to elementary school	3	ELISA, DBS
1999	Ohura *et al.*[8]	2789	1–6 years	2	ELISA, DBS
2001	Hahn *et al.*[11]	3667	3 months to 15 years	1	ELISA, DBS
2002	Owada *et al.*[9]	48,819	Primary school children	2	ELISA, urine
2006	Kroll *et al.*[12]	1380	Newborn to 18 years	0	ELISA, DBS
2008	Nakayama *et al.*[10]	11,362	3 years	1	ELISA, urine
2008	Zappu *et al.*[14]	5290	Newborn (Sardinia)	1:2707	PCR-based, DBS
		397	Newborn (Kalymnos)	27:20,000	

DBS, dried blood spots; ELISA, enzyme-linked immunosorbent assay.

cost-effective biomarkers or methods have yet been developed for Wilson's disease. To date, only a few studies have been conducted using ceruloplasmin as a marker, with limited outcomes (Table 1).

Newborn screening seeks to identify infants with treatable congenital disorders. Recent tandem mass spectrometry (MS/MS) applications have provided the ability to screen for >50 metabolic diseases from a single dried blood spot. The feature that makes metabolic disorders particularly amenable to screening is the presence of abundant small-molecule metabolites in plasma. However, many treatable disorders are characterized by absent or diminished large proteins in plasma or within circulating blood cells, for which there are currently no cost-effective screening methods. The research in our laboratory has focused on developing mass screening methods for these conditions, including Wilson's disease.

Previous experiences from pilot studies

Serum copper and ceruloplasmin are low in the majority of patients with highly elevated copper concentrations in the liver (>250 μg/g of tissue) and in the urine (>100 μg/day).[3] In some patients with Wilson's disease, serum ceruloplasmin is not low, and serum copper could be high in patients suffering from acute liver failure, owing to the release of copper from hepatocytes. Ceruloplasmin, a major copper-carrying protein in the blood, is a secretory enzyme that plays a role in iron metabolism as a ferroxidase. Ceruloplasmin is synthesized mainly by hepatocytes and incorporated with copper by the ATP7B protein before secretion into the blood. Earlier studies indicated that holoceruloplasmin (ceruloplasmin bound with copper) levels in plasma from patients with Wilson's disease were reduced, but the amount of apoprotein (ceruloplasmin without copper) was similar to that observed in normal individuals.[5] Based on this observation, a sandwich enzyme-linked immunosorbent assay (ELISA) kit was developed in Japan using a monoclonal antibody specific to holoceruloplasmin to measure the ceruloplasmin in dried blood spots.[6] Nevertheless, no patients with Wilson's disease were detected after screening over 126,810 newborns.[7] However, three patients were identified from 24,165 children from infancy to elementary school age in Japan. In another subsequent study using the same sandwich ELISA kit, two presymptomatic patients were identified among 2789 children aged from 1 to 6 years.[8] As a result of these studies, 3 years was considered to be the optimal age for Wilson's disease screening.

A mass screening measuring urinary holoceruloplasmin was also conducted in Japan.[9] The investigators found that the quantity of holoceruloplasmin protein in the urine of patients with Wilson's disease was significantly lower than in healthy subjects. However, in 2 of 41 Wilson's disease control samples (5%), urinary ceruloplasmin was not low, supporting previous observations that some patients with Wilson's disease could have normal ceruloplasmin levels. Two cases of Wilson's disease were found by testing urine samples from 48,819 primary school children. The rate of second request was about 0.9%. Another pilot study was conducted in Hokkaido Prefecture in Japan for 3-year-old children to screen for Wilson's disease using an automated assay of ceruloplasmin measurement in urine specimens.[10] A total of 11,362 children were screened, and one

true positive case was identified and confirmed by genetic testing. The urinary ceruloplasmin level in Wilson's disease controls was 13 ± 9.25 ng/mg Cr, while the normal control was 190 ± 154 ng/mg Cr. When the cut-off value was 45.0 ng/mg Cr, the rate of second request was about 1% of total participants.

We also developed a sandwich ELISA method for ceruloplasmin measurement in dried blood spots using specific monoclonal antibodies against ceruloplasmin. In a small pilot study on 3667 children aged 3 months to 15 years, we identified one patient, a 32-month-old boy with a ceruloplasmin concentration of 2.3 mg/dL.[11] The rate of second request of this study was 0.3%. In a subsequent validation study, we were able to retrieve the original newborn blood spots from two affected patients with Wilson's disease along with age-matched controls.[12] The original newborn blood spots were kept in a –20 °C freezer. Ceruloplasmin concentrations in the original newborn blood spots from two patients were indeed very low: 2.6 and 2.8 mg/dL (normal control 47.2 ± 15.6 mg/dL with range from 6.5 to >60). It is conceivable that the ceruloplasmin concentration in Wilson's disease could be much lower during the newborn period compared to normal newborns, but further study will be required to determine the baseline level of ceruloplasmin in newborns with Wilson's disease. Although these findings supported the view that presymptomatic screening for Wilson's disease using dried blood spots could be feasible in the newborn period, subsequent screening over 100,000 newborn blood spots in Minnesota was unsuccessful in identifying a patient (unpublished data), similar to a previous experience in Japan.

We later developed an assay using liquid chromatography–triple quadrupole mass spectrometry (LC-MS/MS) to quantify ceruloplasmin-specific peptides from dried blood spot samples digested by trypsin.[13] Most state laboratories in the United States currently use LC-MS/MS for newborn screening. A method-comparison study on previously tested patient samples for ceruloplasmin gave comparable results, suggesting that it may be feasible to use LC-MS/MS for screening. Lower-limit quantification for ceruloplasmin was around 0.7 mg/dL. Inter- and intra-assay imprecision was acceptable for clinical use; however, this approach has not been studied in a large-scale population.

DNA analysis can be utilized for a genetically homogeneous group with highly prevalent mutations. In Sardinia, a 15-nucleotide deletion in the promoter region of *ATP7B* accounts for 61.7% of alleles. Six common mutations together account for approximately 85% of Wilson's disease in that population. By screening 5290 newborns in Sardinia, Wilson's disease incidence in this population was estimated to be approximately 1 in 2707 live births.[14]

Challenges

Ceruloplasmin is an acute-phase reactant, so the concentration can vary depending on the health status at the time of sample collections. Ceruloplasmin can be in the normal range in some symptomatic patients with Wilson's disease. Low ceruloplasmin can be observed in other conditions such as copper deficiency, Menkes disease, hereditary aceruloplasminemia, severe protein-losing enteropathy, or liver failure. Very little information is available for the baseline concentration of ceruloplasmin in Wilson's disease before patients develop symptoms.

The application of ELISA or immunoassay for ceruloplasmin quantification to newborns, regardless of different antibodies against ceruloplasmin epitopes, may not be sensitive enough to identify newborn patients with Wilson's disease. A substantial number of newborns present with physiologically low ceruloplasmin, which makes this approach difficult. The method using LC-MS/MS developed in our laboratory for ceruloplasmin measurement may also encounter similar problems with sensitivity and detection limit.

However, ceruloplasmin measurement by immunoassay, either on dried blood spots or urine specimens, could be a feasible approach for infants or older children. Indeed, pediatric or infant screening by ceruloplasmin assay seems the only amenable approach for screening Wilson's disease at present. The limited studies in Japan yielded some promising results. The cost is only $2.50 per test, but high false-positive rates, particularly for the urine test, necessitate considerable improvement of the assay. A disadvantage of this screening is that it would be effective only when used in conjunction with a mandatory health care program available at the age of 3 years. This system is not universally available worldwide and may be difficult to establish in the United States with limited federal resources.

Molecular genetic testing is useful in confirming the diagnosis in affected patients, but is also very useful in identifying affected siblings, including

those without definite symptoms.[15,16] Although no single test can permit *de novo* diagnosis of Wilson's disease, the genetic test has been increasingly used in the clinical field and often underscores the important medical decision. Genetic tests play critical roles in the diagnosis of Wilson's disease. A stepwise approach with DNA-based screening in certain ethnic backgrounds or regions, starting with common mutations accounting for the majority of Wilson's disease patients in that region, may be an appropriate strategy.[17] Although the continuous decrease in the cost of DNA testing will render the screening more approachable, the current cost is still prohibitive for mass screening for Wilson's disease.

Recent investigation on 181 patients from the United Kingdom with clinically and biochemically confirmed Wilson's disease showed that overall mutation detection frequency was 98%.[18] The likelihood of mutation in genes other than *ATP7B* causing Wilson's disease is very low. Their study indicates that the frequency of heterozygosity is considerably higher than the previously reported occurrence of 1:90. The calculated frequency of individuals predicted to carry two pathogenic mutations was 1:7026, considerably higher than the reported prevalence of Wilson's disease of 1:30,000. This significant discrepancy between the genetic prevalence and the number of clinically diagnosed cases of Wilson's disease was explained by both a reduced penetrance of *ATP7B* mutations and a failure to diagnose patients. This underlines the need for more reliable biomarkers for screening Wilson's disease to prevent the development of serious complications.

As serum copper is reduced in Wilson's disease, measuring copper in dried blood spots was considered as a potential screening method. Unfortunately, copper is an environmentally abundant heavy metal; we detect very high copper content randomly in dried filter papers, which makes screening for Wilson's disease using dried blood spots unfeasible.

Future development

Many treatable congenital disorders are caused by mutations that result in absent or diminished levels of proteins; thus, protein biomarkers have enormous potential in the diagnosis/screening of congenital disorders. LC-MRM-MS has emerged as a robust technology that enables highly precise, specific, multiplex quantification of signature proteotypic peptides as stoichiometric surrogates of biomarker proteins. As proof of concept, we have developed a novel proteomic screening approach using LC-MRM-MS to simultaneously identify specific signature peptides derived from the transmembrane protein CD3ε (a general marker for T cell number) and the intracellular proteins WASP and BTK (expressed in B and myeloid cells) as markers of three life-threatening primary immunodeficient diseases (PIDD): severe combined immune deficiency (SCID), Wiskott–Aldrich syndrome (WAS), and X-linked agammaglobulinemia (XLA).[19] Blinded patients' peripheral blood mononuclear cell (PBMC) samples with sufficient actin recovery were successfully analyzed for BTK, WASP, and CD3ε signature peptides, using an actin peptide for normalization, to accurately identify patients with PIDD.

With depletion of high-abundance proteins, we were also able to identify signature peptides for BTK and WASP in the dried blood spot extract, along with their isotopically labeled standards. This is compelling evidence that detection of low-abundance proteins in dried blood spots will be feasible by employing specific enrichment techniques using antipeptide antibodies. Our lab is currently exploring the use of peptide immunoaffinity enrichment to overcome a number of caveats, including limited sensitivity and reproducibility, especially for low-abundance proteins or small sample volumes.[20,21] Our preliminary analysis indicates that candidate signature peptides for ATP7B in HepG2 cells can be identified, along with their isotopically labeled internal standards (unpublished data). A substantial proportion of Wilson's disease–associated missense mutations, including p.H1069Q and p.R778L, result in markedly decreased levels of the ATP7B protein caused by enhanced degradation.[22–24] Other prevalent mutations, such as protein-truncating nonsense mutations (~13% of known point mutations)[25] and frameshift mutations,[26] are predicted to result in the absence or decay of mRNA[27,28] or a severely truncated protein, resulting in absent or diminished protein levels. Taken together, it is expected that most patients with Wilson's disease would have absent or significantly reduced levels of ATP7B. Nevertheless, it is still not known whether the MRM-based technique could be sensitive enough or feasible for screening patients or newborns using blood-based specimens. The expression of ATP7B protein

in blood-based specimens may not be high enough to be detected, even with this technology.

In summary, early recognition, diagnosis, and preemptive treatment are critical for improving outcomes in Wilson's disease. Wilson's disease meets the universal criteria for mass population screening, except that there is no cost-effective method yet available. Although the optimum time to screen is disputable, the newborn period has the advantage of an infrastructure that is currently in place for blood spots and screening worldwide. The current ceruloplasmin assay may be suitable for infant or pediatric screening around the age of 3 years, but there is a lack of outpatient-based mandatory programs, which is a significant barrier to implementation. A study using LC-MRM-MS technology is in progress, but at this point, no scientific data is available. New biomarkers and the development of new methods should be continuously explored to provide early and effective preemptive treatment that can drastically improve outcomes in patients with Wilson's disease.

Acknowledgments

This manuscript is partly supported by NIH Grant 1R21HD069890-01A1.

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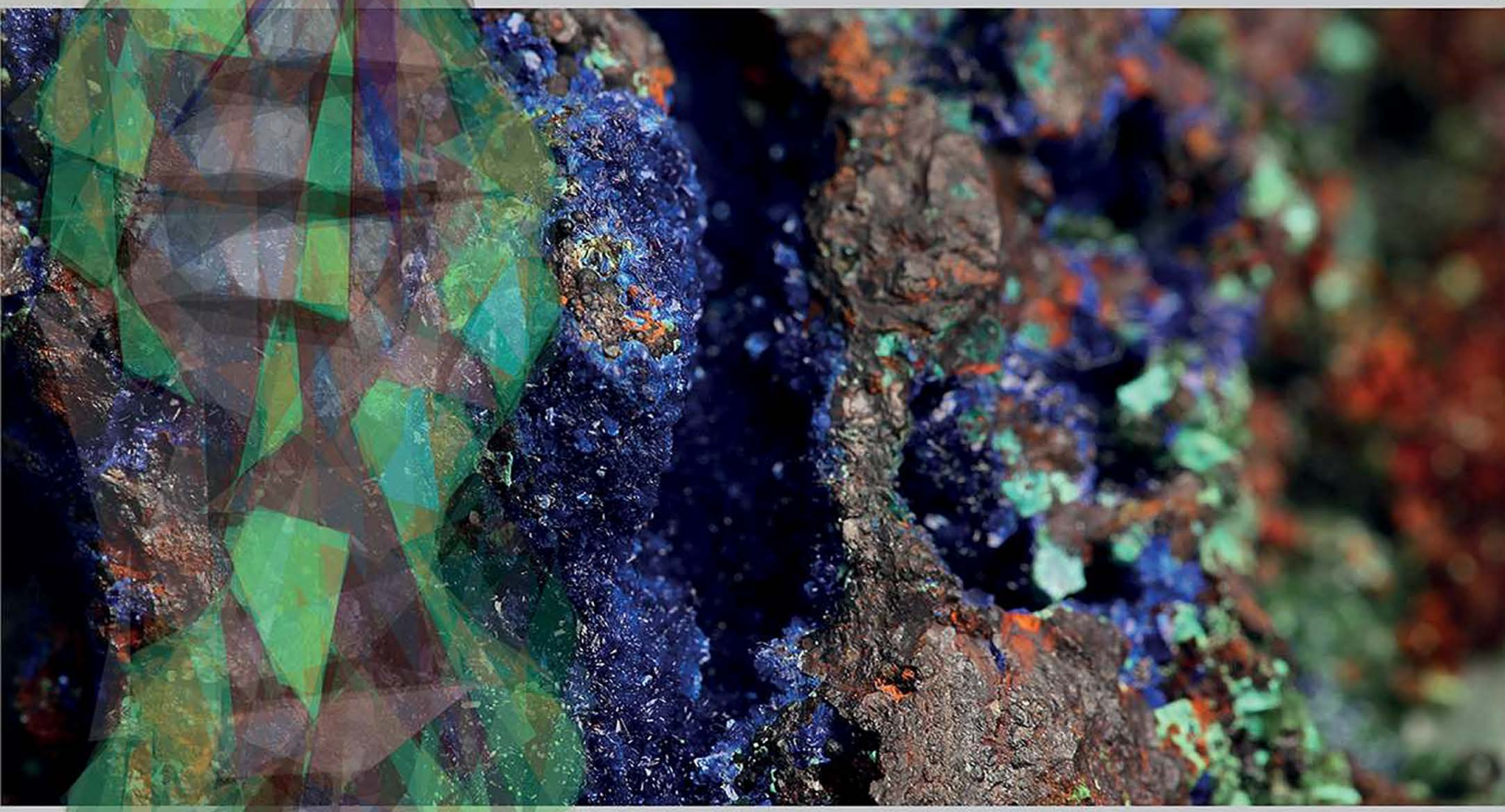
Clinical and Translational Perspectives on WILSON DISEASE

Edited by
Nanda Kerkar
Eve A. Roberts



Chapter 26

Population Screening for Wilson Disease

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BACKGROUND

Wilson disease (WD, OMIM 277900) is an autosomal recessive disorder of copper metabolism with an estimated prevalence of one in 30,000 individuals and carrier frequency of one in 90 individuals in most populations [1]. The only gene known to be associated with WD is *ATP7B*, which encodes a copper-transporting ATPase ATP7B (Wilson ATPase) [2–5]. Over 500 different pathogenic variants and >100 polymorphisms have been identified in *ATP7B* in the Human Genome Mutation Database (http://www.hgmd.cf.ac.uk/ac/gene.php?gene = ATP7B), and most affected patients are compound heterozygotes, with one each of two different mutations [6]. There is wide geographic variation in *ATP7B* mutations [7–9]. These common mutations cause enhanced degradation and decreased levels of ATP7B, the ATPase abnormal in WD [10–13].

Effective treatment for WD is widely available and affordable, highlighting the importance of early detection and intervention. Copper chelators such as penicillamine and trientine bind excess copper and promote excretion, and zinc salts inhibit enteral copper absorption [14–18]. These treatments are highly effective in preventing the downstream morbidity and early mortality associated with WD [15–19]. Zinc monotherapy has also been demonstrated to be an effective long-term maintenance treatment for WD [20–24]. Left untreated, half of affected patients with neurologic symptoms may develop permanent neurologic damage [25]. Patients with chronic liver disease may progress to hepatocellular carcinoma [26,27]. Therefore, the ideal time to detect WD and begin treatment is prior to onset of symptoms for the best long-term clinical outcome.

NEWBORN SCREENING

Newborn screening is implemented by well-established public health programs in most developed countries. Each year, over 4 million newborn babies are screened for congenital disorders in the United States, and over 4000 infants are diagnosed as having a condition [28]. Given the incidence of WD, over 130 infants are likely born each year with WD.

Guidelines for deciding whether a particular condition is a suitable candidate for newborn screening was first formulated by Wilson and Jungner in 1968 and revised by the World Health Organization in 2008 [29,30]. Unfortunately, many aspects of these guidelines are subjective, and there is not always agreement about which disorders to include in the newborn screening panel [31]. Therefore, despite existing guidelines, there are now varying newborn screening panels worldwide. Nonetheless, given the aforementioned factors of high prevalence, severe disease burden, and available treatment, WD has always been an attractive target for newborn screening. However, no cost-effective, specific biomarkers or screening methods have been developed for WD. Previous studies have evaluated the predictive value of low ceruloplasmin, urinary copper, and holoceruloplasmin, with limited outcomes. The last decade has remained largely devoid of new studies on biochemical screening for WD.

The application of tandem mass spectrometry (MS/MS) has recently expanded the ability to screen for >50 metabolic diseases from a single dried blood spot collected on filter paper [32–36]. Inborn errors of metabolism are particularly amenable to screening due to the accumulation of small metabolites, which are detectable by mass spectrometry

Clinical and Translational Perspectives on WILSON DISEASE. DOI: https://doi.org/10.1016/B978-0-12-810532-0.00026-4

[37]. Many treatable disorders such as WD remain difficult to detect as they are characterized by absent or diminished levels of proteins or metabolites present only within leukocytes.

Recently, the emergence of liquid chromatography–selected reaction monitoring–mass spectrometry (LC–SRM–MS) has enabled multiplexed quantification of low-abundance signature proteotypic peptides [38,39]. The application of this technology may help facilitate research on WD for measuring protein abundance, identifying biomarkers, and enabling early diagnosis. Our laboratory has demonstrated a promising method of screening for WD by peptide immuno-SRM assay, raising the possibility of population screening for WD using peptide biomarkers for the ATP7B protein [40,41].

COPPER SCREENING

Most patients with WD have elevated serum non-ceruloplasmin-bound copper (>25 μg/dL), making serum copper quantification a logical potential target for screening in dried blood spots. Relative exchangeable copper (REC = exchangeable copper/total copper) has been proposed as a reliable, sensitive, and specific biomarker for screening and diagnosis of WD in adults (see Chapter 22: Direct Determination of Non-Ceruloplasmin-Bound Copper in Plasma). However, reference ranges have not been established for newborns or children [42]. Copper is also a naturally occurring abundant heavy metal in the environment. In a random sample of commercial filter papers, we detected very high copper concentrations, eliminating the possibility of screening for WD using any copper measurements in dried spots for both blood and urine.

CERULOPLASMIN SCREENING

Ceruloplasmin is an α_2-glycoprotein that contains 90% of circulating copper in the body [43]. Ceruloplasmin is initially synthesized in hepatocytes as apoceruloplasmin. Copper is then incorporated into apoceruloplasmin by the Wilson ATPase to form holoceruloplasmin before secretion into circulation. In WD, the absence of copper binding causes ceruloplasmin to be rapidly degraded by plasma proteases, leading to low plasma ceruloplasmin in most affected patients. Ceruloplasmin is an acute phase reactant, and the presence of low ceruloplasmin levels should be cautiously interpreted as other medical conditions may also lead to low ceruloplasmin (see Chapter 9: Ceruloplasmin). The majority of patients with WD have low plasma ceruloplasmin levels (<20 mg/dL or < 200 mg/L); however, some affected patients have been shown to have borderline or normal ceruloplasmin levels. Up to 20% of heterozygous carriers for WD may also have low-normal range serum ceruloplasmin levels [44]. Previous studies have demonstrated low diagnostic accuracy of serum ceruloplasmin for WD screening [45]. Using the conventional cutoff of 20 mg/dL, the estimated positive predictive value for WD was only 48.3% in adults with WD compared to carriers and controls [46]. A retrospective review of pediatric patients with hepatitis revealed accuracy of a ceruloplasmin level of <20 mg/mL of 84.8% [47].

Previous studies have demonstrated that while holoceruloplasmin levels were reduced in patients with WD, apoceruloplasmin levels were comparable to those observed in unaffected individuals [48]. Based on these findings, Endo and colleagues developed a sandwich enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody specific to holoceruloplasmin to measure ceruloplasmin levels in dried blood spots [49]. Their initial screening of 126,810 newborns did not detect any patients with WD, but their subsequent study of 24,165 infancy to elementary school-aged children identified three affected patients [50]. In another Japanese study using the same sandwich ELISA-based assay, two presymptomatic patients with WD were identified among 2789 children between 1 and 6 years old [51].

A pilot study of mass screening in 3667 children for WD in Korea using the sandwich ELISA assay identified one presymptomatic individual [52]. In a subsequent validation study, retrospective analysis of 353 newborn dried blood spots showed an average ceruloplasmin concentration of 40.0 ± 14.4 mg/dL [53]. Original newborn dried blood spots from two known affected patients were retrieved and ceruloplasmin concentrations were 2.6 and 2.8 mg/dL. A separate ceruloplasmin screening study on dried blood spots of ~100,000 newborns in Minnesota failed to detect any patients with WD (unpublished data). Ceruloplasmin levels in some newborns were unstable and physiologically lower than in older children and adults, and they continue to rise to adult levels 12 months after birth before stabilizing around 3 years of age [50]. Therefore, the optimal age of ceruloplasmin-based screening was thought to be ~3 years.

Other groups have studied population screening for WD by measuring urine holoceruloplasmin levels using sandwich ELISA assay. Owada and colleagues conducted mass screening of 48,819 primary school-aged children in Japan using urinary holoceruloplasmin and identified two presymptomatic individuals with WD [54]. Urinary holoceruloplasmin levels in the two affected patients were low at 1.9 and 9.3 ng/mg creatinine compared to 99.3 ± 77.7 ng/mg creatinine in unaffected 3–5-year-old children. However, two of the 41 WD control samples had higher levels of urinary

TABLE 26.1 Previous Population Screening Studies Using Ceruloplasmin Assays

Year	Study	Number of Subjects	Age Range	Positive Cases	CP in Positive Patients (mg/dL)	Mean CP (mg/dL)	PPV (%)/FP rate (%)	CP Cutoff Value	Methods/ Samples
1993–1995	Yamaguchi et al. [50]	126,810	Newborns	0	N/A	N/A	N/A	−2 to −2.5 SD	ELISA/DBS
1977–1996	Yamaguchi et al. [50]	24,165	Late infancy to elementary school	3	N/A	N/A (98.83% screened had CP > 20)	N/A	−2 to −2.5 SD	ELISA/DBS
1997	Cauza et al. [45]	2867	Adults	1 (then her sister)	N/A	±	PPV 5.9%	20 mg/dL	Radial immunodiffusion/ serum
1999	Ohura et al. [51]	2789	1–6 years	2	Pt1: 4.0,3.5 Pt2: 1.7,1.5	12.4 ± 3.95	FP rate 0.6%	3rd percentile	ELISA/DBS
2001	Hahn et al. [52]	3667	3 months to 15 years	1	2.3	30.5 ± 9.5	PPV 9.1%	15 mg/dL	ELISA/DBS
2002	Owada et al. [54]	48,819	Primary school children	2	Urinary HCP < 1.9, 9.3 ng/mg Cr, serum HCP 2.7, 7.0 mg/dL	99.3 ± 77.7 ng/ mg Cr Serum 21–37	N/A	3rd percentile	ELISA/urine
2006	Kroll et al. [53]	1398	Newborn to 18 years, retrospective	0	N/A, retrospective positive controls 2.6, 2.8	Newborn: 47.2 ± 15.5 Pediatric: 40.0 ± 14.4	N/A	< 15 mg/dL	ELISA/DBS
2008	Nakayama et al. [77]	11,362	3 years	1	13.3, 1.3, 24.9 ng/mg Cr	WD ctrl: 13.2 ± 9.25 ng/ mg Cr Normal ctrl: 190 ± 154ng/ mg Cr	N/A	1% of participants (<45.0 ng/mg Cr), 3 screenings	ELISA/urine
2008	Zappu et al. [70]	5290 397	Newborn (Sardinia) Newborn (Kalymnos)	1:2707 27:20,000	N/A	N/A	N/A	N/A	PCR-based/DBS

DBS, dried blood spots; *ELISA*, enzyme-linked immunosorbent assay; *CP*, ceruloplasmin; *HCP*, holoceruloplasmin; *Cr*, creatinine; *WD*, Wilson disease.; *N/A*, not available; *PPV*, positive predictive value; *FP*, false positive.

holoceruloplasmin within the range of normal controls, consistent with previous reports of patients with WD and normal ceruloplasmin levels. In addition, urinary holoceruloplasmin levels decreased after 3 days of storage in chlorhexidine gluconate at room temperature, and after 7 days at 4 °C. The accuracy and true predictive value were not reported. Furthermore, the study did not include phenotypic data or elaborate on the methodology behind which patients were selected for further testing. A follow-up study of urinary holoceruloplasmin levels in 11,362 primary school children in the island of Hokkaido identified one patient with WD, which was later confirmed molecularly. The wide range of normal values, uncertain accuracy, and instability for storage and transport make urinary holoceruloplasmin less plausible for newborn screening. Previous population screening studies using ceruloplasmin assays and polymerase chain reaction (PCR)-based mutation analysis are summarized in Table 26.1.

At present, assaying ceruloplasmin in late infancy or childhood appears to be the only cost-effective approach to population-based screening for WD. The studies in Japan had an average cost of USD $2.50 per assay, not adjusted for inflation. However, the childhood-based screening model relied on the mandatory healthcare program infrastructure in Japan, which is not universally available in other countries. In the United States, this program would be difficult to implement given limited federal resources, significant population influx and efflux, and lack of compulsory routine pediatric healthcare follow-ups.

From a technical standpoint, ELISA-based immunoassays for ceruloplasmin quantification in plasma and urine, regardless of type of antibody used against different ceruloplasmin epitopes, may not be sensitive enough to detect newborn patients with WD. Taken together, while most patients with WD appear to have low ceruloplasmin levels at baseline, the low positive predictive value, false positive rates in heterozygotes and normal newborns, and variable antibody binding makes ELISA-based assays less than ideal for population screening of WD.

MOLECULAR-BASED SCREENING

Previous studies have failed to detect biallelic pathogenic variants in *ATP7B* in up to 20% of patients with clinically diagnosed WD, raising suspicion for genetic heterogeneity [58]. However, it is more likely that novel variants, reduced penetrance, and unusual mechanisms of inheritance such as uniparental disomy or the presence of three concurrent mutations contribute to this discrepancy. Sequencing of the entire coding region and adjacent splice sites of *ATP7B* in 181 patients with WD in the United Kingdom showed an overall mutation detection frequency of 98%, strongly supporting monogenic disease [59]. The data also suggest a much higher prevalence of biallelic pathogenic variants in *ATP7B* than conventionally reported (that is 1:7026 instead of 1:30,000). Sequencing of all 21 exons of *ATP7B* is a time-consuming and cost-prohibitive approach to population screening for WD. However, targeted DNA analysis can be utilized in genetically homogenous populations with high prevalence of disease and allelic frequency for specific pathogenic variants. For instance, the incidence of WD is particularly high in Sardinia, where a molecular-based screening study of 5290 newborns estimated an incidence of one in 2707 live births [60]. In Sardinia, a 15-nucleotide deletion in the promoter region of *ATP7B* is implicated in 60.5% of patients with WD [61]. In aggregate, six common mutations constitute up to 85% of disease-causing alleles in that isolated population. In the Canary Islands, the L708P mutation is seen in up to 64% of patients [62].

In large, genetically heterogeneous populations such as China, the United States, and India, the most prevalent pathogenic variants have lower combined allelic frequencies; many affected individuals are compound heterozygotes for rare or novel mutations. In China, the two most prevalent mutations (that is, R778L and P992L) were present at a combined allelic frequency of approximately 40%–50% [57,63,64]. In Korea, the two most prevalent mutations (R778L and N1270S) account for 50% of cases [65]. In the United States and Europe, the allelic frequency of H1069Q accounts for approximately 30% of cases alone [8,66,67]. The allelic frequencies of common mutations in India vary largely by geographic region [55,56,68,69].

As the cost of molecular testing decreases and accessibility increases, a stepwise approach starting with DNA-based screening of common mutations accounting for the majority of cases in certain regions may become a feasible and rapid method for diagnosis. Indeed, Zappu and colleagues applied TaqMan technology to initially screen for the six most common mutations in Sardinia, followed by testing for the remaining 16 mutations in heterozygotes. They report a sensitivity of 94.6% with this stepwise screening approach [70]. Mak and colleagues demonstrated detection of 28 common mutations in WD patients in Hong Kong within 3 hours using a gene panel, real-time amplification refractory mutation system PCR, and green fluorescent dye for analysis [71]. In Taiwan, Lin and colleagues employed a three-tiered screening approach in 14 patients with WD and 50 normal controls. First, they screened for

the two most common mutations in exons 8 and 13 using High Resolution Melting analysis, followed by mutation analysis in exons 2, 5, 11, 12, 16, and 18 in negative cases, and finally examining the 5′UTR region and all remaining exons in twice-negative cases. Abnormal melting curves at each step were confirmed with direct DNA sequencing. They report that approximately 67.86% of *ATP7B* mutation alleles could be detected at the first step and 96.43% at the second step [72].

High-throughput technology, such as microarray chips, has also been examined for rapid, cost-effective screening of selective WD mutations [73,74]. However, the detection rate must be improved with follow-up direct sequencing. In China, a large-scale, two-step screening study based on array technology was recently conducted on 1222 patients with WD and 110 healthy controls [57]. Rapid multiplex PCR-MassArray was used to first screen for 110 mutational hotspots common in East Asian populations. Patients who were negative were analyzed with PCR-Sanger sequencing of all exons, flanking regions, and 5′UTR and 3′UTR regions. They identified 88 pathogenic variants and 9 novel mutations, with the six most common mutations accounting for 57.46% of all cases. The authors also report some genotype−phenotype correlation, the strongest being younger age of onset and lower levels of plasma ceruloplasmin and serum copper levels in patients with the R778L mutation. Tiered testing starting with array-based technology customized for common regional mutations offers a promising rapid and sensitive method for WD screening, but it cannot be implemented as a large-scale, universal screening method for WD due to the limited detection rate.

MASS SPECTROMETRY-BASED SCREENING

Liquid chromatography−MS/MS (LC−MS/MS) techniques enable antibody-independent, highly precise, and multiplexed quantification of signature proteolytic peptides as stoichiometric surrogates of biomarker proteins. There are multiple advantages to mass spectrometry-based screening methods on dried blood spots. First, the analysis can be performed on newborn dried blood spots, which are stable without any additional processing, modifications, refrigeration, or special transport. Second, the technology employs existing newborn screening infrastructure, which relies on mass spectrometers already present in most newborn state laboratories. Third, the method offers rapid turnaround. Fourth, the assay directly analyzes affected proteins with high specificity instead of screening for downstream accumulated metabolites. Most importantly, surrogate protein biomarker identification by mass spectrometry offers a plethora of opportunities for application to other treatable congenital disorders characterized by absent or low-abundance proteins such as lysosomal storage diseases and primary immunodeficiency syndromes.

More recently, our laboratory developed a novel proteomic screening assay using peptide immunoaffinity enrichment coupled to selected reaction monitoring (SRM) to improve the detection sensitivity of LC−MS/MS. SRM is a targeted mass spectrometry technique with increased sensitivity compared to profiling modes of analysis while maintaining high specificity for the target analyte and the possibility of multiplexing.

Our laboratory initially harnessed this technology to quantify ceruloplasmin by detecting its tryptic-digest signature peptides. After determining signature peptides, establishing internal standards, and conducting validation studies, our results showed patients with WD had lower ceruloplasmin levels by surrogate biomarker peptides compared to carriers and healthy controls. These findings were consistent with those obtained by ELISA assay, with average ceruloplasmin levels of 0−20 mg/dL in affected individuals, compared to >20 mg/dL in WD carriers. The lower limit quantification for ceruloplasmin was determined to be around 0.7 mg/dL, and inter- and intra-assay precision suggests feasibility for clinical use. However, large-scale studies using this technology have not been performed due to the relatively time-consuming sample preparation and long chromatographic run time [40].

As proof-of-concept, we used LC−SRM−MS to simultaneously identify signature peptides as surrogate biomarkers for three life-threatening primary immunodeficiency diseases—severe combined immune deficiency (SCID), Wiskott−Aldrich syndrome (WAS), and X-linked agammaglobulinemia (XLA). The signature peptides were derived from transmembrane protein CD3ε (a general marker for T-cell number) and intracellular proteins Wiskott−Aldrich syndrome protein (WASP) and Bruton tyrosine kinase (BTK) (expressed in B cells and myeloid cells), respectively [75].

Concentrations for each representative protein (that is, WAS, CD3ε, and BTK) were established in 45 control samples and signature isotopically labeled peptides were customized for each protein. In a blinded fashion, 16 de-identified peripheral blood monocyte samples with sufficient actin recovery from molecularly confirmed affected patients (specifically, 5 with WAS, 5 with SCID, and 5 with XLA) were analyzed for these signature peptides. All three signature peptide biomarkers were quantifiably detectable in unaffected leukocyte samples and absent in

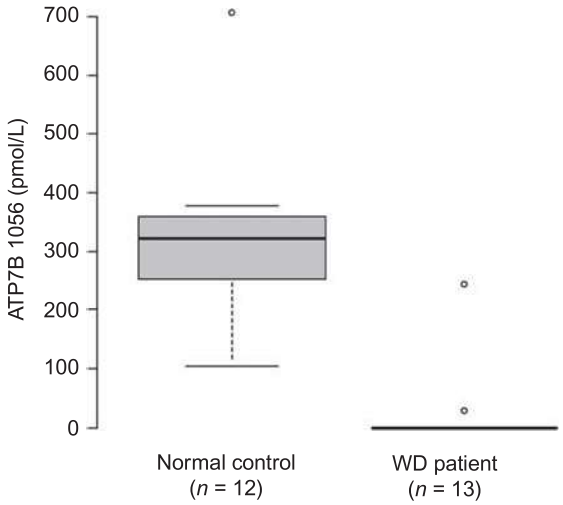

FIGURE 26.1 Distribution of the levels of ATP7B (Wilson ATPase) in dried blood spots from 13 WD patients and 12 normal controls. The bold black line indicates the median, the inner quantiles are represented by boxes, and the whiskers show 95% of the data. *WD*, Wilson disease. *Adapted from Jung S, Whiteaker JR, Zhao L, Yoo HW, Paulovich AG, Hahn SH. Quantification of ATP7B protein in dried blood spots by peptide immuno-SRM as a potential screen for Wilson's disease. J Proteome Res 2017;16:862–71.*

affected leukocyte samples using an actin peptide for normalization. The results were also replicated in T-cell depleted cell lines, demonstrating the ability of LC–SRM–MS to accurately identify patients with primary immunodeficiency disorders. However, the inter- and intra-assay coefficient of variation needed to be lowered for clinical application [75].

To improve the sensitivity and reproducibility of our results for low-abundance proteins and small sample volumes, we enhanced our assay with immunoaffinity enrichment using antipeptide antibodies coupled to SRM (immuno-SRM). Our most recent proof-of-concept study extended this technology to the detection of signature peptides to ATP7B protein in HepG2 cell lines and their isotopically labeled internal standards. This is based on the premise that many missense pathogenic variants associated with WD, including H1069Q and R778L, result in markedly decreased levels of ATP7B protein due to enhanced degradation. Other common WD mutations result in mRNA decay or severely truncated proteins, theoretically resulting in absent or diminished levels of ATP7B in leukocytes. Relative mRNA expression of ATP7B in various tissues, including peripheral leukocytes, has been shown in previous studies (http://biogps.org/#goto = genereport&id = 540) [76]. Therefore, we hypothesized that individuals affected with WD will have lower or absent signature peptides of ATP7B (Wilson ATPase) in their leukocytes and dried blood spots by immuno-SRM compared to carriers or negative controls.

Indeed, our study demonstrated reliable quantification of endogenous ATP7B biomarkers in dried blood spot samples by immuno-SRM. First, we used in silico trypsin digestion to select several signature peptides for ATP7B that are predicted to be well-detected by LC–MS/MS, using criteria previously described [75]. Next, affinity-purified rabbit polyclonal antibodies were generated against four signature peptides. The ATP7B 1056 peptide was selected as a target peptide for quantification in human samples based on good peptide recovery after antibody-enrichment, minimal background signal noise, and the fact that the most common WD mutation in Europeans, H1069Q, occurs within this peptide. Quantification of this ATP7B 1056 peptide was carried out by immuno-SRM on trypsinized dried blood spot samples of patients with WD and unaffected individuals. In 12 out of 13 samples from patients with WD, signature peptides to ATP7B were nearly absent compared to healthy controls, confirming our hypothesis (Fig. 26.1). The genotype and ATP7B 1056 levels in dried blood spots of the 13 WD patients compared to normal controls is depicted in Table 26.2. The one patient with ambiguous results by screening is a compound heterozygote with a variant of uncertain significance in *ATP7B*. Our findings provide compelling evidence that surrogate biomarkers of ATP7B protein may be used as a novel platform to screen for WD in dried blood spots [41]. However, whether the immuno-SRM-based technique is sensitive enough or feasible to screen newborn patients remains to be elucidated. Large-scale studies in newborns with proven carriers and controls are required in the future.

TABLE 26.2 Genotype and ATP7B Peptide 1056 Levels in Dried Blood Spots from 13 Wilson Disease Patients and 12 Normal Controls

Sample	ATP7B 1056 (pmol/L)	Mutation
WD1	29.5	p.R778W and p.T977M
WD2	ND[a]	p.H1069Q and p.R1319*
WD3	ND	p.H1069Q and p.R1319*
WD4	ND	p.G943D and p.T1178A
WD5	ND	p.R778L homozygote
WD6	ND	p.C2304_2305insc and p.L1083F
WD7	NA[b]	p.R778L and p.A874V
WD8	244.8	p.T974M and p.S391L
WD9	ND	p.R778G and p.K175S_fs/p.Q260P_fs
WD10	ND	p.R778G and p.K175S_fs/p.Q260P_fs
WD11	ND	p.R778L and p.E1064A
WD12	ND	p.R778L and p.E1064A
WD13	ND	p.H1069Q and p.Y1331Tfs*61
Controls ($N = 12$)	327.6 ± 147.4	

[a] *ND,* not detected
[b] *NA,* not applicable due to signal to noise ratio (S/N) < 10
Jung S, Whiteaker JR, Zhao L, Yoo HW, Paulovich AG, Hahn SH. Quantification of ATP7B protein in dried blood spots by peptide immuno-SRM as a potential screen for Wilson's disease. J Proteome Res 2017;16:862–71.

SUMMARY

Early detection and treatment of WD is imperative for optimizing long-term clinical outcomes. Diagnosis remains complicated by the nonspecific, broad phenotype of WD and occasionally ambiguous laboratory and molecular studies. WD meets consensus criteria for newborn screening, except that no reliable biomarker or cost-effective screening methods are currently available. Controversy exists on the optimal time to screen, as studies suggest ceruloplasmin assays are most sensitive and specific at around 3 years of age. However, the lack of mandatory outpatient-based pediatric health initiatives presents a challenge for widespread implementation in many countries such as the United States. Screening during the newborn period takes advantage of existing infrastructure for newborn dried blood spots and screening worldwide. Moreover, newborn dried blood spots are sufficiently stable, and they do not require any additional processing, modifications, refrigeration, or special transport. Initial studies utilizing LC–SRM–MS and immuno-SRM technology to quantify signature peptides to target proteins of various metabolic diseases show promising results, but larger studies in newborns compared to carriers and controls are needed. Research into novel biomarkers and honing of DNA- and mass spectrometry-based technology continue to push the frontiers of screening for patients with WD and may result in highly effective strategies for newborn screening.

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Clinical and Translational Perspectives on WILSON DISEASE

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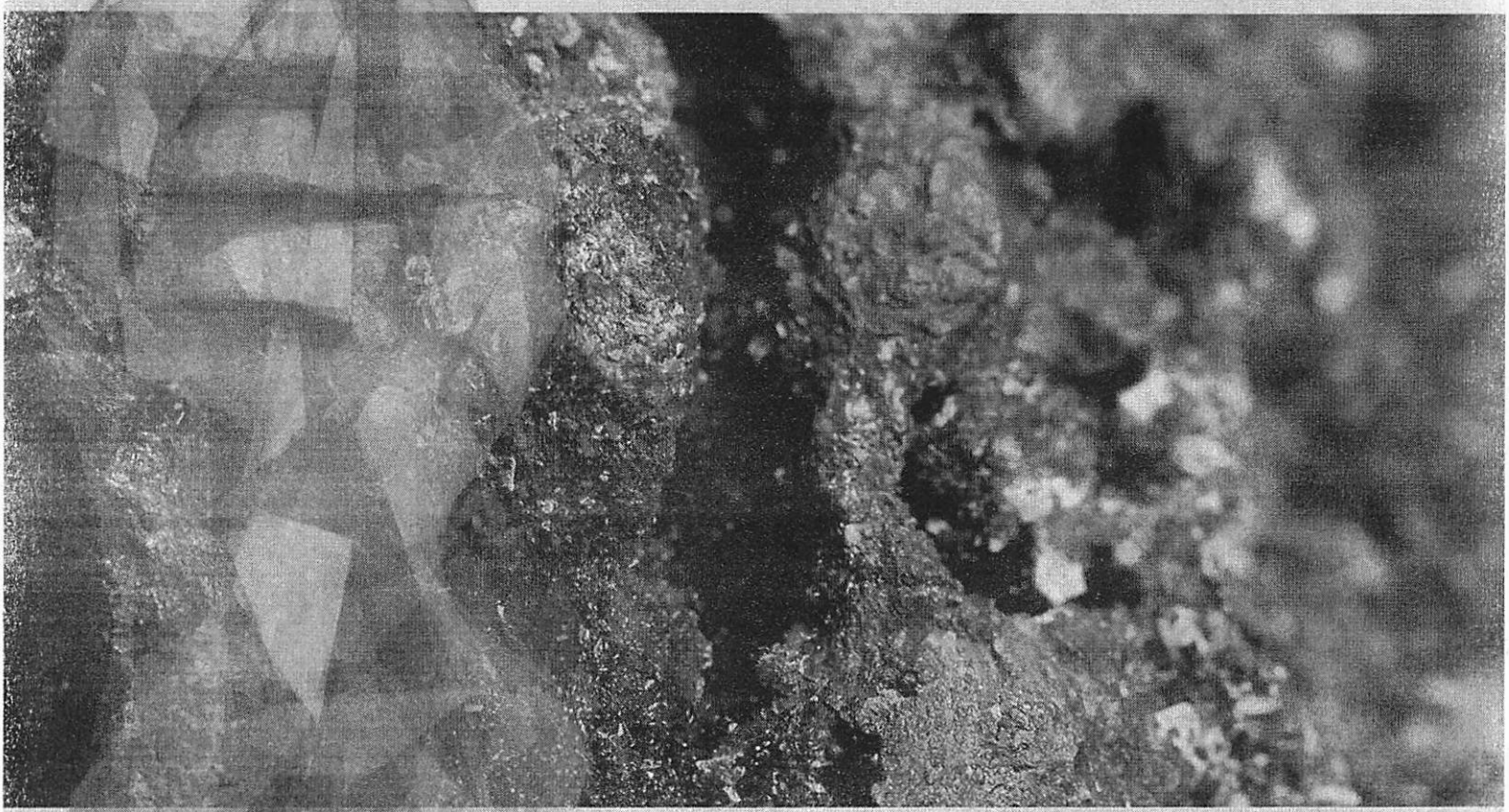

Chapter 17

Wilson Disease in Infancy through Adolescence

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Wilson disease (WD), an inherited disorder mainly of hepatocellular copper disposition, due to dysfunction of the Wilson ATPase, a P_{1B}-ATPase encoded by the gene *ATP7B*, has been regarded as affecting mainly children and young adults. In children, as in older adult patients, WD has protean manifestations. It can involve multiple systems: notably hepatic, neurologic, and psychiatric but also ocular, hematological, renal, skeletal, and cardiac. An important general feature of WD in the pediatric age-bracket is that it often presents with nonspecific features which can be difficult to distinguish from other hepatic or neuropsychiatric disorders. A high level of clinical suspicion, along with effective methodical diagnostic testing, is required. Early diagnosis, preferably before development of clinical symptoms, is associated with normal, healthy life span in patients who are adherent to an effective medical regimen.

WILSON DISEASE IN VERY YOUNG CHILDREN

Classically, in the pediatric age-bracket, WD was most commonly diagnosed in adolescents. It is now well established that WD can present with clinical disease, mainly hepatic, in very young children. Definitions of "very young" children vary slightly among reports. We define "very young" as <5 years-old. Since the first detailed well-documented report of a 3 year-old with cirrhosis [1], numerous further reports have appeared [2–16], as shown in Table 17.1. Some children are diagnosed with WD when they present with an intercurrent health problem such as diarrhea and are found to have elevated serum aminotransferases; others are found through family or general infant screening. An early instance of molecular diagnosis in infancy was an 8-month-old Japanese boy picked up on mass screening [17]. A newborn infant was identified because of established genetic risk of having WD [18]. Among reports predating the identification of *ATP7B*, one appears to be actual WD, not simple copper toxicosis [19]. Many large case series include at least one child <5 years-old. Overall, there appear to be 50–60 such very young children currently reported in the literature. The majority have basal 24-hour urinary copper excretion >0.6 μmol/24 hours (>40 μg/24 hours); however, difficulties in collecting a 24-hour collection in this age-bracket can limit the test's utility. Finding elevated serum aminotransferases in a preschool-aged child is an indication to look for WD.

CLINICAL FEATURES IN CHILDREN AND ADOLESCENTS

The major clinical presentations of WD in children/adolescents are hepatic, neurologic, and psychiatric—and "silent" (no symptoms at all: apparently well). Occurrence of silent WD is extremely important in the pediatric age-bracket. Silent WD includes those patients conventionally described as being asymptomatic or having presymptomatic WD. These terms are often applied interchangeably, although they do not mean the same thing. (Calling a disease state "presymptomatic" makes the assumption that the disease will become clinically manifest.) Silent WD is generally identified

Clinical and Translational Perspectives on WILSON DISEASE. DOI: https://doi.org/10.1016/B978-0-12-810532-0.00017-3

TABLE 17.1 Features of Wilson Disease in the Very Young Child, Based on Available Published Data

Age, years	Number	Sex	Reason for Ascertainment	Serum Ceruloplasmin[a]	Basal 24-hour Urinary Cu[b]
0–1	7	5 M, 2 F	Incidental (diarrhea)→abnl LFTs: 2	No info: 2	No info: 3
			Family screening: 2; mass screening: 1; projected risk: 1; no info: 1	<100: 3	<0.6: 3
				>140: 2	>0.6: 1
1–2	6	1 M, 5 F	Family screening: 3; no info: 3	No info: 1	No info: 3
				<100: 4	<0.6: 3
				100–140: 1	
2–3	13	6 M, 7 F	Clinical liver disease: 1; "hepatic": 6	No info: 1	No info: 5
			Incidental→abnl LFTs: 3; no info: 3	<100: 4	<0.6: 2
				>140: 6	>0.6: 6
3–4	14	8 M, 6 F	Clinical liver disease: 1; "hepatic": 2	No info: 0	No info: 1
			Incidental→abnl LFTs: 3; pre-kindergarten exam→abnl LFTs: 3	<100: 7	<0.6: 3
			Family screening: 3; no info: 2	>140: 5	>0.6: 10
4–5	12	6 M, 6 F	Incidental→abnl LFTs: 2; family screening: 3; pre-kindergarten exam→abnl LFTs: 1; no info: 6	No info: 1	No info: 1
				<100: 8	<0.6: 1
				>140: 3	>0.6: 10
	52	26 M, 26 F			

abnl, abnormal; *LFTs*, liver function tests; *info*, information; *Cu*, copper.
[a] *< 100 mg/L suggests severe mutation; >140 mg/L is above suggested cutoff for ceruloplasmin in WD determined by immunological methodology.*
[b] *< 0.6 μmol/24 hours (<40 μg/24 hours) is below the informative threshold value for diagnosing WD in children.*

by screening or by chance. Individuals with silent WD have a definite diagnosis of WD, either genetically or biochemically (subnormal serum ceruloplasmin, basal 24-hour urinary copper excretion >0.6 μmol/24 hours; elevated hepatic copper concentration, if sought). While free of clinical symptoms, many have biochemical abnormalities indicating organ damage, typically, elevated serum aminotransferases.

Hepatic-WD tends to be evident at an earlier age than WD presenting with neurologic symptoms (called here "neuro-WD"); however, onset of neuro-WD or psychiatric manifestations of WD in childhood is not uncommon [20]. Psychiatric presentation may be very subtle. Based on data from numerous pediatric clinical series, gender distribution shows slight predominance of males over females (M:F = 5:4), whereas in adults, there may be a female predominance; average age at diagnosis is ~10 years-old.

LIVER DISEASE

The symptoms of hepatic-WD in children are frequently nonspecific, ranging from fatigue, anorexia, nausea, vomiting, weight loss to abdominal pain and jaundice. Jaundice is typical of more severe liver involvement but can be due to intravascular hemolysis. Jaundice may indicate liver decompensation in a chronic case or may be the presenting feature in classic Wilsonian acute liver failure (ALF-WD). If the rate of increase in serum bilirubin is high, the prognosis may be poor, as jaundice is then usually accompanied by other signs of liver failure including coagulopathy, ascites, and/or encephalopathy. Perhaps surprisingly, epistaxis is frequently mentioned in pediatric series as a presenting complaint. Hepatic-WD should be considered as a possible diagnosis in any child/adolescent who has hepatomegaly, elevated serum aminotransferases, or evidence of fatty liver.

WD is an important genetic cause of childhood cirrhosis worldwide. Actually determining the prevalence of cirrhosis in pediatric WD is difficult. A scan of the worldwide literature suggests that approximately one-third of children with clinically evident WD present with cirrhosis. The potential for rapid progression to cirrhosis emphasizes the

TABLE 17.2 Reported Clinical Features of Wilson Disease Resembling Autoimmune Hepatitis

		Age, Sex	Onset	IgG > ULN	Antibodies	Ceruloplasmin (mg/dL)	24-hour Urinary Copper (μg)	KF Ring/ DCT	Cirrhosis/ Interface Hepatitis	Liver Copper (μg/g)	Rx	Outcome
1	Milkiewicz [36]	15 years, F	Chronic	−	ANA 1:100, GPC +	1.1	213	−/−	+/+	385	Pred, D-Pen	Stable
2	Milkiewicz [36]	24 years, F	Chronic	+	SMA 1:60, ANA 1:40	1.8	326	−/−	+/+	965	Pred, LTx	Post-LTx
3	Yener [37]	22 years, F	ALF-WD	NA	ANA 1:400, SMA +	11.2	187.5	+/+	Necrosis	−	Pred	Death
4	Deutsch [38]	32 years, M	ALF-WD	+	ANA 1:1280	20	855	−/−	−/+	NA	Pred/AZA	Stable
5	Santos [39]	17 years, F	ALF-WD	−	↓C3, C4	14	10322	−/+	+/+	961	Pred, LTx	Post-LTx
6	Hunt [40]	29 years, M	Chronic	+	ANA 1:40	NA	NA	NA	+/−	1369	Trientine	Stable
7	Dara [41]	10 years, M	Acute Father: IDDM	−	ANA 1:160, SMA 1:80, LKM 1:20	2.8	1600 μg/dL	−/−	−/+	X20ULN	Pred/AZA and D-Pen	Stable
8	Loudianos [42]	15 years, F	ALF-WD	+	ANA 1:320, SMA 1:160	13.7	NA	+/−	+/+	388	Pred, D-Pen, LTx	Post-LTx

IgG, immunogloblin G; *DCT*, direct Coombs test; *KF*, Kayser–Fleischer ring; Rx treatment; *ANA*, anti-nuclear antibody; *GPC*, gastric parietal cell antibody; *SMA*, smooth muscle antibody; *ALF-WD*, classic Wilsonian acute liver failure; *Pred*, prednisolone or prednisone; *D-Pen*, D-penicillamine; *AZA*, azathioprine; *IDDM*, insulin-dependent diabetes mellitus; *LKM*, liver kidney microsomal antibody; *LTx*, liver transplant; *NA*, not applicable; +, positive/present; −, negative/absent.

importance of investigating persistent, even trivial, elevation of serum aminotransferases. In some countries, WD is an important cause of childhood cirrhosis [21,22]. Cirrhosis is uncommon in pediatric patients identified through screening of first-degree relatives [20].

WD can present as acute liver failure. When it is the initial presentation of WD, it may have highly characteristic clinical features. This classic Wilsonian acute liver failure (ALF-WD) typically consists of moderate-to-severe Coombs-negative acute intravascular hemolysis, coagulopathy unresponsive to vitamin K supplementation, some degree of encephalopathy including lethargy, relatively low serum aminotransferases running $\leq$2000 U/L throughout the clinical illness and very low, occasionally undetectable, serum alkaline phosphatase. Why alkaline phosphatase activity is severely depressed is uncertain but may be related to oxidative damage to the active site [23]. Renal failure attributed to renal tubular damage from copper may develop early in the course of illness. Jaundice may be severe, due to bilirubin load released from erythrocytes plus the renal insufficiency. Serum levels of copper not incorporated in ceruloplasmin are high, and consequently, urinary copper excretion is greatly increased. Classic Wilsonian acute liver failure occurs more often in females than in males and can occur in young children. It is worth noting that it is not unusual to see cirrhosis in the hepatic histology of a patient with ALF-WD either when liver biopsy is performed or in the explanted liver at the time of liver transplantation. Much less commonly, a less distinctive acute liver failure may occur with WD.

As obesity has emerged as a worldwide epidemic, nonalcoholic fatty liver disease (NAFLD) has become the most common cause of liver disease in the pediatric population in the United States. The characteristic histology in NAFLD ranges from bland steatosis to steatohepatitis to cirrhosis [24]. Given that steatosis is also a characteristic histologic feature of WD, there is the possibility of missing a diagnosis of WD. A 4.5-year-old girl with fatty liver and nonspecific hepatitis on liver biopsy was diagnosed with WD based on copper studies. Her timely diagnosis was due to early liver biopsy whose histology led to appropriate WD screening [25]. While diffuse fatty infiltration is a known feature of WD [3,6,26], nodular fatty infiltration has also been reported in childhood WD [27]. Concurrence of metabolic syndrome and WD has been reported in adults [28,29]. While NAFLD is much more common than WD, failure to diagnose WD is serious. It is extremely important to rule out WD before labeling a child or adult with NAFLD as a diagnosis. The best test for distinguishing these two conditions is the basal 24-hour urinary copper excretion, which is much lower in NAFLD than in WD [30]. Checking serum ceruloplasmin is informative only if it reveals a distinctly subnormal serum ceruloplasmin, but the test is not very sensitive.

An important clinical presentation mainly in children, but also in young adults, closely resembles autoimmune hepatitis (AIH). This is a chronic inflammatory condition of the liver characterized by elevated serum aminotransferases, hypergammaglobulinemia, presence of nonspecific autoantibodies (anti-nuclear antibody, ANA; smooth muscle antibody, SMA; liver kidney microsomal antibody, LKM), interface hepatitis on liver histology, and response to immunosuppression. Diagnosis of AIH is facilitated by a scoring system, which has been revised and updated, since there is no single pathognomonic feature [31,32]. Classic AIH may have a chronic or an acute presentation. Importantly, the apparent simple acute hepatitis of WD may prove to be identical to an acute autoimmune hepatitis.

Since the early cases [33–35] of WD clinically resembling autoimmune hepatitis, numerous reports document a clinical presentation of pediatric WD resembling that of AIH (Table 17.2) [36–39,41,42]. In some, WD was misdiagnosed as AIH and treated, albeit inadequately, as AIH. For example, an adolescent girl with an 8-month history of jaundice, ascites, hyperglobulinemia, positive ANA, with interface hepatitis and established cirrhosis on liver biopsy, showed improvement with corticosteroid therapy, but a month later, WD screening results returned positive with low serum ceruloplasmin, increased urinary copper and liver copper of 385 μg/g dry weight. She was started on D-penicillamine and tapered off corticosteroids and became clinically stable [36]. ANA-positivity (type 1 AIH) is more frequent than LKM-positivity (type 2 AIH) in AIH-like WD but the latter pattern can occur.

Acute liver failure poses significant diagnostic problems since acute liver failure due to AIH can be difficult to diagnose. Pediatric patients with WD may present a similarly nondescript clinical picture of acute liver failure, not the classic picture of ALF-WD. A 17-year-old girl with acute liver failure was initially started on a trial of corticosteroids based on clinical and histological features of AIH, despite normal IgG and negative autoantibodies. Clinical deterioration prompted urgent liver transplantation. Histopathology of the explant was consistent with WD; her diagnostic basal 24-hour urinary copper excretion was 10,322 μg/24 hours (162.6 μmol/24 hours), results available only after the transplant [39]. A 15-year-old girl with acute liver failure had ANA 1:320, SMA 1:160, hypergammaglobulinemia, and plasma cell infiltrate on liver biopsy suggesting AIH, but she also had low serum ceruloplasmin, Kayser–Fleischer rings, and elevated liver copper concentration. She was started on both corticosteroids and penicillamine, but she underwent liver transplantation as her condition worsened. She had genetically proven WD [42].

A further problem is that possibly some individuals with WD may get autoimmune hepatitis as such. The disease mechanism in such cases might be similar to the situation where an individual with presymptomatic WD gets acute

liver failure from an intercurrent viral infection [43]. In this uncommon circumstance, the affected person develops AIH, possibly but not necessarily due to copper-mediated damage to the hepatocellular plasma membrane. Ulcerative colitis may occur in children who have WD with prominent autoimmune features [7,44]; however, it has also been reported as an adverse event from trientine [45]. Some children have a genetic makeup predisposing them to autoimmune disease: it might coincide with *ATP7B* mutations. In any case, occurrence of each disease needs to be established, or refuted, fully. Diagnostic problems may occur with primary sclerosing cholangitis [46] (Roberts EA, unpublished observations), or notably with autoimmune sclerosing cholangitis (ASC), because stainable copper may be found on liver biopsy. The pattern of distribution of copper in the liver tends to be different between these disorders and WD. In cholestasis, copper accumulation is typically limited to periportal hepatocytes. Early reports confounding WD and AIH may have been mistaking ASC for AIH, since ASC was not conceptualized until the 1990s.

NEUROLOGIC DISEASE

Neurologic disease is less common in pediatric patients with WD than in the adult age-bracket. It has been reported in children as young as 6 years-old [47] and rarely in preschool-aged children [48]. Walshe reported nine children with neuro-WD aged 12–19 years old [49]. A recent series included patients as young as 7 and 9 years old [50]. Neurologic features can develop in a child with hepatic-WD who is non-adherent to treatment. The neurologic changes of WD are predominantly extrapyramidal. Three distinct neurologic patterns have been described including dystonic, ataxic, and Parkinson-like syndromes, each with characteristic changes in magnetic resonance imaging (MRI) of the brain in each group [51]. The presence of "wing beating" or flapping tremor in association with dysarthria is characteristic of WD. "Risus sardonicus" or the involuntary grimace with an open mouth and contracted upper lip has also been described. These are classic and somewhat late findings. More commonly, in the pediatric population, subtle problems such behavioral changes, deterioration in handwriting, and inability to perform activities requiring good hand-eye coordination are noted. Pseudobulbar palsy can occur with either dystonia or ataxia: features include drooling, dysarthria, and problems with swallowing. Recent pediatric series feature dystonia, dysarthria, cognitive decline, tremor, gait abnormalities, and a Parkinson-like movement disorder, dysphagia, and drooling [50,52]. The majority of these pediatric patients with neuro-WD have Kayser–Fleischer rings. Seizures are atypical but may occur in some adolescents.

Diagnostic problems arise because of the subtlety of clinical presentation. Initial symptoms may be extremely nonspecific. One child presented initially with "learning disabilities" whose cause was discovered only in the course of investigating abdominal pain [53]. A teenager developed tremor, cramps in hands and feet, indecisiveness, and poor school performance [54]. Tremors, gait disorders, clumsiness, and school aversion may be too subtle to be noticed or may be attributed to teenage awkwardness.

PSYCHIATRIC DISORDERS

In Wilson's 1912 monograph, 8 of the 12 patients had psychiatric symptoms. Psychiatric symptoms can occur in both untreated and treated WD patients. Behavioral and psychiatric symptoms are more common in patients with neurologic involvement than in patients with hepatic involvement. Psychiatric symptoms may precede the recognition of hepatic or neurologic WD by a significant period of time, and there may be a delay in diagnosis of WD for several years [55,56]. This is likely because behavioral and psychiatric symptoms due to WD are often misdiagnosed. For example, they may be erroneously attributed to emotional stresses associated with puberty. The mechanism of development of psychiatric manifestations is not clear. Since many patients have the symptoms prior to diagnosis, it cannot be merely due to the psychological impact of the diagnosis. Other factors that have been explored are dopamine dysregulation from basal ganglia involvement, and the role of copper and trace elements in schizophrenia and bipolar illness [57,58]. Changes in circadian rhythm may be relevant [59].

The most common psychiatric features are abnormal behavior (typically increased irritability or disinhibition), personality changes, anxiety, and depression. Psychosis and schizophrenia have also been reported in WD [60,61]. Psychiatric presentation of WD may be more common in the pediatric age-bracket than is generally appreciated. Worsening performance at school or at work can be regarded as a psychiatric feature. A child whose academic performance drops off for no apparent reason requires consideration with respect to WD. Psychiatric aspects of WD in children presenting with hepatic-WD may not be prominent; moreover, development of psychiatric issues after the diagnosis of WD has received relatively little attention. It may have an impact on adherence (see Chapter 19: Wilson Disease: Psychiatric Aspects).

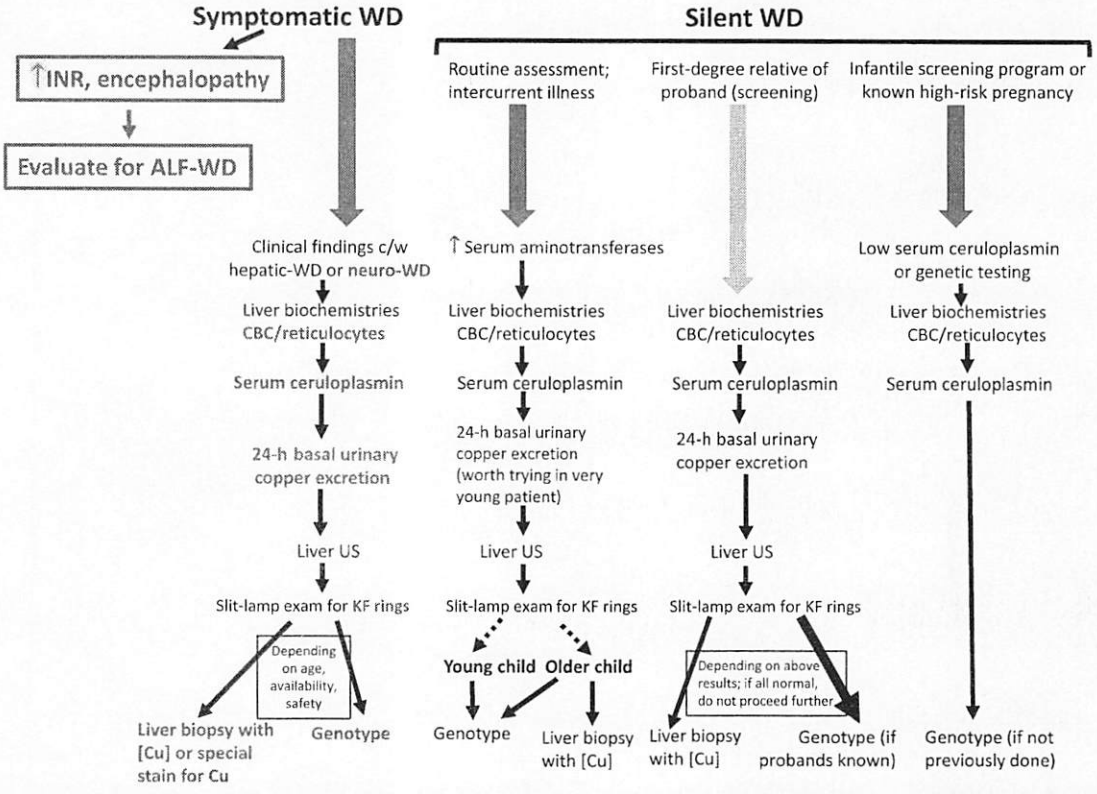

FIGURE 17.1 **A general approach to diagnosis of Wilson disease in the pediatric age-bracket, following comprehensive review of patient's medical history and a full physical examination.** Symptomatic WD comprises a spectrum of hepatic and neurologic disease: red lettering denotes inclusion in the Leipzig score for diagnosing WD. Silent WD includes children found incidentally to have nonspecific liver abnormalities (typically, elevated serum aminotransferases, measured as part of routine screening, or with an intercurrent illness) as well as infants/children identified through general population screening, based on ceruloplasmin or genetic testing. Silent WD also includes first-degree relatives of a WD patient for whom no preliminary data are available. "Liver biochemistries" signifies full liver panel including (at minimum) liver enzymes, total and conjugated bilirubin, albumin, and a coagulation test such as INR. Arrows indicate general progression of investigations, which can be grouped; dotted arrows indicate age dependence; heavy arrow indicates preferred investigation. *c/w*, consistent with; *24-h*, 24-hour; *US*, ultrasonography; *KF*, Kayser–Fleischer; *CBC*, complete blood count; *[Cu]*, parenchymal copper concentration; *ALF-WD*, classic Wilsonian acute liver failure.

OTHER CLINICAL FEATURES

Eye signs include the Kayser–Fleischer ring and sunflower cataracts. The latter are quite rare in children but were reported in a neurologically normal 8-year-old girl with WD [62]. Kayser–Fleischer rings are present in only ~40% of affected children. They are highly informative of the diagnosis when present. Slit-lamp examination by a skilled pediatric ophthalmologist is required for identifying them, especially in very young children. Neither Kayser–Fleischer rings nor sunflower cataracts affect vision. Both resolve with successful treatment.

Transient episodes of intravascular hemolysis are frequently reported in children, mainly as brief self-limited episodes of jaundice. Hemolysis may be an initial presentation of WD. In some cases presenting with hepatic or neurologic features, medical history reveals one or more previously undiagnosed hemolytic episodes. Hemolysis in WD is sometimes apparently precipitated by infection or drugs. A recent retrospective analysis of 321 WD patients revealed presentation with acute hemolysis in 22 (7%). The patients' age ranged from 7 to 20 years with an average onset of 12.6 years. Delayed diagnosis resulted in progression to severe hepatic disease and neurologic deterioration; 4 of these 22 patients may have had classic Wilsonian acute liver failure. [63]. Chronic hemolytic anemia may also occur in pediatric WD [64]. The mechanism is direct damage to the erythrocyte membrane by copper: hence, it is Coombs-negative. As previously noted, severe acute Coombs-negative intravascular hemolysis is prominent in classic Wilsonian acute liver failure.

Renal Fanconi syndrome is typical of WD in children. It may lead to nephrolithiasis. Renal tubular acidosis may also be present. In a Chinese series, five children aged 4–11 years old presented with a renal disorder causing pedal

edema [65]. Evident cardiac involvement is uncommon. Subtle asymptomatic myocardial dysfunction [66] or frank cardiomyopathy may be found [65].

Pediatric patients may have "osseomuscular" WD: arthralgia or less commonly arthritis, proximal muscle weakness, bony deformities, and pathological fractures [67]. These patients are mainly from the Indian subcontinent. Osseomuscular WD seems to be less common elsewhere. Individual musculoskeletal involvement including arthritis, myopathy [68,69], noteworthy muscle cramps [54,70], and rhabdomyolysis [71] has been reported. Osteochondritis dissecans have also been reported [72]. Diagnosis can be delayed if these complaints are misattributed: for example, confused with benign "growing pains" [73].

Other relatively rare organ involvement includes hypoparathyroidism and pancreatitis [74]. Skin signs in children are nonspecific but include extremely dry skin [75]. A clinical picture resembling Henoch–Schönlein purpura with palpable purpura was reported in pediatric WD [65].

DIAGNOSIS

Diagnosis of WD in children can be difficult (Fig. 17.1). The problems of diagnosing WD in a child with acute liver failure may be mitigated by finding Coombs-negative acute intravascular hemolysis, cholestasis in combination with a strikingly low serum alkaline phosphatase, and comparatively low serum aminotransferases from the onset of clinical illness. Very low serum alkaline phosphatase may also be found in WD with severe liver disease not quite qualifying as acute liver failure. For the child with less severe liver disease, such as seronegative acute hepatitis or unexplained elevation of serum aminotransferases or unexplained hemolysis producing jaundice, the most important biochemical criteria are serum ceruloplasmin and basal 24-hour urinary copper excretion, which is an indirect quantification of non-ceruloplasmin-bound copper in the plasma compartment. Measurement of hepatic parenchymal copper concentration provides critical data but involves an invasive procedure. For most patients, genotype determination demonstrating homozygosity for one disease-causing *ATP7B* alteration, or compound heterozygosity for two, is conclusive of the diagnosis. Genotype interpretation can be complex (see Chapter 14: The *ATP7B* Gene).

Among biochemical tests, serum ceruloplasmin remains an important parameter, despite being insufficient as a single test to rule in or exclude WD. Basically, the immunologically based analytical methods for measuring serum ceruloplasmin now in general use for automated measurement of serum ceruloplasmin have changed how that parameter supports the diagnosis of WD. Immunological measurement of serum ceruloplasmin measures apo- as well as holo- (copper-containing) ceruloplasmin. Fully functional holoceruloplasmin is produced as a function of the Wilson ATPase itself; its half-life is ~5.5 days. In contrast, apoceruloplasmin has a relatively short half-life of about 5 hours [76]: serum levels tend to end up lower than normal. Levels may be exceedingly low if the mutation is truncating. Thus, very low serum ceruloplasmin (<50 mg/L, or <5 mg/dL, measured immunologically) may be taken as strong evidence in favor of the diagnosis of WD. One clinical approach is to regard 140 mg/L (14 mg/dL) as the informative cutoff [77]. A more pragmatic approach is to investigate anyone with a subnormal serum ceruloplasmin. As many as one-third of pediatric WD patients may have a normal serum ceruloplasmin. In children with congenital nephrotic syndrome, serum ceruloplasmin may be subnormal due to excessive urinary loss, but anemia is present, as well as features of renal dysfunction [78]. Serum ceruloplasmin level is elevated by inflammation or the oral contraceptive pill since ceruloplasmin is an acute phase reactant.

Measurement of the basal 24-hour urinary copper excretion is valuable. Urine must be collected in copper-free containers; completeness of a full 24-hour collection should be confirmed by measuring urinary creatinine excretion (normal: 10–20 mg in 24 hours). The reference value of 100 μg/24 hours (1.6 μmol/24 hours) is insensitive for diagnosing WD in any age group [79]. The value >0.6 μmol/24 hours (>40 μg/24 hours) is preferable for children. This recommendation is based on a series of 29 pediatric patients of whom 27 had basal 24-hour urinary copper excretion exceeding 0.6 μmol/24 hours (>40 μg/24 hours); in that series, 8 of 29 (28%) had values <1.6 μmol/24 hours (<100 μg/24 hours) [26]. Systematic assessment by ROC analysis showed that 0.6 μmol/24 hours (40 μg/24 hours) is the best cutoff (sensitivity = 79%; specificity = 88%), at least in children [8]. In this latter study, patients who did not reach this level of cupruria were girls under the age of 4 years-old. Clearly, this test is limited by potential difficulties in getting an accurate 24-hour urine collection in very young children. Otherwise, it has physiological rationale and works well. For the purposes of screening, erring toward false positives is acceptable. Chronic cholestatic disorders such as MDR3 deficiency causing chronic cholestasis [80] can produce cupruria >0.6 μmol/24 hours (>40 μg/24 hours): genetic diagnosis is required.

The penicillamine challenge test, in which 500 mg D-penicillamine is given by mouth as a 24-hour urine collection is commenced and then again 12 hours later at the halfway point of the collection, was recommended for diagnosing

WD in children [81]. The diagnosis was established if urinary copper excretion equaled/exceeded 25 μmol/24-hours (>1600 μg/24-hours). Over time, this test has proved less reliable. In various series, 40%–50% or more of patients failed to meet that diagnostic criterion and yet were shown to have WD. It appears that asymptomatic patients may not be detected [82], possibly because the test was developed based on findings in WD children with established disease. The practical problems of testing very young children apply, and the best dose of D-penicillamine for testing very young children is not determined.

Liver biopsy can be highly informative for the diagnosis of WD in children, but it immediately entails the risk and cost of an invasive procedure. The time-honored criterion of hepatic parenchymal copper concentration >250 μg/g dry weight as diagnostic for WD is actually based on an early small study. Thus, the criterion has been subject to reassessment [30,83]. For children with WD, the proposed threshold of 75 μg/g dry weight requires further evaluation: thus, the cutoff of >250 μg/g dry weight remains preferred. Further diagnostic testing is required for children whose hepatic parenchymal copper is between 75 and 250 μg/g dry weight. Some risk of misdiagnosis of WD exists when liver copper content is used as the only test [84]. While normal hepatic parenchymal copper concentration (<50 μg/g dry weight) argues against the diagnosis of WD in the symptomatic patient, erroneously low concentrations can be found if the sample is too small. Presence of cirrhosis may also compromise this test. Sometimes hepatic copper content is not dramatically increased in toddlers with WD. Likewise, in ALF-WD, hepatic parenchymal copper shown by special stains may be misleadingly low.

Identifying an individual as homozygous for a disease-causing *ATP7B* mutation establishes the diagnosis of WD. Many individuals with WD have one each of two different disease-causing mutations. More complex patterns of inheritance may occur (see Chapter 14: The *ATP7B* Gene). In the context of first-degree-relative screening, if the genotype of the proband is known, genetic testing is the most efficient method for identifying siblings with WD. (Genetic screening of a pediatric proband's offspring may be more complicated—at least, logistically.) Generally in children, genetic testing is invaluable for diagnosing cases with atypical clinical findings because it provides the definitive diagnosis.

Scoring systems fall broadly into two categories: for diagnosis and for estimating prognosis (see Chapter 25: Role of Scoring Systems in Wilson Disease). The Leipzig criteria developed for adult patients [85,86] have been modified for use in the pediatric age-bracket [8]. A problem in the pediatric age-bracket is that often genotype is the best, or only, route to a Leipzig score diagnostic of WD. A methodical approach to diagnosis of WD in children may be the best strategy, as opposed to depending on a scoring system (see Fig. 17.1). Among scoring systems for predicting prognosis, the modified King's College Hospital score (new Wilson Index) is in wide use [87] (see Chapter 25: Role of Scoring Systems in Wilson Disease).

WILSON DISEASE-MIMICS

At the present time, mutations in the gene *ATP7B* appear to be the only genetic etiopathogenesis of WD. However, a few disorders have some claim to being a WD-mimic: namely, a different genetic defect produces a highly similar disorder. These include MEDNIK syndrome (mental retardation–enteropathy–deafness–neuropathy–ichthyosis–keratodermia), certain congenital disorders of glycosylation, and a hepatic disorder characterized by manganese retention in hepatocytes (see Chapter 41: Disorders that Mimic Wilson Disease). Indian Childhood Cirrhosis and similar infantile copper toxicoses (see Chapter 44: Indian Childhood Cirrhosis and Other Disorders of Copper Handling) feature hepatic copper accumulation but can be distinguished clinically.

SCREENING NEWBORNS AND PEDIATRIC FIRST-DEGREE RELATIVES

WD fits the criteria of a disease suitable for general newborn and infant screening; however, finding reliable and cost-effective methodology for such screening has proven challenging (see Chapter 26: Population Screening for Wilson Disease). A sensitive immunoaffinity enrichment mass spectrometry test to quantify Wilson ATPase in dried blood spots of newborns may serve effectively to screen for WD [88]

Retrospective reviews have shown that screening brothers and sisters of a child diagnosed with WD and initiating treatment in those found to have WD before they manifest symptomatic disease prevent clinical WD [89,90]. Screening involves physical examination, liver biochemistries, serum ceruloplasmin, and basal 24-hour urinary copper excretion. If positive, liver biopsy with measurement of parenchymal copper should be performed. If the genotype of the proband is known, genetic testing of first-degree relatives is an efficient option, when available and affordable (cost covered by insurance for those who cannot pay). A problem for managing these patients is that they may not fully understand that they have a serious disease: adherence to treatment may be poor [89,91,92] (see Chapter 37: Transition of Care and Adherence in Patients with Wilson Disease).

PROGNOSIS

Early diagnosis, preferably before a child/adolescent has symptomatic hepatic or neurologic disease, is most likely to result in near-normal longevity with generally good health so long as the patient tolerates effective medication, is adherent to the lifelong treatment regimen, and has consistent access to the medication. Families/Patients need to understand that stopping therapy completely may be fatal. An urgent contemporary social justice problem for children with WD is to assure life-long accessibility to effective drug treatment.

MANAGEMENT

Availability of oral chelators revolutionized the management of WD and changed its dire prognosis to a good outlook. Penicillamine is the amino acid cysteine to which two methyl groups have been attached. Extensive clinical experience attests to the efficacy, and relative safety, of D-penicillamine in children [87,93]. The dosage of D-penicillamine in children is 20 mg/kg/day, divided into two to three equal doses. If possible, D-penicillamine should be taken well away from mealtime. Vitamin B6 supplementation (25–50 mg daily) is customary. Adverse side effects, such as pancytopenia and proteinuria, are a major problem: the drug has to be stopped in ~30% of patients. As with adults, clinical deterioration of hepatic or neurologic disease when D-penicillamine is started has been reported in children [94,95]. Starting D-penicillamine at gradually increasing doses over the first 10–14 days of treatment may minimize the risk of the so-called hypersensitivity syndrome. In the first 2–3 months of treatment with D-penicillamine, very close monitoring is required to identify any adverse reactions immediately. Trientine is structurally very different form D-penicillamine. It is safe and effective for WD patients intolerant of D-penicillamine, including children [91,96,97]. Many in the United States advocate it as the preferred first-line oral chelator for any pediatric WD patient, not merely children predicted to be at risk for adverse effects associated with D-penicillamine. Trientine itself has few adverse side effects. Dosage in children is also 20 mg/kg/day, divided into two to three equal doses. In general, trientine should be taken away from meals as food can interfere with its already limited absorption.

Zinc influences copper homeostasis in the body by altering expression of metallothioneins in gut and possibly in liver. It interferes with copper absorption and increases fecal copper excretion. In general, hepatic copper concentrations do not change. Zinc is regarded as effective and well tolerated in children [3,98,99]. Recent data cast some doubt on this enthusiasm [100]. Lack of very long-term follow-up is problematic. Dosage in children is 25 mg elemental zinc three times daily, or twice daily in 5-year olds [101]; teenagers can take the adult dose (50 mg elemental zinc three times daily); optimal dose for toddlers and preschool-aged children remains undetermined. While the actual zinc salt is unimportant, zinc acetate and gluconate are well tolerated, and zinc sulfate seems to be the least well tolerated. It has been associated with gastric or duodenal mucosal erosion/ulceration [102]. Zinc should not be taken with food. Zinc may be more effective for neuro-WD than for hepatic-WD, a relevant consideration since hepatic-WD is more common in children. A child whose hepatic status deteriorates on zinc monotherapy requires oral chelation instead. With pediatric WD, establishing a consistent routine of taking zinc may be difficult because it must be taken three times a day and away from meals/snacks. Zinc salts can cause nausea, vomiting, and epigastric pain—further disincentives to taking it. Changing to a different salt or taking the zinc with a little food may solve this problem.

Tetrathiomolybdate (TTM) is a strong anticopper drug which works by chelation and also by interfering with copper absorption. It is an attractive option for treatment of neuro-WD. A new formulation of tetrathiomolybdate as the bis-choline salt is in development, but it is not yet available for treatment in children.

MEDICAL MANAGEMENT OF CLINICALLY EVIDENT WILSON DISEASE

Most children with WD who have clinical evidence of hepatic or neurologic damage require treatment with an oral chelator. Primary treatment with zinc is often reserved for those who have prominent neurologic involvement. Imaging of the central nervous system, preferably by MRI, should be performed in any child with neurologic WD prior to commencing treatment.

For very severe clinical presentations, mainly decompensated cirrhosis, an induction regimen consists of an oral chelator (D-penicillamine or trientine) and zinc given on a temporally dispersed regimen where some drug is given every 5–6 hours, chelator alternating with zinc. This regimen provides a full dose of chelator plus an adequate dose of zinc in each 24-hour period. It is unwieldy for long-term treatment: after 2–3 months, patients who respond should be transitioned to monotherapy. This regimen appears effective [103–105]. For the patient with decompensated cirrhosis, backup liver transplantation must be arranged because medical treatment failure may occur.

Zinc acetate was approved in the United States as maintenance therapy for WD in 1997. Pediatric patients may be suitable for conversion from oral chelator to zinc, usually after 1–5 years of oral chelation. The patient should be clinically stable with normal liver chemistries and biochemical evidence of good response to chelator therapy. The switch can be immediate (no tapering or ramping up), but close monitoring is required during conversion [83]. Alternatively, pediatric patients have been treated initially with chelation (trientine) for 4–8 months, followed by gradual conversion to zinc including a brief period of combined therapy [96].

MEDICAL MANAGEMENT OF SILENT WILSON DISEASE, INCLUDING THE VERY YOUNG CHILD WITH SILENT WILSON DISEASE

Since many children have silent WD, finding the best treatment for them is important. It is not a "one-size-fits-all" situation. Several biological considerations attain: actual mutation(s), evidence of liver damage, and age. Economic or social considerations may force a treatment decision independent of pathobiology.

Current treatment strategies focus on toxic copper overload. Oral chelators are regarded as aggressive treatment: the aim is to "de-copper" the liver. Zinc is considered gentler, but it is well established that zinc does not radically alter hepatic copper concentration. Extended experience in adults, while limited [106], suggests that zinc may not be effective for hepatic-WD long-term. Overall, pharmacological management of WD requires monitoring and flexibility in drug treatment.

Any child with silent WD who has mutation(s) resulting in failure to produce the Wilson ATPase protein altogether requires aggressive treatment and close monitoring. These individuals likely approximate to the rapidly progressive deterioration of the knockout mouse. Likewise, a child with silent WD and, additionally, silent cirrhosis probably needs oral chelation since evidence for histological improvement in children is based on chelation [107]. Silent WD with abnormal liver biochemistries may respond adequately to zinc or may be recalcitrant. Accruing pediatric experience suggests that close monitoring is mandatory [100]. The patient's age poses various problems. It cannot be assumed that teenagers necessarily have greater hepatic copper concentrations than toddlers. Dosing may be problematic in young children. Adherence may be a game-changer in adolescents.

Specifically for very young children (<5 years-old) with definite WD, best treatment remains undetermined. Although zinc is currently preferred for treatment of very young asymptomatic children, the optimal dose has not been established. The commonsense determination is 25 mg elemental zinc once (toddlers) or twice daily. Early chelation therapy to reduce the hepatic parenchymal copper concentration deserves serious consideration. Any treatment regimen in the very young child must be assessed for risks associated with copper depletion or zinc surfeit in a critical period of growth. For example, if WD is diagnosed in the neonatal period, immediate restriction of dietary copper may interfere with neurologic development. A regular baby formula may thus be appropriate. Delaying treatment to the second year of life may be reasonable if the very young child has no evidence of hepatic or neurologic disorder. Dosing becomes easy once the child weighs 10–12 kg.

In the future, there may be a second therapeutic focus, namely rehabilitation of the defective Wilson ATPase. Decoppering would then take place through the normal function of the rehabilitated protein. This strategy may apply to only selected mutations, for example, H1069Q, but it will enhance the need for genotypic diagnosis.

DIETARY CONSIDERATIONS FOR CHILDREN WITH WILSON DISEASE

WD cannot be treated successfully under any circumstances by dietary manipulation alone. Opinion is divided about the importance of excluding dietary copper in pediatric patients on pharmacological treatment [108]. Some restriction in the first year of treatment may be appropriate for children with abnormal liver tests or clinical illness. The problem is that dietary limitations reinforce the stigma of being unwell. In any case, if a child's dietary copper is to be limited, guidelines must be extremely simple. Patients should avoid liver and organ meats, shellfish, mushrooms, nuts, and chocolate. Vegetarian and vegan patients do require supervision by a dietitian, possibly an issue if an adolescent adopts such dietary practices after achieving clinical stability. If drinking water is suspected to be high in copper, it should be checked: filtered water can be substituted, if necessary. No systematic data are available in children regarding the merits of supplementation with dietary antioxidants such as vitamin E. Other complementary/alternative medicines and dietary supplements [109] may be high in copper.

FOLLOW-UP STRATEGIES FOR THE PEDIATRIC PATIENT ON TREATMENT

Follow-up is similar to that for adults (see Chapter 16: Wilson Disease in Adults: Clinical Presentations, Diagnosis, and Medical Management), with at least twice yearly clinical review. When starting therapy, patients should be advised to report any adverse events; they should have complete blood count, liver and renal biochemistries performed at each review. Pediatric patients maintained on zinc need to be followed specifically for signs of advancing hepatic disease (elevated aminotransferases, thrombocytopenia, ultrasonographic abnormalities). Measuring 24-hour urinary copper excretion is useful in monitoring therapy; measuring 24-hour urinary zinc is useful for monitoring adherence to zinc intake. On oral chelators, urinary copper <200 μg/24 hours (<3 μmol/24 hours) suggests non-adherence or overtreatment. Estimate of the non-ceruloplasmin-bound copper may distinguish between these situations, as it is subnormal (<5 μg/dL; <50 μg/L) in overtreatment and elevated (>15 μg/dL; >150 μg/L) with inadequate treatment or non-adherence [83]. Issues relating to how non-ceruloplasmin-bound copper is estimated limit the utility of this approach (see Chapter 22: Direct Determination of Non-Ceruloplasmin-Bound Copper in Plasma). If the non-ceruloplasmin-bound copper is consistently 0–5 μg/dL (0–50 μg/L), dose of chelator should be gradually reduced and patient monitored. Serial ophthalmologic examinations may be helpful in documenting that the Kayser–Fleischer rings have resolved and vanished, versus development of Kayser–Fleischer rings in those with poor adherence.

CLASSIC WILSONIAN ACUTE LIVER FAILURE AND LIVER TRANSPLANTATION

The child with acute liver failure in WD may benefit from specific interventions as a bridge to transplant, in addition to meticulous supportive care. Specifically, albumin dialysis [110] and related techniques [111,112] may stabilize the pediatric patient. In an adolescent, this type of intervention has on occasion obviated the need for transplant [113]; however, this outcome is exceptional with classic Wilsonian acute liver failure.

Prognostic scoring systems can assist in determining who needs a liver transplant [87]. Liver transplantation can be lifesaving in classic Wilsonian acute liver failure and true treatment failures. For the patient with decompensated cirrhosis, backup liver transplantation should be arranged because medical treatment failure may occur. Living-related donors who are obligate heterozygote carriers are acceptable as donors [114]. Review of the United Network for Organ Sharing database in the United States has shown that those transplanted for WD-related chronic liver disease have higher patient survival rates than those with classic Wilsonian acute liver failure and that children had better survival rates than adults undergoing transplantation for WD [115]. Overall, outcomes for liver transplantation in WD are excellent and comparable to transplantation for other indications (see Chapter 38: Liver Transplantation in Wilson Disease).

CONCLUSION

With improved methods of diagnosis, WD has become an important disease in childhood. The hepatic manifestations are varied, and the neuropsychiatric manifestations may be subtle or nonspecific. Treatment needs to be individualized; comprehensive follow-up is important for all patients. Scoring systems may assist with diagnosis or clinical decisions relating to need for liver transplantation. A recently published position paper complements the views expressed in this chapter [116].

SUMMARY

WD can affect children of all ages. In the current era of increased availability of genetic testing, WD is being diagnosed earlier in life. Indeed, there are over 50 cases of WD in children under 5 years of age documented in the literature. The need to be aware of the possibility of WD as the cause of liver dysfunction regardless of age is important. Clinical presentation may be chronic liver disease, acute liver failure with distinctive features (ALF-WD) or it may be "silent" liver disease. A broad spectrum of neurologic or psychiatric conditions can occur in childhood or adolescence. While hepatic presentation is more common than neurologic in the pediatric population, manifestations of WD are characteristically multisystemic. WD can resemble autoimmune liver disease clinically. Particularly when there is inadequate response to immunosuppression therapy in autoimmune hepatitis, every effort must be made to ensure that a diagnosis of WD is not being missed. A combination of biochemical tests including serum ceruloplasmin, 24-hour urinary copper excretion, and liver copper concentration as well as genetic tests is used to establish diagnosis. Scoring systems to assist diagnosis and to assess prognosis are available. Current management includes chelators like D-penicillamine or trientine; zinc has multiple effects exclusive of chelation. Economic and social factors often dictate drug therapy. Foods containing high

amounts of copper like shellfish and chocolates may be restricted, at least in the first year of treatment. After a diagnosis of WD, first-degree relatives of the child should be screened. Therapy is lifelong. Adherence to medical recommendations is necessary for good outcomes. Parents should be made aware that stopping therapy suddenly may prove fatal for the child. Liver transplantation is required in classic Wilsonian acute liver failure and when medical therapy fails. Newer therapies are being investigated. Newborn screening may become routine in the future.

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Handbook of Clinical Neurology, Vol. 142 (3rd series)
Wilson Disease
A. Członkowska and M.L. Schilsky, Editors
http://dx.doi.org/10.1016/B978-0-444-63625-6.00003-3

Chapter 3

The genetics of Wilson disease

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Abstract

Wilson disease (WD) is an autosomal-recessive disorder of hepatocellular copper deposition caused by pathogenic variants in the copper-transporting gene, *ATP7B*. Early detection and treatment are critical to prevent lifelong neuropsychiatric, hepatic, and systemic disabilities. Due to the marked heterogeneity in age of onset and clinical presentation, the diagnosis of Wilson disease remains challenging to physicians today. Direct sequencing of the *ATP7B* gene is the most sensitive and widely used confirmatory testing method, and concurrent biochemical testing improves diagnostic accuracy. More than 600 pathogenic variants in *ATP7B* have been identified, with single-nucleotide missense and nonsense mutations being the most common, followed by insertions/deletions, and, rarely, splice site mutations. The prevalence of Wilson disease varies by geographic region, with higher frequency of certain mutations occurring in specific ethnic groups. Wilson disease has poor genotype–phenotype correlation, although a few possible modifiers have been proposed. Improving molecular genetic studies continue to advance our understanding of the pathogenesis, diagnosis, and screening for Wilson disease.

INTRODUCTION

In this chapter, we will discuss the inheritance, gene frequency, variants, genotype–phenotype correlation, and modifiers of the *ATP7B* gene, and the clinical molecular diagnosis and population screening for Wilson disease.

INHERITANCE

Wilson disease is a monogenic autosomal-recessive condition and carriers do not manifest any symptoms. Autosomal-recessive conditions are not usually present in consecutive generations, but may occur in populations with particularly high carrier frequency of Wilson disease (Wu et al., 2015). Our group and others have reported the presence of Wilson disease in two or more successive generations within the same family, reflecting a "pseudo-dominant" inheritance (Dziezyc et al., 2011, 2014; Bennett et al., 2013; H. Park et al., 2015). Therefore, the diagnosis of Wilson disease should not be excluded simply due to a misleading family history consistent with an autosomal-dominant inheritance pattern. Furthermore, recent studies have also identified Wilson disease due to atypical forms of inheritance, such as the presence of three concurrent mutations in a single patient or segmental uniparental disomy (Coffey et al., 2013). Uniparental disomy occurs when both homologs of a chromosome originate from a single parent. These findings have implications for clinical practice and genetic counseling, as clinicians may need to consider genotyping asymptomatic parents or obtaining full sequencing of *ATP7B* to confirm that pathogenic variants occur in *trans*.

ATP7B GENE AND ATPASE

Wilson disease is caused by homozygous or compound heterozygous mutations in the *ATP7B* gene (OMIM# 606882), which encodes a transmembrane copper-transporting P-type ATPase of the same name. Currently,

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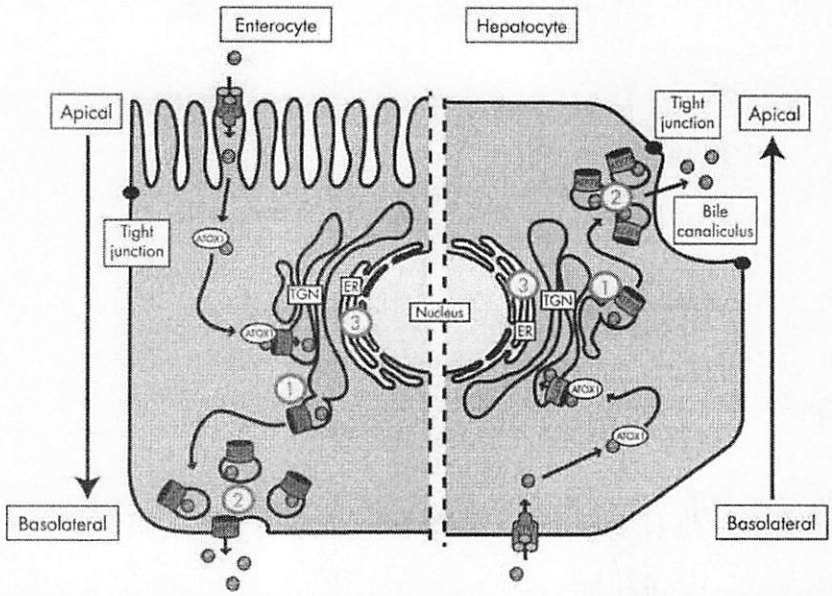

Fig. 3.1. Schematic representation of copper-induced relocalization of ATP7A and ATP7B. The left side of the diagram represents an enterocyte and the right side represents a hepatocyte. On both sides, copper enters the cell through copper transporter 1 (CTR1) and is escorted by copper chaperone antioxidant protein 1 (ATOX1) to ATP7A or ATP7B in the trans-Golgi network (TGN). When copper levels rise above a certain threshold, ATP7A and ATP7B excrete copper into the plasma on the basolateral side of the enterocyte and into the bile on the apical side of the hepatocyte. Defects in localization of ATP7B may lead to copper accumulation at the (1) TGN due to unresponsiveness, (2) cell periphery, and (3) endoplasmic reticulum (ER) due to misfolding. (Reproduced from de Bie et al., 2007.)

ATP7B is the only identified gene known to cause Wil son disease (Bull et al., 1993; Petrukhin et al., 1993; Tanzi et al., 1993). Mutations in the *ATP7B* gene have been reported in almost all exons. Previous studies have reported individuals with both biochemical and clinical diagnosis of Wilson disease in the absence of two *ATP7B* mutations, raising the possibility of a second causative gene (Lovicu et al., 2006; Kenney and Cox, 2007; S. Park et al., 2007; Mak and Lam, 2008; Nicastro et al., 2010; Coffey et al., 2013). Nonetheless, *ATP7B* remains the only known gene responsible for Wilson disease.

Human dietary intake of copper is about 1.5–2.5 mg/day, which is absorbed in the stomach and duodenum, bound to circulating albumin, and transported to the liver for regulation and excretion (Culotta and Scott, 2016). The uptake of copper occurs on the basolateral side of hepatocytes via copper transporter 1 (CTR1), as illustrated in Figure 3.1. A specific copper chaperone, antioxidant protein 1 (ATOX1), delivers copper to the Wilson disease protein, ATP7B, by copper-dependent protein–protein interactions (Walker et al., 2004). Within hepatocytes, ATP7B performs two important functions in either the trans-Golgi network (TGN) or in cytoplasmic vesicles. In the TGN, ATP7B activates ceruloplasmin by packaging six copper molecules into apoceruloplasmin, which is then secreted into the plasma. In the cytoplasm, ATP7B sequesters excess copper into vesicles and excretes it via exocytosis across the apical canalicular membrane into bile (Bull et al., 1993; Tanzi et al., 1993; Yamaguchi et al., 1999; Cater et al., 2007). Due to the binary role of the ATP7B transporter in both the synthesis and excretion of copper, defects in its function lead to copper accumulation and the progressive features of Wilson disease (Fig. 3.1).

MOLECULAR STRUCTURE OF *ATP7B*

ATP7B is located on 13q14.3 and contains 20 introns and 21 exons, for a total genomic length of 80 kb (Bull et al., 1993; Petrukhin et al., 1993; Tanzi et al., 1993). The gene is synthesized in the endoplasmic reticulum, then relocated to the TGN within hepatocytes. *ATP7B* is most highly expressed in the liver, but is also found in the kidney, placenta, mammary glands, brain, and lung.

ATP7B (P-TYPE ATPASE) PROTEIN STRUCTURE AND FUNCTION

ATP7B belongs to class 1B (PIB) of the highly conserved P-type ATPase superfamily, which is responsible for the transport of copper and other heavy metals across cellular membranes (Gourdon et al., 2011). The protein contains 1465 amino acids, a phosphatase domain (A-domain), phosphorylation domain (P-domain, amino acid residues 971–1035), nucleotide-binding domain (N-domain, amino acid residues 1240–1291), and

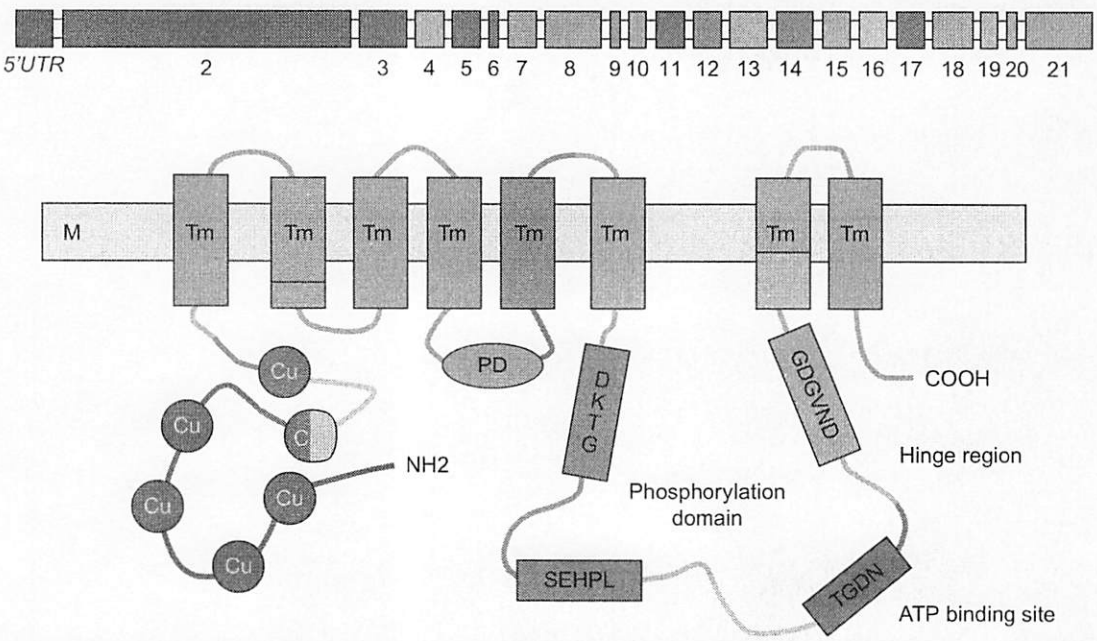

Fig. 3.2. Schematic representation of *ATP7B* gene and corresponding human ATP7B protein. Top diagram shows 5'UTR promoter region and exons separated by introns. Bottom diagram shows the domain organization of human copper ATPase. Conserved amino acid motifs are present at the core structure of each functional domain, i.e., TGDN and GDGVND at the A-domain, DKTG at the P-domain, and SEHPL in the N-domain. M, phospholipidic bilayer of the membrane; Cu, the metal-binding domains of the trasmembrane cation channel; Tm, transmembrane domains; PD, phosphatase domain. (Reproduced from Fanni et al., 2005.)

M-domain, which comprises eight transmembrane ion channels (Fig. 3.2) (Cater et al., 2004, 2007; Lenartowicz and Krzeptowski, 2010).

Unique amino acid motifs are present at the core structure of each domain, such as TGEA at the A-domain, DKTGT at the P-domain, and SEHPL in the N-domain. Specifically, the N-terminal metal-binding domain (MBD) is composed of six copper-binding sites, each with the conserved sequence motif GMXCXXC (Fatemi and Sarkar, 2002; Sazinsky et al., 2006). These MBDs play a central role in accepting copper from copper chaperone ATOX1 through protein–protein interactions. Previous studies have demonstrated unequal impact of MBDs on ATP7B activity, with MBD 5 and 6 having stronger effects on the catalytic activation of ATP7B than MBDs 1–4 (Lutsenko et al.,1997).

The active transport of copper across membranes is a complex process that begins with ATP7B binding copper at the N-terminal domain and transporting it across cellular membranes, using ATP as an energy source (Fig. 3.2). Next, free copper binds intracellularly to GG motifs in the MBDs, followed by transport on to the Cys-Pro-Cys (CPC) sequence motifs in MBD 6. Finally, dephosphorylation of acyl-phosphate at the A-domain discharges copper across the cellular membrane. Mutations causing copper accumulation may occur at any of these steps (Huster et al., 2006; Schushan et al., 2012).

Although the mechanism by which the histadine-containing SEHPL motif affects copper transport remains to be elucidated, it is clear that histidine-to-glutamate substitution at amino acid 1069 (p.H1069Q) in this motif is the most common cause of Wilson disease in northern Europeans. In the hepatocytes of patients homozygous for p.H1069Q, ATP7B was found in the endoplasmic reticulum instead of its usual TGN location, suggesting abnormal protein trafficking (Huster et al., 2003). Insect models with the p.H1069Q mutation in SF9 cells showed decreased ATP-mediated catalytic phosphorylation but no major protein misfolding, suggesting a role for p.H1069Q in the orientation of the ATP7B catalytic site for ATP binding prior to hydrolysis (Tsivkovskii et al., 2003).

VARIANTS IN THE *ATP7B* GENE

More than 600 pathogenic variants in *ATP7B* have been identified, with single-nucleotide missense and nonsense mutations being the most common, followed by insertions/deletions and splice site mutations (Human Gene Mutation Database, accessed 29 April 2016; Stenson et al., 2014). Other rare genetic mechanisms that have been reported in the literature include whole-exon deletions, promoter region mutations, three concurrent pathogenic variants, and monogenic disomy (Coffey et al., 2013; Bandmann et al., 2015). Mutation "hotspots" in

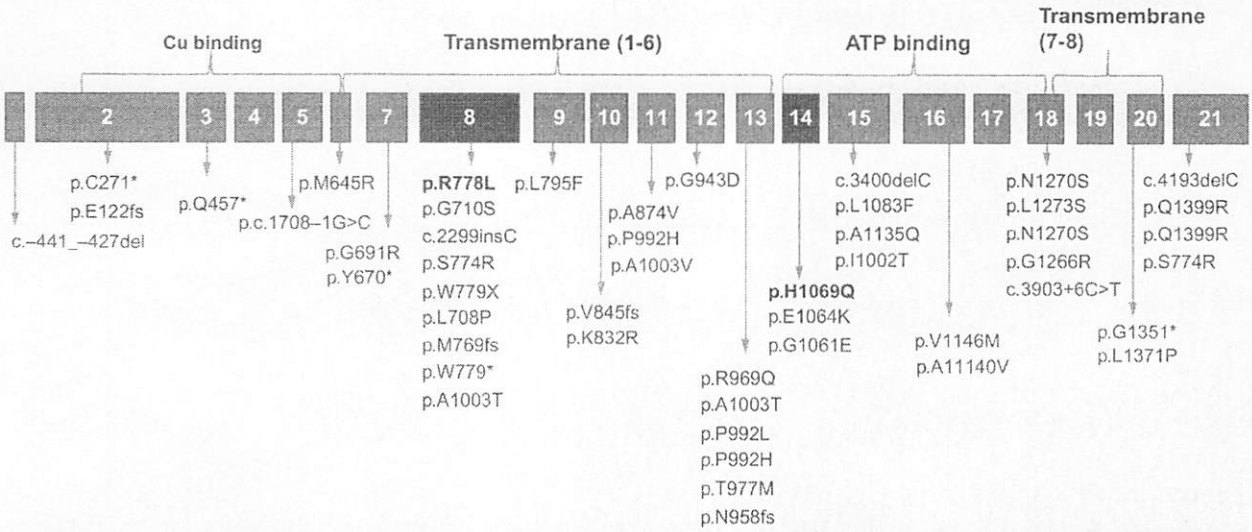

Fig. 3.3. Schematic of the *ATP7B* gene with common mutation sites, including p.H1069Q (rs76151636), p.R778L (rs28942074), p.E1064K (rs376910645), c.3400delC, and p.Ala1135fs (rs137853281). Please refer to Table 3.1 for more details.

ATP7B have also been reported to vary by geographic region (see regional gene frequency section, below). The majority of pathogenic mutations are located in the M- and N-domains in presymptomatic patients or in those with hepatic symptoms (S. Park et al., 2007). The common mutations in *ATP7B* seen in various populations are listed in Figure 3.3.

The p.H1069Q mutation is one of the most common mutations, with a population allelic frequency of 10–40% (30–70% among Caucasians). Most patients are compound heterozygotes, carrying different mutations on each copy of the chromosome (Usta et al., 2014). The p.H1069Q mutation occurs when histidine of the conserved SEHPL motif in the N-domain of *ATP7B* is replaced by glutamic acid, resulting in N-domain protein misfolding, abnormal phosphorylation in the P-domain, and decreased ATP binding affinity (Rodriguez-Granillo et al., 2008). This mutation also leads to decreased heat stability and abnormal localization of the protein to the TGN (Ralle et al., 2010).

Other common mutations in *ATP7B* include p.E1064A, p.R778L, p.G943S, and p.M769V. Mutations in p.E1064A, also found in the SEHPL motif, completely disable ATP binding affinity but do not result in protein misfolding, transport abnormalities, or thermal instability. The p.R778L mutation affects transmembrane transport of copper (Dmitriev et al., 2011). The p.G943S and p.M769V mutations result in defective copper metabolism but preserved ceruloplasmin levels (Okada et al., 2010).

A substantial proportion of Wilson disease-associated missense mutations, including p.H1069Q and p.R778L, result in markedly decreased level of the protein caused by enhanced degradation (Payne et al., 1998; de Bie et al., 2007; van den Berghe et al., 2009). Other prevalent mutations, such as protein-truncating nonsense mutations (~13% of known point mutations) (Merle et al., 2010) and frameshift mutations (Vrabelova et al., 2005), are predicted to cause decay of mRNA (Mendell et al., 2004; Chang et al., 2007) or a severely truncated protein, resulting in absent or diminished levels of protein. It is therefore expected that most patients with Wilson disease have absent or significantly reduced levels of ATP7B.

REGIONAL GENE FREQUENCY

The prevalence of Wilson disease varies by geographic region, with higher prevalence of specific mutations reported in certain populations (Ferenci, 2006) (see Chapter 2 for more details). A list of the common regional variants of ATP7B mutations and geographic clustering of mutations are shown in Table 3.1 and Figure 3.4, respectively.

GENOTYPE–PHENOTYPE CORRELATION

Direct genotype–phenotype relationships in Wilson disease have been difficult to establish, despite several studies examining correlation (Panagiotakaki et al., 2004; Vrabelova et al., 2005; Nicastro et al., 2010; Cocoş et al., 2014; Usta et al., 2014). The numerous low-frequency and compound heterozygous nature of Wilson disease obfuscate the process of characterizing its numerous genetic variants and their clinical consequences. Descriptions of phenotypes are limited to age of onset and presenting symptoms, both of which may be affected by inaccurate diagnostic criteria, delayed diagnosis, and practitioner selection bias. Therefore,

Table 3.1

Regional distribution of common Wilson disease mutations by geographic location

Region	AF (%)	Prevalent mutations					
		Protein	Nucleotide	RS	Exon	Type	Domain
Europe							
Austria (**Ferenci**, 2006)	34.1	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
	6.4	p.Gly710Ser	c.2128G>A		8	Missense	TM2
	3.6	p.Met769fs	c.2298_2299insC	rs137853287	8	Premature stop	TM4
Benelux (**Ferenci**, 2006)	53	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
Bulgaria (**Todorov** et al., 2005)	58.8	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
Canary Islands (**García Villarreal** et al., 2000)	64	p.Leu708Pro	c.2123 T>C		8	Missense	TM2
Czech Republic (**Vrabelova** et al., 2005)	57	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
Denmark (**Møller** et al., 2011)	18	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
	16	p.Trp779*	c.2336G>A	rs137853283	8	Nonsense	TM4
France (**Bost** et al., 2012)	15	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
Germany (**Ferenci**, 2006)	47.9	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
Germany (East, former) (**Caca** et al., 2000)	63	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
Greece (**Panagiotakaki** et al., 2004; **Dedoussis** et al., 2005; **Gomes** and **Dedoussis**, 2016)	35	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
	12	p.Arg969Gln	c.2906G>A	rs774028495	13	Missense	TM6
Hungary (**Firneisz** et al., 2002; **Folhoffer** et al., 2007)	42.9	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
Iceland (**Thomas** et al., 1995a; **Hofer** et al., 2012)	100	p.Tyr670*	c.2007_2013del		7	Nonsense	TM1
Italy (**Loudianos** et al., 1999)	17.5	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
	9	p.Val845fs	c.2530delA	rs755709270	10	Premature stop	Td
	6	p.Met769fs	c.2298_2299insC	rs137853287	8	Premature stop	TM4
Netherlands (**Stapelbroek** et al., 2004)	33	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop

Continued

Table 3.1

Continued

Region	AF (%)	Prevalent mutations		RS	Exon	Type	Domain
		Protein	Nucleotide				
Poland (**Gromadzka** et al., 2005)	72	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
	7.3	p.Ala1135fs	c.3400delC	rs137853281	15	Premature stop	ATP loop
	3.7	p.Gln1351*	c.4051C>T		20	Nonsense	
Romania (**Iacob** et al., 2012)	38.1	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
Russia (**Ivanova-Smolenskaya** et al., 1997)	49	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
Sardinia (**Figus** et al., 1995)	60.5		c.-441_-427del		5prime	Unknown	Promoter
	8.5	p.Met822fs	c.2463delC		10	Deletion	TM4/Td
	7.9	p.Val1146Met	c.3436G>A		16	Missense	ATP loop
Serbia (**Tomić** et al., 2013)	38.4	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
	11.6	p.Met769fs	c.2304dupC		8	Missense	TM4
	9.3	p.Ala1003Thr	c.3007G>A	rs1801247	13	Missense	TM6/Ph
Spain (**Margarit** et al., 2005)	27	p.Met645Arg	c.1934 T>G	rs121907998	6	Missense	Cu6/TM1
Sweden (**Shah** et al., 1997)	38	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
Turkey (**Ferenci**, 2006; **Simsek Papur** et al., 2013)	17.4	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
	5.3	p.Gly710Ser	c.2128G>A	rs772595172	8	Missense	TM2
	4.53	p.Gln457*	c.1369C>T		3	Nonsense	Cu4/Cu5
UK (**Coffey** et al., 2013)	19	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
	8	p.Met769Val	c.2305A>G		8	Missense	TM4
Yugoslavia (former) (**Loudianos** et al., 1999)	48.9	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
	11.4	p.Met769fs	c.2298_2299insC	rs137853287	8	Premature stop	TM4
Asia							
China (**Gu** et al., 2003; **Z.-Y. Wu et al.**, 2003; **Wang** et al., 2011; **Wei** et al., 2014)	31	p.Arg778Leu	c.2332C>T	rs28942074	8	Missense	TM4
	10	p.Pro992Leu	c.2975C>T	rs201038679	13	Missense	TM6/Ph
	9.6	p.Ile1148Thr	c.3443 T>C	rs60431989	16	Missense	ATP loop
	3.3	p.Thr935Met	c.2804C>T		12	Missense	TM5
	19	p.Arg778Leu	c.2332C>T	rs28942074	8	Missense	TM4

North India (**S. Kumar** et al., 2006; **Gupta** et al., 2007)							
	12	p.Ile1102Thr	c.3305 T>C	rs560952220	15	Missense	ATP loop
	9	p.Pro992His	c.2975C>A		13	Missense	TM6/Ph
South India	11	p.Ala1003Val	c.3008C>T		13	Missense	TM6/Ph
(**Santhosh** et al., 2006; **S. S. Kumar** et al., 2012)							
	11	p.Cys271*	c.813C>A	rs572147914	2	Nonsense	Cu3
	9	p.Pro768Leu	c.2303C>T		8	Missense	TM4
	9	p.Arg969Gln	c.2906G>A	rs121907996	13	Missense	TM6
East India	16	p.Cys271*	c.813C>A	rs572147914	2	Nonsense	Cu3
(**Gupta** et al., 2005)							
	11	p.Gly1061Glu	c.3182G>A		14	Missense	ATP loop
	8.5		c.1708-1G>C	rs137853280	5	Splice	Cu6
West India	20	p.Cys271*	c.813C>A	rs572147914	2	Nonsense	Cu3
(**Aggarwal** and **Bhatt**, 2013; **Aggarwal** et al., 2013)							
	11	p.Glu122fs	c.365_366delins TTCGAAGC		2	Ins/Del	Cu1
	6	p.Thr977Met	c.2930C>T	rs72552255	13	Missense	TM6
	6	p.Leu795Phe	c.2383C>T		9	Missense	TM4/Td
Japan	17.95	p.Asn958fs	c.2871delC		13	Premature stop	TM5/TM6
(**Okada** et al., 2000; **Tatsumi** et al., 2010)							
	16.7	p.Arg778Leu	c.2332C>T	rs28942074	8	Missense	TM4
	10.5		c.1708-5 T>G		5	Splice	Cu6
Korea	37.9	p.Arg778Leu	c.2332C>T	rs28942074	8	Missense	TM4
(**E. K. Kim** et al., 1998; **Yoo**, 2002; **G.-H. Kim** et al., 2008; **Song** et al., 2012)							
	12.1	p.Asn1270Ser	c.3809A>G	rs121907990	18	Missense	ATP hinge
	9.4	p.Ala874Val	c.2621C>T	rs376355660	11	Missense	TM5
	8	p.Leu1083Phe	c.3247C>T		15	Missense	ATP loop
Lebanon	44.7	p. Ala1003Thr	c.2299insC	rs137853287	8	Missense	TM4
(**Usta** et al., 2014)							
Saudi Arabia	32	p.Gln1399Arg	c.4196A>G		21	Missense	After TM8
(**Al Jumah** et al., 2004; **Majumdar** et al., 2004)							
	16	p.Ser774Arg	c.2230 T>C	rs535217574	21	Missense	TM3
Taiwan	29.6	p.Arg778Leu	c.2332C>T	rs28942074	8	Missense	TM4
(**Lee** et al., 2000; **Wan** et al., 2006)							
	8.9	p.Pro992Leu	c.2975C>T	rs201038679	13	Missense	TM6
	4.8	p.Gly943Asp	c.2828G>A		12	Missense	TM5

Continued

Table 3.1

Continued

Region	AF (%)	Prevalent mutations					
		Protein	Nucleotide	RS	Exon	Type	Domain
Thailand (**Panichareon** et al., 2011)	10.52	p.Arg778Leu	c.2332C>T	rs28942074	8	Missense	TM4
	7.89	p.Leu1371Pro	c.4112 T>C		20	Missense	TM8
Iran (**Zali** et al., 2011)	19	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
Africa							
Egypt (**Abdelghaffar** et al., 2008; **Abdel Ghaffar** et al., 2011)	42.2	IVS18+6 T>C	c.3903+6C>T	rs2282057	18	Splice	
	40.6	p.Ala11140Val	c.3419C>T		16	Missense	ATP loop
	26.5	p.Lys832Arg	c.2495A>G	rs1061472	10	Missense	TM4/Td
Americas							
USA (**Kuppala** et al., 2009)	40.3	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
	1.9	p.Asn1270Ser	c.3809A>G	rs121907990	18	Missense	ATP hinge
	1.9	p.Gly1266Arg	c.3796G>A	rs121907992	18	Missense	ATP hinge
Brazil (**Deguti** et al., 2004; **Machado** et al., 2008; **Bem** et al., 2013)	37.1	p.His1069Gln	c.3207C>A	rs76151636	14	Missense	ATP loop
	31.25	p.Ala1135fs	c.3400delC	rs137853281	15	Premature stop	ATP loop
	11.4	p.Ala1135GlnfsX13	c.3402delC	rs137853281	15	Premature stop	ATP loop
		p.Leu708Pro	c.2123 T>C		8	Missense	TM2
Costa Rica (**Shah** et al., 1997)	61	p.Asn1270Ser	c.3809A>G	rs121907990	18	Missense	ATP hinge
Venezuela (**Paradisi** et al., 2015)	26.9	p.Ala1135GlnfsX13	c.3402delC	rs137853281	15	Premature stop	ATP loop
	9.6	p.Gly691Arg	c.2071G>A		7	Missense	TM2

AF, allelic frequency; RS, Reference single nucleotide polymorphism (SNP) cluster identification number.

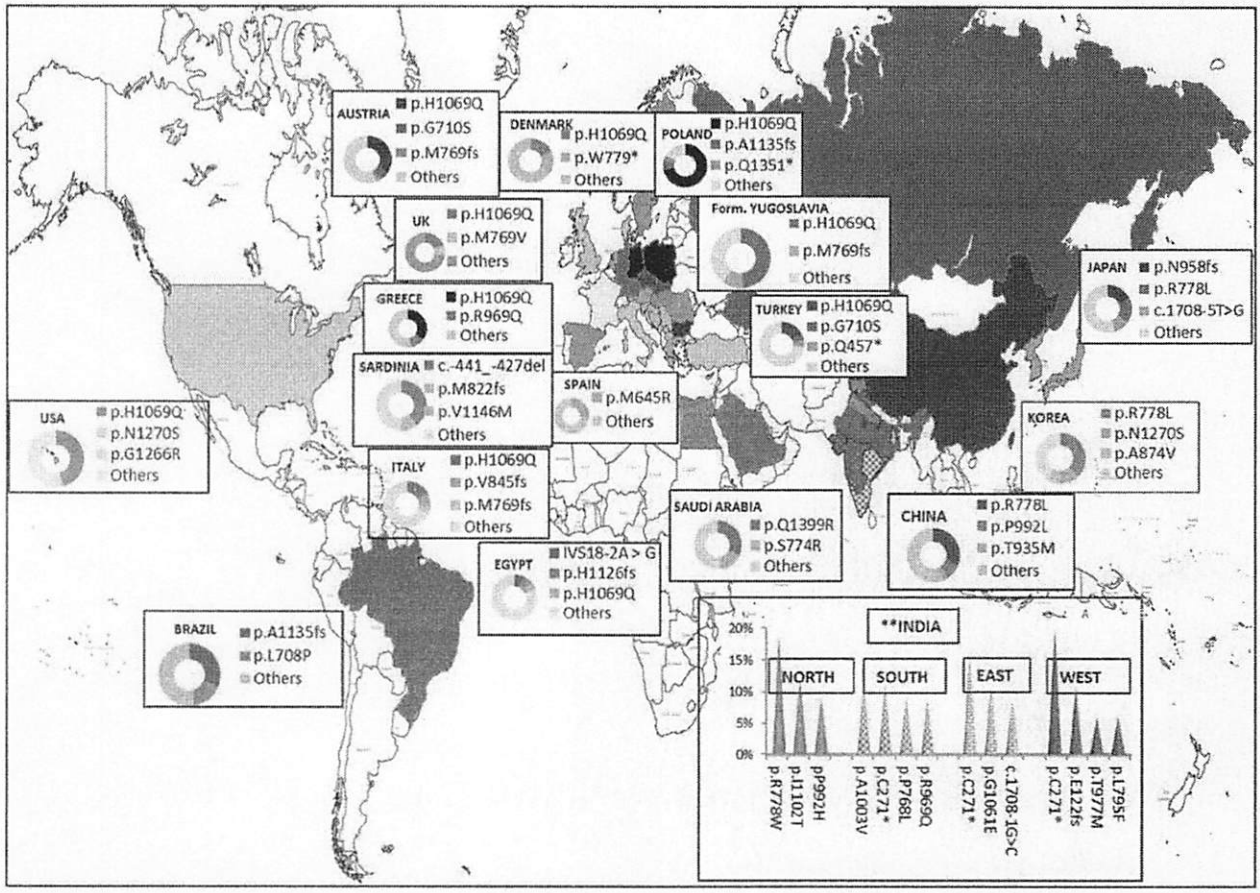

Fig. 3.4. Prevalence of *ATP7B* mutation by geographic region; the darker the gradient, the higher the allelic frequency. (Reproduced from Gomes and Dedoussis, 2016, with permission from Taylor and Francis.)

the marked variability in phenotype of Wilson disease is likely attributable to an amalgamation of genetic, metabolic, and environmental factors (Leggio et al., 2006).

The most consistent genotype–phenotype correlation in Wilson disease is that the most severe, early-onset disease with predominantly hepatic presentation is associated with mutations causing absent ATPase activity. Convincing studies have demonstrated fulminant hepatic disease in mouse models such as the toxic milk (tx) mouse and the Jackson tx mouse (tx^J), which harbor point mutations causing loss of ATP7B function, but not affecting ATP7B synthesis (Theophilos et al., 1996; Coronado et al., 2001; La Fontaine et al., 2001; Huster et al., 2006).

Genetic polymorphisms in *ATP7B*, other genes, and epigenetic factors have been shown to impact disease phenotype by affecting ATP7B protein structure and function. Of the over 600 mutations associated with Wilson disease, the majority are missense mutations that completely inactivate the copper-transporting function of ATP7B (Lutsenko, 2014). In general, individuals with protein-truncating mutations have earlier onset of disease due to decreased protein stability and quantity (Merle et al., 2010). However, other studies have demonstrated partial preservation of copper-transporting function, perhaps explaining the milder phenotypes associated with certain mutations (Rodriguez-Granillo et al., 2008; Dmitriev et al., 2011; Huster et al., 2012). Individuals with the R778L mutation have been shown to have an earlier onset of disease and predominantly hepatic presentation (Z. Y. Wu et al., 2003). In contrast, individuals with the H1069Q mutation have a mean onset of symptoms between 20–22 years old and predominantly neurologic phenotype (Stapelbroek et al., 2004; Kalita et al., 2010). There is also some evidence that Kayser–Fleischer rings are more common in H1069Q homozygous patients in Hungary at time of diagnosis than in compound heterozygous individuals (Folhoffer et al., 2007).

Moreover, pathogenic variants may affect ATP7B targeting from the TGN to cytosolic vesicles. For instance, the p.Met875Val mutation results in a less stable protein and causes reversible ATP7B localization defects. Under a low-copper environment, the p.Gly875Arg variant is sequestered in the endoplasmic reticulum. However, addition of exogenous copper to the cellular growth medium stabilizes the protein, allowing it to complete

its intended journey to the TGN and overcoming its disease-causing phenotype. Theoretically, patients with this specific variant may be more sensitive to dietary copper deficiency (Gupta et al., 2011).

The timing and location of copper buildup can also preferentially alter the hepatic transcriptome, based on homozygous *ATP7B*$^{-/-}$ mouse models. Proteomic analyses of mRNA profiles at each of these disease stages reflect unique patterns (Huster et al., 2006; Ralle et al., 2010). In the initial stage, mRNA for proteins responsible for cell cycle regulation, splicing, and cholesterol synthesis is present (Burkhead et al., 2011). This leads to early accumulation of copper bound to metallothioneins in the cytosol and free copper in the nuclei. In the progressive stage, mRNA changes throughout the cell are present, including the endoplasmic reticulum, mitochondria, and endocytic pathways, causing copper to pathologically accumulate within hepatocytes. In the later stages, mRNA for lysosomal and endosomal proteins is upregulated. In these final stages, copper concentrations decrease in the cytosol and nuclei, and accumulate in the membranous cellular compartment, causing bile duct proliferation and hepatic neoplastic changes. Therefore, the location of copper accumulation may convey more specific prognostic information about disease progression rather than total copper levels.

Other studies have compared homozygotes to compound heterozygotes of the same mutation to establish genotype–phenotype correlations. A study of 76 members of a large, consanguineous Lebanese family showed an association between c.2299insC and hepatic disease and between the p.Ala1003Thr mutation and neurologic disease (Usta et al., 2014).

Other candidate polymorphisms that are thought to modify the clinical phenotype of Wilson disease include MTHFR (Gromadzka et al., 2005), COMMD1 (Weiss, 2006), ATOX1 (Simon, 2008), XIAP (Weiss et al., 2010), PNPLA3 and hepatic steatosis (Stättermayer et al., 2012), and DMT1 (Przybyłkowski et al., 2014), although none of these genes has been demonstrated to have significant diagnostic or predictive value.

Significant phenotypic variation of Wilson disease exists between individuals with the same mutation, individuals within the same family, and even between monozygotic twins (Członkowska et al., 2009; Kegley et al., 2010). While some studies have documented high intrafamilial concordance of clinical symptoms and biochemical results (Hofer et al., 2012; Chabik et al., 2014; Ferenci et al., 2015), others have reported a wide range in age of onset and presenting symptoms amongst siblings (Ala et al., 2007; Taly et al., 2007) and families carrying the same mutation (Takeshita et al., 2002). Indeed, disparate clinical presentations in monozygotic twins raise the suspicion for epigenetic modifiers in Wilson disease. See Chapter 4 for more details about the genetic and environmental modifiers of Wilson disease.

CLINICAL MOLECULAR DIAGNOSIS

The current gold standard of diagnosis for Wilson disease is direct Sanger sequencing of the *ATP7B* gene or molecular testing for previously identified familial mutations. Historically, most pathogenic variants in *ATP7B* were identified using a combination of polymerase chain reaction (PCR)/restriction fragment length polymorphism (RFLP), single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), temporal temperature gradient electrophoresis (TTGE), denaturing high-performance liquid chromatography (DHPLC), and Sanger sequencing (Loudianos et al., 1999; Shimizu et al., 1999; Margarit et al., 2005; Vrabelova et al., 2005; G. H. Kim et al., 2008). The critical demerits of this complex tiered approach are that the detection rate is not high enough to find most mutations and the turnaround time is often extended. Although regional clusters of specific mutations have been well described, a customized screening approach taking into account these regional variants may be complicated by ethnically diverse populations and inaccurate clinical information provided with samples. Biochemical results are often imprecise, as elevations in urinary copper excretion tend to occur late in the disease process and fewer than 40% of presymptomatic patients excrete copper less than 100 μg/day (Sternlieb and Scheinberg, 1968; Nakayama et al., 2008). For these reasons, direct sequencing of the *ATP7B* gene has become the preferred standard and provides the greatest yield in clinical molecular diagnosis. Please refer to Chapter 14 for details about the diagnosis of Wilson disease.

Starting the diagnostic process with molecular testing may significantly reduce the need for invasive liver biopsy. Liver copper content alone was found to be insufficient to exclude Wilson disease, as levels may not be elevated in some affected patients. Based on several previous studies, biallelic pathogenic variants were identified in about 80% of patients with biochemical and clinical tests suggestive of Wilson disease. Currently available screening tests may not definitively rule out the disease, and no single test could permit *de novo* diagnosis. Of note, many patients may not possess the characteristic findings and may present when their clinical disease is relatively mild. Inappropriate treatment for false-positive cases has the potential of inducing copper deficiency, which can result in hematologic and neurologic sequelae (N. Kumar et al., 2003). These findings reinforce the need for reliable clinical diagnostic criteria

and underscore the benefits of DNA testing prior to invasive procedures (Ferenci, 2005).

Multiplex PCR is used to amplify all 21 exons and splice sites of *ATP7B*, including promoter regions. Although the large deletions or duplications cannot be detected with this conventional Sanger sequencing method, the chance of these being present in Wilson disease appears low (Stenson et al., 2012). If clinical suspicion is still high with only one pathogenic variant identified, then multiplex ligation-dependent probe amplification (MLPA) test should be considered. Microarray-based comparative genomic hybridization is another option to evaluate partial or full gene deletions or duplications with higher sensitivity. Cases with only one pathogenic variant present should be carefully reviewed in the context of other biochemical and clinical findings. Molecular genetic testing using direct mutation analysis is very effective in identifying affected patients and presymptomatic siblings of probands (Manolaki et al., 2009).

Wilson disease is an autosomal-recessive disorder, which means that there is a 25% chance that a full sibling of the index case is also affected. Once homozygous or compound heterozygous mutations in *ATP7B* have been established in the index patient, mutation detection becomes valuable in family screening. The same genotype in asymptomatic family members confirms diagnosis of the disease, thus allowing for early treatment before the onset of complications. In family members in whom clinical and biochemical features are uncertain, the demonstration of either heterozygous (carrier) or wild-type gene sequence prevents unnecessary treatment (Chang et al., 2007).

If the proband has secured a diagnosis of Wilson disease on the basis of clinical and biochemical evidence, but testing for *ATP7B* mutations is not available, family screening can be done by haplotype analysis of polymorphic markers flanking the disease gene (Thomas et al., 1995b; Gupta et al., 2005; Przybyłkowski et al., 2014). In this instance, the rare possibility of recombination events (typically 0.5–5% of cases) needs to be considered. The rate of recombination is dependent on which flanking markers are studied. Microsatellite or single-nucleotide polymorphisms in the *ATP7B* lateral wing are used for haplotyping, which is useful for screening relatives of patients with previously identified familial mutations. False-positive results may occur if haplotyping is used on patients with low-probability gene recombinations.

Genetic testing for *ATP7B* mutations can be valuable to confirm a diagnosis of Wilson disease, especially when presentation is unusual (Caprai et al., 2006). Attention has been drawn to this situation by the molecular confirmation of early-onset hepatic disease in a 3-year-old child (Wilson et al., 2000). Mutation analysis has also confirmed late-onset disease, including the case of two siblings in their 70s – the oldest reported patients so far at time of diagnosis (Nanji et al., 1997; Gupta et al., 2005; Perri et al., 2005; Weitzman et al., 2014).

ATP7B mutation analysis makes an important contribution to clinical practice. Unfortunately, systematic genetic testing for Wilson disease is still difficult and fairly expensive due to the plethora of different mutations, the occurrence of regulatory mutations in noncoding sequence, the large size of the gene, and the limitations of currently available methods. However, technical advances allowing high-throughput screening could be applied to the disease (Bost et al., 2012; Lepori et al., 2012). This new apparatus can sequence six million basepairs of DNA per hour with accuracy greater than 99%. Such advances might permit specialized laboratories to detect all variants by sequencing the entire genomic Wilson disease gene from patients, including not only the translated exons, but also the important noncoding sequences that are not normally investigated.

Interpretation of variants of uncertain significance has become a major challenge for accurate interpretation, genetic counseling, and prevention. Screening family members may help with the interpretation of variants of uncertain significance, but not all variants can be resolved with this approach. Functional analysis is often necessary; however, no clinical functional analysis is currently available. A computational approach to predict significance of mutations is often helpful, but a further concrete model is required to demonstrate the efficacy in guiding clinical decisions.

POPULATION SCREENING

The purpose of newborn screening is to identify treatable congenital conditions that can affect a child's long-term health and development. Recent tandem mass spectrometry (MS/MS) applications have markedly expanded the ability to screen for >50 metabolic diseases from a single dried blood spot. In addition to the original Wilson–Jungner classic screening criteria (Wilson and Jungner, 1968), the American College of Medical Genetics convened the Newborn Screening Expert Group to develop a uniform screening panel in 2006 (American College of Medical Genetics Newborn Screening Expert Group, 2006). Of the primary tenants, Wilson disease is an ideal target for screening, given its relatively high prevalence and availability of effective treatment (Hahn et al., 2002; Roberts et al., 2008). Unfortunately, despite extensive discussion on the need for population screening, no cost-effective biomarkers or methods for early detection have been developed for Wilson disease yet. Several

small pilot studies have been conducted using ceruloplasmin as a biomarker for screening, with limited findings (Yamaguchi et al., 1999; Hahn et al., 2002; Owada et al., 2002; Schilsky and Shneider, 2002; Kroll et al., 2006). Ceruloplasmin alone is not sufficient to screen for Wilson disease in newborns, as a substantial number of newborns present with physiologically low ceruloplasmin. Ceruloplasmin assay around 3 years of age may be the most appropriate population-screening method, but mandatory health checkups at this age are not universally available in the USA and worldwide.

Many treatable congenital disorders are caused by mutations that result in absent or diminished levels of proteins; thus, protein biomarkers have enormous potential in the diagnosis/screening of congenital disorders. Liquid chromatography mass spectrometry with multiple reaction monitoring (LC-MRM-MS) has emerged as a robust technology that enables highly precise, specific, multiplex quantification of signature proteotypic peptides as stoichiometric surrogates of biomarker proteins.

Our lab is currently exploring the use of peptide immunoaffinity enrichment (Whiteaker et al., 2010, 2011) to quantify ATP7B in dried blood spot (DBS). These promising proof-of-concept data open up the possibility of screening for Wilson disease in newborns. Further clinical validation on a large-scale study will be required to determine the efficacy of the assay.

CONCLUSION

Wilson disease is an autosomal-recessive disease due to pathogenic mutations in *ATP7B*. *ATP7B* is the only identified gene known to cause Wilson disease, and encodes a transmembrane copper-transporting ATPase of the same name. While biochemical testing and clinical criteria may assist in the early diagnosis and treatment, the current gold standard for Wilson disease diagnosis is direct Sanger sequencing of ATP7B or molecular testing for known familial mutations. Genotype–phenotype correlations have been studied extensively but direct causations remain nebulous. Modifier genes may affect the penetrance and phenotypes but a large-scale study for clinical validation is warranted. The overall worldwide prevalence of Wilson disease is 1 in 30 000 individuals, with significant geographic variation. The most common mutation in Northern America and Europe is the missense mutation p.H1069Q and the most common mutation in East Asian populations is the missense p.R778L. Ceruloplasmin alone is insufficient to screen for Wilson disease in newborns. While peptide immunoaffinity assays show promise for newborn screening, further large-scale clinical studies are required to determine efficacy of these population-based screening methods for Wilson's disease.

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Quantification of ATP7B Protein in Dried Blood Spots by Peptide Immuno-SRM as a Potential Screen for Wilson's Disease

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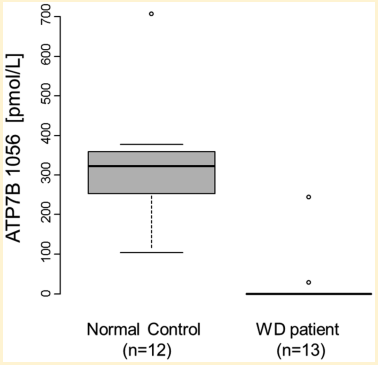
ABSTRACT: Wilson's Disease (WD), a copper transport disorder caused by a genetic defect in the *ATP7B* gene, has been a long time strong candidate for newborn screening (NBS), since early interventions can give better results by preventing irreversible neurological disability or liver cirrhosis. Several previous pilot studies measuring ceruloplasmin (CP) in infants or children showed that this marker alone was insufficient to meet the universal screening for WD. WD results from mutations that cause absent or markedly diminished levels of ATP7B. Therefore, ATP7B could serve as a marker for the screening of WD, if the protein can be detected from dried blood spots (DBS). This study demonstrates that the immuno-SRM platform can quantify ATP7B in DBS in the picomolar range, and that the assay readily distinguishes affected cases from normal controls ($p < 0.0001$). The assay precision was <10% CV, and the protein was stable for a week in DBS at room temperature. These promising proof-of-concept data open up the possibility of screening WD in newborns and the potential for a multiplexed assay for screening a variety of congenital disorders using proteins as biomarkers in DBS.



KEYWORDS: *Wilson's disease, WD, newborn screening, NBS, ATP7B, dried blood spots, DBS, immuno-SRM, peptide immunoaffinity enrichment, mass spectrometry*

INTRODUCTION

Wilson's Disease (WD) is an autosomal recessive disorder caused by mutations in *ATP7B* gene *(OMIM *606882)*.[1−3] *ATP7B* encodes a transmembrane protein ATPase (ATP7B), which is highly expressed in the liver and kidney and functions as a copper-dependent P-type ATPase. *ATP7B* is required for transmembrane transport of copper from hepatocytes into the biliary system. Absent or reduced function of ATP7B protein results in copper accumulation in the liver and subsequently in the brain, kidneys, and other organs. The impaired function of ATP7B protein also fails to incorporate copper into apoceruloplasmin, resulting in the decreased blood level of ceruloplasmin (CP) in the majority of patients with WD.[4−6]

The prevalence of WD is ∼1 in 30 000 newborns, with a carrier frequency of 1 in 90 (higher in certain populations).[7] However, regional variations exist. In particular, Costa Rica, Sardinia, the Canary Island, and Crete have all reported to have increased incidence.[8−11] WD is a slow, progressive disease. Although the biochemical defects are present from birth, patients with WD typically present with chronic hepatitis, cirrhosis, or acute liver failure in the first or second decade of life. They may have tremors, ataxia, dysarthria, and difficulty swallowing. WD was fatal until treatments were developed a half century ago. In 1955, the identification of D-penicillamine by John Walshe dramatically improved the outcome of WD by increasing the urinary excretion of copper.[12] Other treatments have since been introduced, including trientine and zinc salts, and have proven efficacious.[13−17] Unfortunately, despite the fact that effective medical treatments have been available for over 50 years and can prevent a fatal outcome if patients are diagnosed early, clinically recognizing WD remains difficult because of its slow progression and the broad clinical spectrum of symptoms. Therefore, many patients still present with irreversible multiorgan damage at the time of diagnosis.

Ideally, patients should be recognized in the presymptomatic stage. There has been a consensus that the best way to achieve early diagnosis of WD before the onset of serious symptoms is through NBS.[18−20] The current gold standard for diagnosis of WD includes multiple laboratory tests, such as copper determination in the urine and liver tissue, followed by confirmation with genetic testing of the *ATP7B* gene. These current diagnostic tools, however, are not suitable for large-scale screening.

With the discovery that CP was reduced in the majority (∼85%) of patients with WD,[5,6,20] several methods were developed to measure CP, a proposed marker for WD, in DBS or urine using different analytical platforms such as a sandwich

Received: September 14, 2016
Published: November 22, 2016

DOI: 10.1021/acs.jproteome.6b00828
J. Proteome Res. 2017, 16, 862−871

ELISA assay and an LC−MS/MS assay.[19,21−25] Unfortunately, from the few pilot studies published measuring CP in infants or children, CP determination alone was insufficient to screen for WD because of high false discovery rates resulting from the fact that (1) CP is physiologically low in some unaffected newborn babies and (2) some heterozygote carriers for WD have reduced levels of CP. Pediatric screening around 3 years of age has been proposed in Japan, but it is practically difficult to screen the entire population.[26] Despite a strong need for more reliable markers or methods to meet the requirement for the universal screening of WD, no further developments have been made in attempting to screen for WD in recent years.

Most WD mutations have been shown to disrupt ATP7B stability, resulting in absent or diminished levels of the protein; thus quantifying ATP7B levels has enormous potential in the screening of WD. There are >370 mutations reported worldwide;[27,28] most are rare and infrequent, except the two most common mutations, p.H1069Q (~37−63% of the white population) and p.R778L (57% of the East Asian population).[8,29−31] In line with previous observations for disease-causing missense mutations,[32−35] some WD-associated missense mutations including p.H1069Q and p.R778L resulted in a markedly decreased level of the ATP7B protein caused by enhanced degradation.[31,36,37] Other prevalent mutations such as protein-truncating nonsense mutations (~13% of known point mutations)[38] and frameshift mutations[30] are predicted to result in the absence or decay of mRNA[39,40] or a severely truncated protein, resulting in absent or diminished levels of the protein. Taken together, it is expected that most patients with WD would have absence of or reduced levels of ATP7B. While these findings suggest that absent or diminished ATP7B levels can be an indicator for WD, it has not yet been tested.

Targeted mass spectrometry, in particular, multiple/selected reaction monitoring (M/SRM), has been used for rapid development of quantitative assays with high specificity, high-throughput, precision, and robustness,[41−45] and cross-laboratory (including international) transferability of SRM-based assays has been achieved.[45] The combination of DBS with SRM is the standard analytical approach in clinical or NBS laboratories for a variety of small metabolites that accumulate as a result of inborn errors of metabolism.[46,47] Although SRM is capable of quantifying proteins present in μg/mL and higher concentrations, many potential protein markers of greatest interest are often in the low ng/mL range. Quantification of proteins and peptides in complex samples (e.g., plasma) in the ng/mL range by SRM is challenging because of the complexity and large dynamic range of the matrix. The sensitivity of SRM is not sufficient to measure low abundance protein markers directly from DBS without an enrichment process due to interferences from more abundant analytes present in the matrix.

In recent years, peptide immunoaffinity enrichment coupled to SRM (immuno-SRM) has emerged as a promising technique for the quantification of low abundance proteins in complex matrices.[48−57] The benefits of immunoaffinity enrichment of the target peptide analyte from digests of complex samples are to greatly enrich the peptide of interest before LC−MS/MS, reducing ion suppression from background components and greatly enhancing the sensitivity of the method. Immuno-SRM has been successfully implemented to address the detection-limit challenges associated with measuring low-abundance protein biomarkers in the low- and sub-μg/L range in a wide array of studies.[48−51,58−62]

While the immuno-SRM technology has been demonstrated on clinical samples,[63,64] it has not yet been adapted for measuring low-abundance proteins from dried blood spots on filter paper. In this study, we proposed to evaluate ATP7B as a marker for WD. As proof-of-concept, we investigated the immuno-SRM methodology and applied this assay to determine the concentrations of ATP7B in DBS from unaffected and WD-affected individuals. The results demonstrate that immuno-SRM is a high-sensitivity platform for DBS analysis of proteins in the low-picomolar range and ATP7B is a potential marker for screening WD in newborns.

EXPERIMENTAL PROCEDURES

Study Approval

Blood samples involved in this study included a total of 13 WD patients and 12 healthy volunteers. Of these, 10 WD and 12 healthy subjects were from Seattle Children's Hospital and 3 WD subjects were from Asan Medical Center in Seoul, Korea. All patients signed informed consent and all research procedures were approved by the institutional review boards of aforementioned institutions. Whole blood from each subject was collected in ACD (acid citrate dextrose) tubes. Dried blood spots were prepared by pipetting 70 μL of blood/spot onto filter paper card (Protein Saver 903 Card, Whatman, Piscataway, NJ), allowed to dry at room temperature overnight, and then stored in sealed plastic bags at −80 °C until use. Fifteen year old DBS samples from proven carriers and affected patient from a previous study[21] were retrieved from −20 °C and tested as described.

Materials

ProteaseMAX Surfactant (no. V2072) was purchased from Promega (Madison, WI). Proteomics grade trypsin, bovine serum albumin protein standard (200 mg/mL), (3-[3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) (CHAPS, no. PI28300) detergent, and ammonium bicarbonate (XX) were obtained from Sigma Life Science (St. Louis, MO). Acetonitrile (no. A955) and water (no. W6, LCMS optima grade), formic acid (no. PI28905), and phosphate-buffered saline (PBS, no. 10010-023) were obtained from Thermo Fisher Scientific (Waltham, MA). HepG2 cell line was obtained from the ATCC (Manassas, VA).

Generation of Immuno-SRM Assay Reagents

Rabbit polyclonal antibodies were produced by Pacific Immunology (Ramona, CA). Polyclonal antibodies were affinity purified from 25 mL of antiserum. Purified (>95% by HPLC) heavy stable isotope-labeled peptides were obtained from Anaspec (Fremont, CA). For stable isotope-labeled peptides, the C-terminal arginine or lysine was labeled with [^{13}C and ^{15}N] labeled atoms, resulting in a mass shift of +8 or +10 Da, respectively. Aliquots were stored in 5% acetonitrile/0.1% formic acid at −20 °C until use. The antibody was coupled and immobilized to 2.8 μm Protein G magnetic beads (no. 10004D, Invitrogen, Carlsbad, CA) in a 1 μg antibody-to-2.5 μL of beads ratio. In brief, 250 μL of the beads was added to 1.6 mL Eppendorf tubes and washed once with 250 μL of 1× PBS, followed by the addition of 100 μg of antibody and 1× PBS + 0.03% CHAPS (no. 28300, Thermo Scientific) to yield a total 250 μL of volume. The antibodies were allowed to couple to the beads overnight with tumbling at 4 °C. The next day, the antibodies were immobilized onto the beads as follows (the work was performed in a fume hood). The supernatant was

DOI: 10.1021/acs.jproteome.6b00828
J. Proteome Res. 2017, 16, 862−871

Table 1. List of Candidate Peptides

peptide	sequence	molecular weight	parent ion	daughter ions[a]
ATP7B 301−313	YDPSCTSPVALQR	1435.7	718.8	579.8 (y11 + 2), 683.4 (y6), 871.5 (y8), 974.5 (y9)
ATP7B 325−339	VSLPDGAEGSGTDHR	1496.7	499.9	599.8 (y12 + 2), 656.30 (y13 + 2), 729.33 (y7), 858.37 (y8)
ATP7B 887−901	ATHVGNDTTLAQIVK	1566.8	523.3	558.4 (y5), 671.4 (y6), 772.5 (y7), 897.4 (b9)
ATP7B 1056−1077	VLAVVGTAEASSE HPLGVAVTK	2134.2	712.4	827.4 (y17 + 2), 876.9 (y18 + 2), 926.5 (y19 + 2), 966.0 (y20 + 2)

[a]Ion type for daughter ions is in parentheses.

discarded, and 250 μL of freshly prepared 20 mM DMP (dimethyl pimelimidate dihydrochloride, no. D8388, Sigma) in 200 mM triethanolamine, pH 8.5 (no. T1377, Sigma) was added. The samples were tumbled for 30 min at room temperature, and the DMP in triethanolamine was discarded. To quench the reaction, 250 μL of 150 mM monoethanolamine (no. 411000, Sigma) was added and the beads were tumbled at room temperature for 30 min. The antibody beads were washed twice using 250 μL of 5% acetic acid + 0.03% CHAPS (5 min of tumbling at room temperate each time), and washed once more using 250 μL of 1× PBS + 0.03% CHAPS. The antibody-beads suspension was finally resuspended in 250 μL of 1× PBS and stored at 4 °C until use.

Trypsin Digestion

From each DBS sample, twenty 3 mm punches (containing ∼3.7 μL of blood per disc) were obtained with a standard leather punch and placed into a 1.7 mL microcentrifuge, followed by the addition of 500 μL of 0.1% ProteaseMax in 50 mM ammonium biocarbonate (pH 8). Tubes were vortex-mixed for 1 h on Eppendorf MixMate (Eppendorf, Hamburg, Germany). At this point, aliquots were reserved for a Bradford assay. Disulfide bond reduction and trypsin digestion were performed in a single step with 2 M DTT and acetonitrile added to final concentrations of 5 mM and 15%, respectively. Trypsin was used in a 1:50 enzyme to protein ratio (w/w). The mixture was incubated in a 37 °C water bath overnight. After a 10 min centrifugation, the supernatant was transferred to a new tube, dried under a nitrogen stream, and stored at −80 °C until use.

Peptide Immunoaffinity Enrichment and Liquid Chromatography−Mass Spectrometry

The DBS digests were resuspended in 1× PBS + 0.03% CHAPS to yield a 1 μg/μL nominal protein digest concentration. Next, ∼2 mg of protein digest was combined with 4.8 μg of the antibody (immobilized on beads) in each tube and tubes were incubated overnight at 4 °C with tumbling. (The total capture volume was 500 μL.) The beads with immobilized antibodies and captured peptides were washed twice in PBS buffer + 0.03% CHAPS and washed once in PBS diluted 1:10, and peptides were eluted in 30 μL of 5% acetic acid/3% acetonitrile. The elution was frozen at −80 °C until analysis. An Eksigent Ultra nanoLC 2D system (Eksigent Technologies, Dublin, CA) with a nano autosampler was used for liquid chromatography. The peptides were loaded on a trap column (0.3 × 5 mm, C18, LCPackings, Dionex) at 10 μL/min and the LC gradient was delivered at 300 nL/min and consisted of a linear gradient of mobile phase B developed from 2 to 40% B in 18 min on a 10 cm × 75 μm column (Reprosil AQ C18 particles, 3 μm; Dr. Maisch, Germany). The nanoLC system was connected to a hybrid triple quadrupole/ion trap mass spectrometer (6500 QTRAP, ABSciex, Foster City, CA) equipped with a nano-electrospray interface operated in the positive ion SRM mode. Parameters for declustering potential (DP) and collision energy (CE) were taken from a linear regression of previously optimized values in Skyline.[65] SRM transitions were acquired at unit/unit resolution in both the Q1 and Q3 quadrupoles with 5 ms dwell time and 3 ms pause between mass ranges, resulting in a cycle time of 1.5 s. All samples were run in a blinded fashion.

Data Analysis

All SRM data were analyzed using Skyline. The presence of multiple transitions and retention time alignment with standard peptides were manually reviewed to verify detection of the correct peptide analyte. Data were exported from Skyline for analysis and plotting. The amount of the peptide in each DBS sample was determined by calculating the ratio of the peak areas for the signature peptide to that of its labeled IS present at a known concentration.

Method Assessment

Response curves were generated in a DBS matrix. The heavy stable isotope−labeled peptides were added to the tryptic digests of DBS covering the following concentrations: 0.03, 0.13, 0.67, 3.35, and 16.77 fmol/uL. Three process replicates were prepared and analyzed at all concentration points. Repeatability was determined using two samples: (i) DBS from normal control and (ii) DBS pooled from two WD affected siblings. Complete process triplicates were prepared and analyzed on three independent days. Intraassay variation was calculated as the average CV obtained within each day. Inter-assay variation was the CV calculated from the average values of the 3 days. The stability of analytes in DBS was tested by using the same DBS card stored at room temperature and at −20 °C for 0, 3, and 7 days. For each DBS sample, samples were prepared in triplicate.

RESULTS

Selection of Target Peptides

To develop a quantitative method for the quantification of ATP7B in DBS, candidate peptides for ATP7B were screened by in silico trypsin digestion, using criteria previously described,[44] followed by BLAST searching to ensure that the sequences are unique within the human genome. These peptides were screened using the tryptic digests of HepG2 cells to empirically determine which ATP7B peptides could be best detected and quantified by LC−MS/MS. Several peptides were chosen based on the intensity of the extracted ion chromatogram and their fragmentation pattern in SRM mode. Data for the four most abundant peptides are presented in Table 1, and an example of full-MS and MS/MS spectra of ATP7B 1056−1077 is shown in Figure 1. Affinity-purified, rabbit polyclonal antibodies were generated against all four peptides. Because the sequence for ATP7B 1056 is highly hydrophobic in the N-terminal region (the first five amino acids in particular), the shortened sequence, ATP7B 1061−1077, was

DOI: 10.1021/acs.jproteome.6b00828
J. Proteome Res. 2017, 16, 862−871

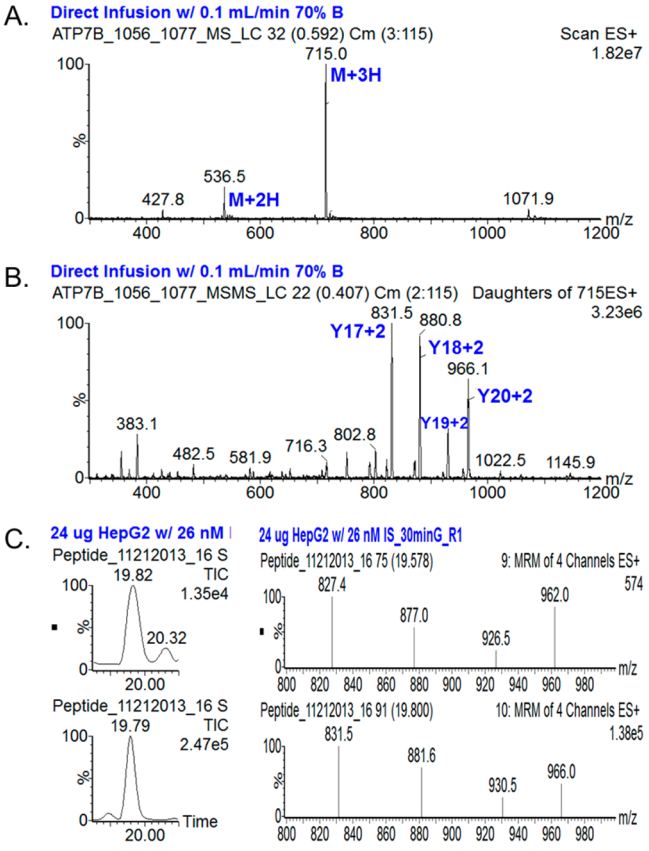


Figure 1. (A) Mass spectrum of heavy peptide 1056 for ATP7B and (B) tandem mass spectrum of the most abundant parent ion (M+3H). Abundant fragments are selected and optimized for SRM analysis. (C) Total ion chromatogram (TIC) and SRM spectra of endogenous (top) and heavy (bottom) peptide 1056 observed in HepG2 cell extract. Chromatographic peaks overlap and SRM patterns are compatible.

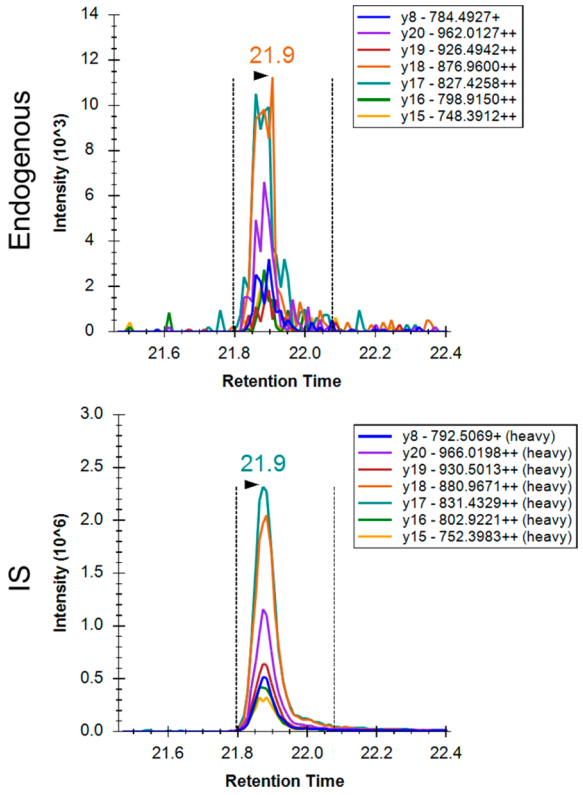


Figure 2. Extracted ion chromatograms for ATP7B 1056 peptide after peptide capture in normal PBMC. Top panel is a signature peptide found in the PBMC. Bottom panel is the isotopically labeled internal standard. Chromatographic peaks overlap, and SRM patterns are comparable. Transition labels refer to the precursor charge, fragment ion, fragment *m/z*, and fragment charge state.

used as a target for polyclonal antibody generation. While polyclonal antibodies for ATP7B 301 and 887 allowed very weak recovery of the peptides, ATP7B 325 and 1056 peptides showed recovery efficiencies ranging from ~50 to 70%. Of these two peptides, the ATP7B 1056 peptide was pursued further as a target peptide to quantify ATP7B in subsequent human samples because: (i) there was no background signal resulting from carrier peptides copurified with the antibodies[49,50,66] and (ii) the most common mutation, p.H1069Q, occurs in this peptide, taking advantage of absence of this peptide in WD patient with p.H1069Q.

Quantification of the Target Peptide by Immunoaffinity Peptide Enrichment and LC–MS/MS

While ATP7B is highly abundant in several tissues including liver, kidney, and placenta,[67] it is known to present at very low abundance in white blood cells, and it has also been observed in blood including lymphocytes and erythrocytes (gpmdb.thegpm.org). Thus ATP7B 1056 peptide was first analyzed in peripheral blood mononuclear cells (PBMCs) to see if we could identify the peptide. Isolated PBMCs were lysed and digested by trypsin, and the ATP7B 1056 immuno-SRM assay was used to enrich the peptide upstream of LC–SRM. A chromatogram of the sample eluate showing the ATP7B 1056 peptide identified in PBMC is shown in Figure 2. We next tested the feasibility of measuring ATP7B 1056 peptide in DBS. These results show the feasibility of developing an assay in DBS; we then characterized and assessed the assay for use in DBS samples.

Assay Assessment

The linearity, imprecision, and stability of the assay was assessed by following fit-for-purpose guidelines (detailed in the Experimental Procedures section).[68] The linear dynamic range was determined by generating a five-point response curve using synthetic standard peptides. Three DBS samples for each concentration level were prepared to account for any variation in protein extraction from the DBS card. These samples were analyzed in the order of increasing concentration with one blank injection between different sample concentrations. The assay showed a linear response (r^2 = 0.99) for all peptide amounts tested, spanning the peptide concentration of 27 to 16 765 pmol/L (0.7 to 417 femtomoles) (Figure 3). Results with a signal-to-noise ratio (S/N) < 10 were considered unreliable data. The low limit of quantification (LOQ) was estimated to be ~27 pmol/L based on the lowest concentration of the response curve (27 pmol/L) and patient sample no. 1 (29.5 pmol/L). The average CV was <12% for the three replicates of DBS samples prepared and analyzed at each concentration level. The linear response with the high reproducibility shows constant protein recovery and protein digestion efficiency for the target peptide. We assessed intra- and inter-assay imprecision by the LC–SRM analysis of complete process triplicates prepared from a healthy subject and a pooled patient sample and analyzed on three independent days. The results are summarized in Table 2.

DOI: 10.1021/acs.jproteome.6b00828
J. Proteome Res. 2017, 16, 862–871

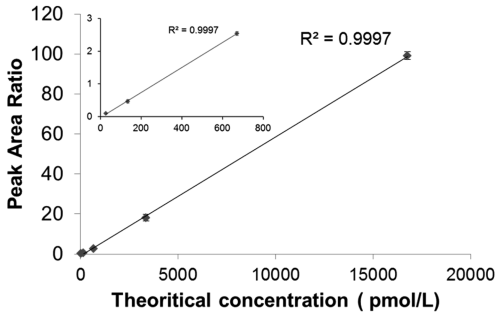


Figure 3. Response curve for ATP7B 1056 peptide. Curves are plotted for the sum of all transitions. The inset plot shows more detail of the lower end of the concentration range. Error bars are the standard deviation of three process replicates.

Table 2. Intra-Assay and Inter-Assay Imprecision of Immuno-SRM Assay for ATP7B 1056 Peptide[a]

sample	intra-assay, %	inter-assay, %
normal control	8.42	2.9
WD patient	NA	NA

[a]NA, not applicable.

Intra- and inter-assay imprecision from the normal control were 8.4% CV and 2.9% CV, respectively, suggesting the high reproducibility of both sample preparation and the method of analysis. The assay imprecision from the pooled WD patients was not calculated because most peaks observed were below the LOQ. The stability data for the ATP7B peptide is presented in Figure 4. The ATP7B peptide was stable for at least 7 days in DBS at RT and −20 °C.

Evaluation of ATP7B as a Marker for Early Screening of WD

To determine if the chosen target peptide could be used as a screening marker for WD, a total of 25 DBS samples from 12 controls and 13 confirmed WD patients were analyzed in a blinded fashion for ATP7B. Representative SRM traces

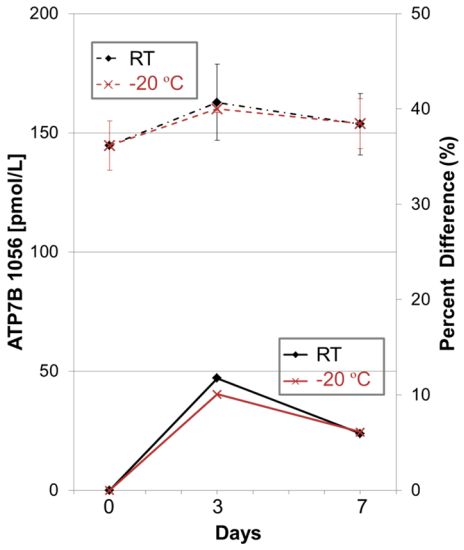


Figure 4. Stability of ATP7B 1056 peptide in normal control DBS at room temperature and −20 °C for 0, 3, and 7 days. The data represent the average of three replicates. Dashed and solid lines represent ATP7B concentrations and percent difference, respectively. Error bars are the standard deviation of three process replicates.

obtained from a healthy control and a WD patient are presented in Figure 5. The results are summarized in Table 3

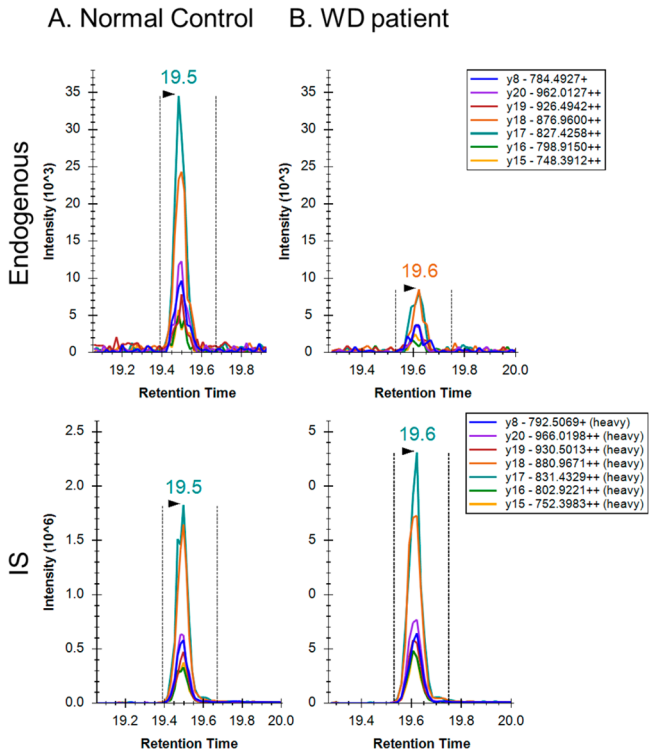


Figure 5. Extracted ion chromatograms for ATP7B 1056 peptide after peptide capture in DBS from (A) normal control and (B) WD patient. Top panel is a signature peptide found in DBS. Bottom panel is the isotopically labeled internal standard. Chromatographic peaks overlap and SRM patterns are comparable. Transition labels refer to the precursor charge, fragment ion, fragment m/z, and fragment charge state.

and Figure 6. As shown, the assay readily distinguished affected patients from controls ($p < 0.0001$). While we were able to reliably detect endogenous ATP7B ranging from 105 to 708 pmol/L in normal controls, the analyte response from WD patients was either not detected or below 29.5 pmol/L, except for one case, WD08. Of note, there was no ATP7B 1056 peptide detected in either of the WD patients who carried p.H1069Q mutation, as predicted. Case 8 presented with presumably alcoholic liver cirrhosis at the age of 56 years with a history of chronic jaundice, ascites, and hepatosplenomegaly. The copper content in the liver biopsy was marginally elevated at 116 μg/g dry weight tissue (control <35 μg/g), which prompted a genetic test for *ATP7B* gene. She was found to carry one known pathogenic variant, p.Thr974Met, and one variant of unknown significance, p.Ser391Leu in trans. Her 24 h urine copper was normal at 17 μg/24 h, serum CP was within the normal range, and no Kayser−Fleischer ring was detected in her eyes. Given her clinical and biochemical evaluations, she was suspected to be a presumtive carrier for Wilson's Disease, and the VUS was predicted to be benign in nature. Her mildly elevated copper content in the liver tissue was considered most likely secondary to long-standing liver disease.

The levels of ATP7B 1056 peptide were determined to see if the assay can distinguish between WD patients and proven carriers. Because of the high prevalence of carriers, it is important that a screening test should be able to limit or avoid

DOI: 10.1021/acs.jproteome.6b00828
J. Proteome Res. 2017, 16, 862−871

Table 3. ATP7B 1056 Peptide Concentration in 13 DBS Samples from WD Patients

sample	ATP7B 1056 (pmol/L)	mutation
WD01	29.5	p.R778W and p.T977M
WD02	ND[a]	p.H1069Q and p.R1319*
WD03	ND	p.H1069Q and p.R1319*
WD04	ND	not available at this time
WD05	ND	p.R778L homozygote
WD06	ND	p.C2304_2305insc and p.L1083F
WD07	NA[b]	p.R778L and p.A874 V
WD08	244.8	p.Thr974Met and p.Ser391Leu
WD09	ND	p.R778G and p.K175S_fs/p.Q260P_fs
WD10	ND	p.R778G and p.K175S_fs/p.Q260P_fs
WD11	ND	p.R778L and p.E1064A
WD12	ND	p.R778L and p.E1064A
WD13	ND	p.H1069Q and p.Y1331Tfs*61
controls (N = 12)	320.9 ± 147.4	

[a]ND, not detected. [b]NA, not applicable due to S/N < 10.

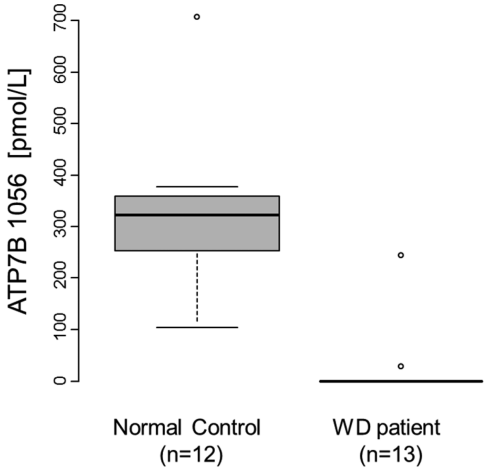


Figure 6. Distribution of the levels of ATP7B in DBS from 13 WD patients and 12 normal controls. The bold black line indicates the median, the inner quartiles are represented by boxes, and the whiskers show 95% of the data.

carrier detections. A set of DBS specimens used for this test included a WD patient, two proven carriers (the WD patient's mother and father), and two age-matched normal controls. These DBS had been stored at −20 °C for ∼15 years. The results are shown in Table 4. ATP7B 1056 peptide in the WD patient was markedly reduced compared with the two carriers and two age-matched controls. The levels of ATP7B 1056 peptide did not differ between the carriers and age-matched controls. Of note, the levels of ATP7B from the WD patient and the four controls were in the range of the WD patients and the control groups tested in this study, indicating that the protein in DBS could be stable for many years.

Table 4. ATP7B 1056 Peptide Concentration in 15 year old DBS

sample	ATP7B 1056 (pmol/L)
WD patient	60.3
Carrier 1 (mother)	270.9
Carrier 2 (father)	251.4
age-matched NC 1	369.5
age-matched NC 2	452.6

DISCUSSION

WD is a progressive and fatal disorder that is treatable, where early detection can make a significant impact on disease outcome and even be life-saving. However, there is currently no suitable marker and method available for population screening. In this study, we: (i) propose ATP7B as a potential marker to screen WD, taking advantage of an absence or decrease in the amount of the ATP7B protein in most WD patients; (ii) provide a sensitive immuno-SRM assay for the quantification of ATP7B in DBS, demonstrating the feasibility of DBS/immuno-SRM formats for screening congenital disorders lacking marker proteins; and (iii) suggest our approach can be further applied to aid diagnostics in conjunction with clinical and other biochemical test results.

We were able to reliably detect endogenous ATP7B in DBS from normal controls in the range of 105−708 pmol/L. To our knowledge, the detection and quantification of ATP7B protein in DBS has never been achieved with any method before. In 12 out of 13WD patients, the amount of the deficient ATP7B was below the control range, suggesting the feasibility of the use of ATP7B as a potential marker for WD. This approach is unique as most studies are looking for accumulated metabolites or markers, whereas this assay directly analyzes the affected protein. Although the number of samples tested in this study is quite limited, the results on the patients carrying the two most common mutations (p.R778L and p.H1069Q) are promising and demonstrate the feasibility of the immuno-SRM approach for mass screening.

The result on case 8 highlights the use of this assay for those patients with ambiguous genetic or biochemical test results, which is not an uncommon situation in the clinic. The variant of uncertain significance is not uncommon in ATP7B gene, while there is no definite diagnostic test available. The diagnosis for WD could be challenging, especially with those ambiguous or borderline results that could potentially lead to unnecessary treatment. Our result seems very promising in aiding the appropriate and accurate diagnosis in conjunction with other genetic and biochemical test results, although further studies on many clinical samples are necessary.

While the data presented in this study indicate the possible use of ATP7B in DBS for screening WD, we acknowledge that the findings are preliminary. Larger studies including both controls with proven carriers and patient samples with a broad mutational spectrum will be required to determine more

DOI: 10.1021/acs.jproteome.6b00828
J. Proteome Res. 2017, 16, 862−871

accurate reference, disease ranges, and cutoff. In addition, patient samples tested in this study are limited to children or adults due to the difficulty of identifying newborn samples from affected patients. Although we expect no significant age dependence of its abundance, the effect of age on the level of ATP7B protein (in particular for newborns) is not known. As with all NBS assays, the implementation of this assay will need to be tested in the newborns or infants on whom the testing would be carried out.

As anticipated, there was no detectable ATP7B 1056 peptide in either of the WD patients who carried the p.H1069Q mutation that occurs within this peptide. However, it can be argued that the absence of this peptide could be due to sequence variations (mutations/polymorphisms) from presumed healthy subjects. This could be resolved by monitoring multiple peptides for each target protein, which will help ensure that negative results are truly negative. The reduced/absence of multiple peptides in a SRM assay could increase the confidence of a negative result. This applies to the quantification of any protein to prevent underestimation due to single nucleotide polymorphisms and posttranslational modifications.

Becaue of the nature of the individual variants, measurement of a target protein, ATP7B, may not be sensitive enough to identify all affected individuals. For example, some patients with mutations that affect protein function/structure but not quantity may not be detected. One approach to address this limitation is the use of additional/secondary markers such as CP. When the primary marker shows ambiguous or undetermined result, the application of secondary markers can help improve both the sensitivity and specificity of the assay. It is important to note that the ability to measure multiple analytes in the same analysis would not require additional sample process or sample collection.

DBS offers many advantages such as a less invasive sampling, a simpler storage, and an easier transfer. However, there are several variables that may affect how uniformly blood and analytes spread across the filter paper, influencing precision in quantitative analysis. These include the hematocrit values, blood volume spotted, and chromatographic effects. There are contradicting reports in the literature as to whether these variables have a direct impact on the precision of the analytical result.[69,70] Note that in our proof-of-principle study we chose to reduce the effects of these variables by spotting the same volume of blood for all DBS samples used in experiments in this study and using nearly the entire blood spots for analysis.

The performance characteristics of our immuno-SRM assay were found to be compatible with expectations for clinical sample analysis. The inter-assay and intra-assay CV of ATP7B assay using DBS from a healthy control specimen were <10%, demonstrating that the assay is precise. The response curve was linear, with an R^2 of 0.99 and the dynamic range from 27 to 16 765 pmol/L. The use of immuno-SRM also offers other compelling advantages with respect to NBS. These advantages include: (i) this assay can be multiplexed without loss of specificity and sensitivity, enabling simultaneous analysis of multiple proteins from a single sample and a single injection, permitting greater statistical power to be achieved and robust cross-correlations to be made and (ii) screening programs already have been using MS/MS technology, in particular, SRM.

DBS is an attractive alternative to the collection of plasma or serum, particularly for NBS. Analytes are known to be more stable in DBS compared with those in plasma, blood, or other solutions,[71] likely because of the dehydration of the sample on the card and consequent minimization of chemical and enzymatic hydrolysis of the analytes.[71] Note that in our bottom-up approach to quantify the protein we focused on the integrity of the targeted peptide that serves as surrogates for the protein and not the intact protein itself. Our stability test showed that the ATP7B peptide is fairly stable in DBS for a week at room temperature and −20 °C. Additionally, results from the ATP7B peptide from DBS stored at −20 °C for ∼15 years were comparable to fresh samples, suggesting good long-term stability. Our data strongly suggest that peptides in DBS may be stable, opening up the possibility of application of immuno-SRM to NBS for testing for other genetic conditions such as primary immunodeficiencies or cystinosis.

While screening programs already utilize MS/MS technology for small molecules, immuno-SRM for quantification of proteins is a novel platform in the clinical and NBS laboratories. The method for measuring proteins uses quite different procedures from measuring small metabolites. The assay requires a relatively large number of steps before LC−MS/MS: (i) proteolytic digestion of proteins in the DBS; (ii) immunoaffinity enrichment of target peptides; and (iii) MS/MS coupled to liquid chromatography. Although this is a relatively complex assay format, the implementation of robotic sample preparation for trypsin digestion and peptide enrichment and a robust chromatography configuration[45] should enable this technology to advance into routine clinical analysis for thousands of samples.

A limitation for setting up an immuno-SRM assay may be the time required and likelihood of success in generating an antipeptide antibody.[66] Monoclonal antibodies are preferable for clinical or screening assays because they provide a renewable resource for long-term supply with acceptable consistency and reproducibility. Some monoclonal antibodies outperform the polyclonal antibodies with increased recovery efficiency and sensitivity of the immuno-SRM assay.

■ CONCLUSIONS

We developed a novel, sensitive immunoaffinity LC−MS/MS assay for quantitative measurement of ATP7B levels in DBS. This study demonstrates the feasibility of the use of immuno-SRM to quantify ATP7B in DBS to screen for WD. To the best of our knowledge, this is the first published report of employing immuno-SRM strategy for measuring a clinically important, low-abundance protein in DBS. The described method opens up future opportunities for the analysis of other protein markers in DBS for many other life-threatening congenital disorders that are currently not a part of the NBS.

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The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the patients and their parents for their generous blood donations for this study. S.J. and S.H. were funded by NIH R21HD069890-01A1, and J.R.W, L.Z., and A.G.P were

DOI: 10.1021/acs.jproteome.6b00828
J. Proteome Res. 2017, 16, 862−871

supported by the National Cancer Institute of the National Institutes of Health (NIH) Clinical Proteomics Tumor Analysis Consortium Initiative (U24CA160034).

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DOI: 10.1021/acs.jproteome.6b00828
J. Proteome Res. 2017, 16, 862−871

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DOI: 10.1021/acs.jproteome.6b00828
J. Proteome Res. 2017, 16, 862−871

Gastroenterology 2021;160:2367–2382

CLINICAL—LIVER

Direct Measurement of ATP7B Peptides Is Highly Effective in the Diagnosis of Wilson Disease

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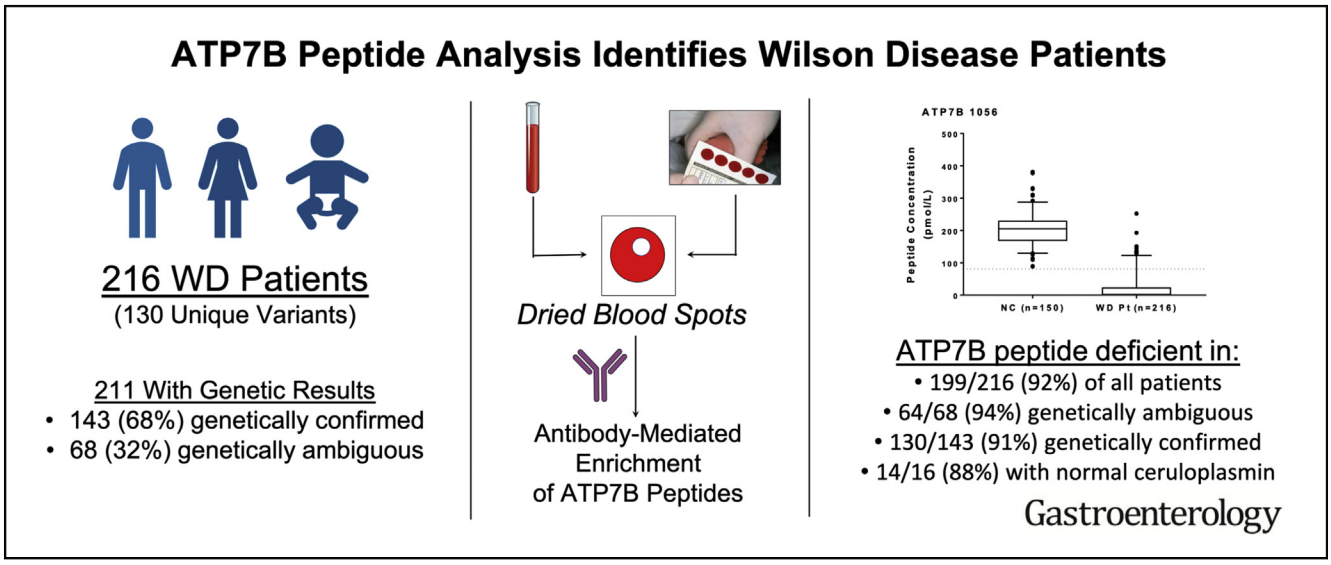

See Covering the Cover synopsis on page 2231; See editorial on page 2249.

BACKGROUND & AIMS: Both existing clinical criteria and genetic testing have significant limitations for the diagnosis of Wilson disease (WD), often creating ambiguities in patient identification and leading to delayed diagnosis and ineffective management. ATP7B protein concentration, indicated by direct measurement of surrogate peptides from patient dried blood spot samples, could provide primary evidence of WD. ATP7B concentrations were measured in patient samples from diverse backgrounds, diagnostic potential is determined, and results are compared with biochemical and genetic results from individual patients. **METHODS:** Two hundred and sixty-four samples from biorepositories at 3 international and 2 domestic academic centers and 150 normal controls were obtained after Institutional Review Board approval. Genetically or clinically confirmed WD patients with a Leipzig score >3 and obligate heterozygote (carriers) from affected family members were included. ATP7B peptide measurements were made by immunoaffinity enrichment mass spectrometry. **RESULTS:** Two ATP7B peptides were used to measure ATP7B protein concentration. Receiver operating characteristics curve analysis generates an area under the curve of 0.98. ATP7B peptide analysis of the sequence ATP7B 887 was found to have a sensitivity of 91.2%, specificity of 98.1%, positive predictive value of 98.0%, and a negative predictive value of 91.5%. In patients with normal ceruloplasmin concentrations (>20 mg/dL), 14 of 16 (87.5%) were ATP7B-deficient. In patients without clear genetic results, 94% were ATP7B-deficient. **CONCLUSIONS:** Quantification of ATP7B peptide effectively identified WD patients in 92.1% of presented cases and reduced ambiguities resulting from ceruloplasmin and genetic analysis. Clarity is brought to patients with ambiguous genetic results, significantly aiding in noninvasive diagnosis. A proposed diagnostic score and algorithm incorporating ATP7B peptide concentrations can be rapidly diagnostic and supplemental to current Leipzig scoring systems.

Keywords: Wilson disease; Leipzig Score; Immuno-SRM; ATP7B.

Wilson disease (WD) is named for Dr Samuel Alexander Kinnier Wilson, who first described the disorder in his 1912 doctoral thesis. Since then, treatments have been developed for WD and, importantly, WD has become a preventable disease. WD is an autosomal recessive disorder of copper metabolism due to mutations in the *ATP7B* gene that encodes copper-transporting P-type ATPase (EC # 7.2.2.8).[1–3] WD has an estimated prevalence of 1:30,000 and a carrier frequency of 1:90 with regional variation.[4,5] Although guidelines for the diagnosis of WD have been developed,[6,7] patient identification remains a challenge resulting in delayed diagnosis and development of irreversible severe complications, such as permanent brain or liver damage, which render treatments ineffective.[5]

The key features of WD are liver disease, neuropsychiatric abnormalities, and Kayser-Fleischer (KF) rings. The presence of KF rings with neurologic manifestation and/or low serum ceruloplasmin (Cp) is considered enough to establish WD diagnosis. However, most cases require a combination of clinical symptoms and laboratory evaluations.[8] Currently, no single test permits de novo WD diagnosis in every potential patient.[7] Serum Cp is decreased in neurologic WD, but can be in the low-normal range in up to 50% of adult patients with active liver disease[9,10] and the positive predictive value of serum Cp for diagnosis of WD is poor.[9,11,12] In children with WD, 15%–36% had Cp in the normal range.[13] Serum Cp alone is not sufficient to diagnose or exclude WD. A diagnostic score (Leipzig score, 2003) was proposed to guide clinical diagnosis and has been adopted in the clinical practice guidelines for the European Association for the Study of the Liver.[14] Although recent advances in clinical molecular diagnosis have greatly improved the accuracy of WD diagnosis in affected patients and their siblings, traditional Sanger sequencing cannot detect large deletion or duplications. In addition, there are many single nucleotide polymorphisms and variants of unknown significance (VUS) in the ATP7B gene. Interpretation of genetic sequencing results, particularly in the presence of only one identified mutation or VUS, create ambiguity in WD patient identification.

We evaluated the direct measurement of ATP7B protein from WD patient dried blood spots (DBS), through surrogate ATP7B peptides, as a diagnostic tool.[15] As reported in our previous studies for multiple primary immunodeficiency conditions, peptide measurements are made using immunoaffinity enrichment coupled to selected reaction monitoring (immuno-SRM) mass spectrometry.[16,17] This method uses antipeptide antibodies to concentrate and quantify extremely-low-concentration peptide targets from complex matrices, including DBS.[15–21] Analysis of ATP7B concentration in DBS from WD patients with a broad range of genetic backgrounds shows that ATP7B peptide levels are greatly reduced. Analysis of ATP7B protein concentration can identify WD with high diagnostic accuracy.

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Identification of patients with Wilson disease remains a challenge, resulting in delayed diagnosis and development of irreversible severe complications, such as permanent brain or liver damage, which render treatments ineffective.

NEW FINDING

Directly measuring ATP7B from dried blood spots of patients with Wilson disease with diverse genetic backgrounds showed ATP7B peptide concentrations have a high diagnostic potential.

LIMITATIONS

The study is limited in that data are mostly obtained from White patients. Sensitivity and specificity may be variable geographically.

IMPACT

ATP7B peptide analysis identifies most patients with Wilson disease, reducing ambiguities resulting from genetic analysis, and is expected to advance the use of proteomics, a promising but largely clinically untapped technology.

Methods

Dried Blood Spot Samples

This protocol was approved by the Institutional Review Board of Seattle Children's Hospital (SCH) and each of the participating institutes. All subjects gave written informed consent. Patient and carrier samples were provided by SCH, Seattle, WA; Medical University of Vienna, Austria; Medical University Innsbruck, Austria; University of Medicine and Pharmacy, Iuliu Hatieganu, Cluj-Napoca, Romania; Wilhelmina Children's Hospital, University Medical Center, Utrecht, The Netherlands; Yale University, New Haven, CT; University of Heidelberg, Germany; and Asan Medical Center, Seoul, South Korea. Samples were prepared either by fingerstick or by pipetting 70 μL of blood (per 12-mm spot) onto filter paper cards (903 ProteinSaver; Whatman, Piscataway, NJ). The samples were then dried overnight at room temperature, delivered to SCH, and stored at −80°C until use. One hundred and fifty normal control DBS samples (BioIVT, Westbury, NY) were analyzed to establish the normal reference range and cut-off.

Abbreviations used in this paper: **ACN, acetonitrile; AF, allele frequency; CHAPS, 3-([3-cholamidopropyl] dimethylammonio)-1-propanesulfonate; Cp, ceruloplasmin; CV, coefficient of variation; DBS, dried blood spot; FA, formic acid; immuno-SRM, immunoaffinity enrichment coupled to selected reaction monitoring; IS, internal standard; KF, Kayser-Fleischer; LC/MS, liquid chromatography/mass spectrometry; LLOD, lower limits of detection; LLOQ, lower limits of quantification; mAb, monoclonal antibody; SCH, Seattle Children's Hospital; VCI, variant with conflicting interpretations; VUS, variants of unknown significance; WD, Wilson disease; WT, wild-type.**

Most current article

0016-5085
https://doi.org/10.1053/j.gastro.2021.02.052

Immunoaffinity Enrichment Coupled to Selected Reaction Monitoring Reagents

Triton X-100 (T9284, 100 mL) and Ammonium bicarbonate (A6141-25G) were purchased from Sigma-Aldrich (St. Louis, MO). TPCK-treated Worthington trypsin (LS003740) was purchased from Worthington (Lakewood, NJ). 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate (CHAPS) (no. 28300), acetonitrile (ACN) (liquid chromatography/mass spectrometry [LC/MS] grade), acetic acid (LC/MS grade), water (Optima LC/MS grade), formic acid (FA) (Optima LC/MS grade), 1× phosphate-buffered saline (no. 10010-023), dithiothreitol (no. 20290), and 1M Tris-(hydroxymethyl)aminomethane (pH 8) (no. 15568-025) buffer were obtained from Fisher Scientific (Waltham, MA). Protein G–coated magnetic beads (Dynabeads, no. 10004D) were purchased from Invitrogen (Carlsbad, CA).

Isotope-labeled internal standard (IS) peptides were purchased from either Atlantic Peptides (Lewisburg, PA) or Life Technologies Corporation (Carlsbad, CA). IS peptides were >95% pure and incorporated heavy stable isotope-labeled (^{13}C and ^{15}N) C-terminal lysine (+8 Da) or arginine (+10 Da). IS stock solutions are stored frozen as 500× mixtures in 1× phosphate-buffered saline + 15% ACN + 0.1% FA + 0.03% CHAPS in H_2O and diluted to 1× immediately before use.

Selection of Signature Peptides and Antibody Production

Selection and production of ATP7B 1056 peptide and antibody has been described in previous reports.[15–17]

Using the same guidelines, ATP7B 887 was selected. Antibody production was performed by Excel Biopharm (San Francisco, CA), Pacific Immunology (Ramona, CA), and ExonBio (San Diego, CA). Enzyme-linked immunosorbent assays were performed, by the companies mentioned, in bleed samples after immunization and in supernatant samples after monoclonal antibody production. Selections were further confirmed at each step by the immuno-SRM method.[22]

Antibody Bead Reagent Production

Monoclonal antibody (mAb) beads were produced by overnight 4°C incubation of Protein G Dynabeads with mAb, as reported previously.[15–17]

Dried Blood Spot Extraction, Trypsin Digestion, and Immunoaffinity Enrichment

Protein extraction and tryptic digestion were performed as reported previously, with slight modifications.[16] One 6.35-mm diameter DBS punch was placed into 96-well plates (ThermoScientific, Chicago, IL), covered with an adhesive seal (Genesee Scientific, San Diego, CA), and extracted using 0.1% Triton X-100 in 50 mM ammonium bicarbonate (200 μL) with dithiothreitol (final concentration 0.2M). After 30 minutes incubation at 37°C with agitation, trypsin (37.5 μg) was added and incubated for 2 hours at 37°C. For enrichment, 10 μL of 1M TRIS (pH 8) and 10 μL of 1× ATP7B IS mix were added (final concentrations = 0.25 nM). Extracted supernatant (200 μL) was transferred to a new plate and incubated with 2.5 μL of each mAb bead overnight at 4°C with agitation.

After incubation, mAb beads were isolated using a 96-well magnetic plate (Alpaqua Magnum EX, Beverly, MA), washed twice with 1× phosphate-buffered saline + 0.01% CHAPS, and magnetically isolated. Peptides were eluted with 30 μL of H_2O with 5% acetic acid and 3% ACN for 5 minutes and transferred to a new 96-well plate for analysis (Abegene, Chicago, IL).

Liquid Chromatography-Mass Spectrometry

LC-MS/MS was performed using a Waters Xevo TQ-XS with Ionkey source and dual M-Class chromatography pumps (Milford, MA). Chromatographic solvents were A: H_2O + 0.1% FA and B: ACN + 0.1% FA. Peptides are loaded onto an M-Class Trap Symmetry C18 column (300 μM x 25 mm, 100A, 5 μM) for 3 minutes with a constant flow of 98:2 A:B at 20 μL/min. After loading, the flow is reversed and peptides are separated using a 150 μM × 100 mm BEH C18 ionkey (130 Å, 1.7 μM). The gradients used are summarized in Supplementary Table 1 and were reported previously.[16] Precursor mass, fragment mass, and collision energy were tuned to optimize the generated signal (Supplementary Table 2). Representative chromatograms for both ATP7B 887 and ATP7B 1056 peptides were shown in Supplementary Figure 1*A–D*.

Concentration Calculation and Data Analysis

Selected reaction monitoring data captured in the MS were analyzed using Skyline (MacCoss Lab, Seattle, WA, https://skyline.ms/project/home/begin.view).[23] Specificity was assured by monitoring retention times and relative transition intensities of endogenous and IS peptides. Concentrations of endogenous signature peptides were calculated using endogenous/IS signal ratio. DBS spots are assumed to contain 70 μL of evenly distributed whole blood. The volume of blood in the punch area is calculated as 17.5 μL. Concentrations are calculated from blood volume, ratio, and IS concentration. Statistical analyses and receiver operating characteristics curves were generated using GraphPad Prism (San Diego, CA).

Method Performance Assessment

Response curves were generated for each peptide to establish assay linearity and determine the lower limits of detection (LLOD) and quantification (LLOQ). Seven concentrations (0×, 0.05×, 0.1×, 0.5×, 1×, 5×, and 50×) of IS were added across the set of pooled digest samples in triplicate. LLOD was calculated as: $LLOD = mean_{blank} + 3 \times SD_{Low}$ ($mean_{blank}$: mean signal from a triplicate blank injection, SD_{Low}: SD of IS injection below the LLOQ). LLOQ is the lowest concentration with a coefficient of variation (CV) of <20%. The linearity curves of ATP7B 1056 and ATP7B 887 are shown in Supplementary Figure 1*E* and *F*.

The assay precision and accuracy were evaluated by within-day (intra-) and between-day (inter-) assay CV, respectively. The intra- and inter-assay CV were determined using 5 replicates of an identical pooled blood DBS sample each day over a course of 5 days (Table 1).

Internal Sample Quality Control

Measurement of endogenous peptides unrelated to WD and, therefore, assumed to be present at normal concentrations, was used as an internal quality control to monitor the successful

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Table 1. Analytical and Diagnostic Performance for ATP7B Peptides

Peptide	LLOD, *pmol/L*	LLOQ, *pmol/L*	Intra-assay CV, %	Inter-assay CV, %	PPV, %	NPV, %	AUC
ATP7B 1056	3.81	71.43	12.9	15.3	96.1	91.3	0.98
ATP7B 887	2.17	7.14	11.0	13.0	98.0	91.5	0.98

AUC, area under the curve; NPV, negative predictive value; PPV, positive predictive value.

extraction, digestion, and enrichment of target peptides. These peptides are ADA 93, representing adenosine deaminase, CD42 128, representing glycoprotein Ib, and IDUA 462, representing α-L-iduronidase.[16] A sample run was assumed to be of sufficient quality if the measured concentration of 2 of these 3 peptides was within 1.75 SD of the mean for the cohort (Supplementary Tables 3–5). Samples failing these acceptance requirements were repeated. With confirmatory test failure, the DBS was removed from the sample cohort due to inadequate matrix and an additional sample was requested.

Results

Characteristics of Patient Cohort

WD DBS samples were obtained from 198 White and 18 Far Eastern Asian patients with WD (114 male and 102 female) and 48 obligate carriers (Supplementary Tables 3 and 4). All carriers are obligate heterozygote from the family members of index case patients with 2 confirmed variants. Patient age range spanned from 2 months to 73 years. For the purpose of stability validation, samples from 11 patients and 1 healthy normal subject were collected from both fresh and blood samples stored up to 11 years prior (Supplementary Table 7). Clinical information including Cp concentrations, Leipzig scores, liver copper content, presence of KF rings, initial presentation, and presence of cirrhosis are presented where available (Supplementary Table 3). Control DBS samples (n = 150) were obtained from healthy subjects ranging from 18 to 73 years of age.

An analysis of the specific variants in the sample set showed that the cohort contained 130 unique variants (Figure 1*B*, Supplementary Table 6), including 83 pathogenic or likely pathogenic variants, 43 VUS, 3 benign or likely benign variants, and 1 with conflicting interpretations (VCI). In affected patient samples, 143 exhibited only pathogenic or likely pathogenic mutations according to a public database (ClinVar, gnomAD [Genome Aggregation Database]), including 31 patients homozygous for p.H1069Q, the most common variant in WD patients (Figure 1*A*, Supplementary Table 3). In addition, 20 patients were homozygotes for other variants. Seven patients exhibited 2 VUS (2 of them are homozygotes). In addition, 37 patients were compound heterozygous for 1 VUS and 1 pathogenic or likely pathogenic mutation. Eighteen patients had only 1 variant detected by Sanger sequencing. No second variant was detected. Three clinically suspected patients were compound heterozygotes, with 1 likely benign variants according to gnomAD and the second variant with likely pathogenic, pathogenic, and unknown, respectively.

Forty-eight samples from obligate carriers, all of them family members of affected patients, presented with 2 variants, had a single pathogenic variant and a single wild-type allele or a benign variant (Supplementary Table 4).

Surrogate Peptide Markers for ATP7B

The mean ± SD signature peptide concentration in normal control was 257.7 ± 57.5 pmol/L for ATP7B 887 (range, 136.4–447.0 pmol/L; 5^{th}–95^{th} percentile range, 165.0–359.6 pmol/L) and 203.0 ± 48.9 for ATP7B 1056 (range, 88.2–381.3 pmol/L; 5^{th}–95^{th} percentile range, 129.7–287.3 pmol/L). These cut-offs were set at –2.5 (114.0 pmol/L) and –2.5 SD (80.8 pmol/L) below the mean normal concentration for ATP7B 887 and ATP7B 1056, respectively.

Analytical Performance

The analytical figures of merit for immuno-SRM quantification of ATP7B peptides are given in Table 1. The LLODs of ATP7B quantification were determined to be 3.81 pmol/L and 2.17 pmol/L for ATP7B 1056 and ATP7B 887,

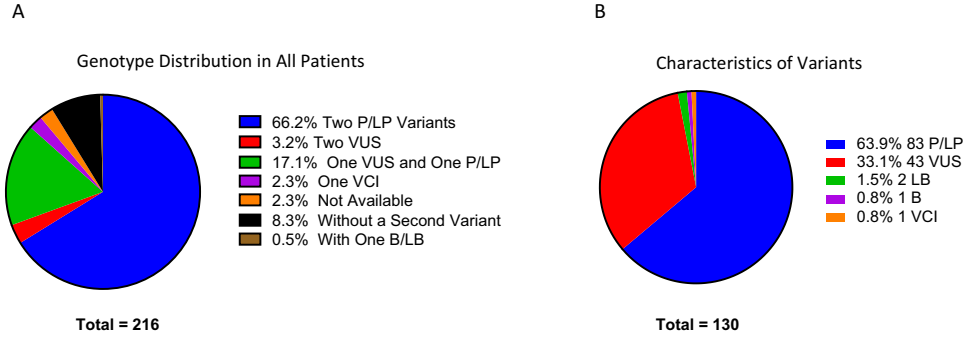


Figure 1. Patient cohort characteristics. Analysis of the genotypes of patients (*A*) and the characteristics of the variants present (*B*) show the diversity of variants and variant combinations present.

respectively. LLOQs were determined to be 71.43 pmol/L and 7.14 pmol/L. The intra-assay CVs were 12.9% and 11.0% for ATP7B 1056 and ATP7B 887. The inter-assay CVs were 15.3% and 13.0%, respectively.

ATP7B Concentration Measurements and Primary Diagnostic Performance

Signature peptide levels in patient DBS were below cut-off in 195 of 216 (90.3%) of samples for both ATP7B 1056 and ATP7B 887 (Figure 2*A* and *B*). There were 17 WD patients (7.9%) that had ATP7B level above the cut-off for both ATP7B peptides. There were 2 WD patients had only ATP7B 887 and 2 WD patients had only ATP7B 1056 levels above the cut-off. In all, 199 of 216 patients (92.1%) had at least 1 peptide below cut-off. In WD carriers, 8 of 48 (16.7%) and 4 of 48 (8.3%) samples were below diagnostic cut-offs for ATP7B 1056 and ATP7B 887, respectively. These patient samples generate potential false positives.

As a primary diagnostic, receiver operating characteristics curve analysis (Figure 2*C* and *D*) constructed from this DBS sample cohort found that both ATP7B 1056 and ATP7B 887 peptide analysis have an area under the curve of 0.98 (ATP7B 1056 [SE = 0.006; 95% confidence interval, 0.97–0.99; $P < .0001$] and ATP7B 887 [SE = 0.007; 95% confidence interval, 0.96–0.99; $P < .0001$]). ATP7B 887 analysis was found to have a sensitivity of 91.2%, specificity of 98.1%, positive predictive value of 98.0%, and a negative predictive value of 91.5%. ATP7B 1056 showed positive predictive value of 96.1% and negative predictive value of 91.3% (Table 1).

Effects of Common Variants

In the cohort of 216 patients with WD, a total of 130 variants were identified (Supplementary Table 6). Many common pathogenic variants, including p.H1069Q (allele frequency [AF], 0.103%), p.R778L (AF, 0.013%), p.M645R (AF, 0.047%), and p.E1064A (AF, 0.015%), were associated with either an undetectable or significantly reduced level of ATP7B (Figure 3*A*–*D*).

Variants Leading to Potential False Negatives

In this cohort, 17 of 216 patients (7.9%) have ATP7B concentrations above the cut-off for both signature peptides. The genetic information is summarized in Table 2. In these 17 WD patients, 13 variants were commonly involved and could contribute to normal levels of ATP7B. According to gnomAD (https://gnomad.broadinstitute.org), these variants are rare, with an AF <0.0089%; the remaining variant, p.M665I, has a VCI designation. Of note, the 4 variants above (p.R616W, p.G710S p.M769V, and p.R969Q) have been reported to show the ATP7B protein distribution similar to wild-type (WT) in an in vitro study (Figure 3*E* and *F*).[24]

ATP7B Analysis and Variant Pathogenicity

Of the 216 WD patients, 211 had genetic test results available (Figure 2*E*). One hundred and forty-three were genetically confirmed to be WD patients by being compound heterozygous or homozygous for known pathogenic or likely pathogenic mutations (Figure 1*A*). One hundred and thirty of these patients (91.0%) had concentrations of at least 1 signature peptide below established cut-offs (Figure 4*D*). Alternatively, 68 patients had ambiguous genetic test results preventing straightforward genetic identification. Sixty-four (94%) of these patients were deficient in ATP7B by peptide analysis (Figure 2*E*). Seven patients were compound heterozygous or homozygous for 2 VUS. All samples (100%) contained significant reductions in ATP7B peptides (Table 2). Thirty-seven patients were compound heterozygous for 1 VUS and 1 known pathogenic or likely pathogenic variant (Table 2, Figure 4*E*). ATP7B concentrations were below cut-off in 35 of 37 (94.6%) of these cases. One VCI, p.M665I, was found in 5 patients. In this case, 3 patients had peptide concentrations below established cut-offs and the remaining 2 were compound heterozygous p.G710S, known to cause false negatives. Three patients had known benign or likely benign in combination with known pathogenic or likely pathogenic mutations. Two are likely carriers with normal ATP7B, but 1 patient with a likely benign mutation in combination with a known pathogenic mutation had nondetectable ATP7B, indicating possible misannotation (#64). Finally, in 18 WD cases that have only 1 variant with no second mutation detected, their ATP7B peptide levels were all (100% of samples) reduced below the cut-off (Table 2, Figure 4*F*).

ATP7B Analysis and Ceruloplasmin Concentration

The 200 patients for whom Cp values were provided were stratified into the following 3 subgroups: 107 patients with Cp <10 mg/dL, 77 patients with Cp between 10 and 20 mg/dL, and 16 patients with Cp >20 mg/dL (Figure 4*A*–*C*). Within these groups, 101 of 107 (94.3%) with Cp <10 mg/dL, 70 of 77 (90.9%) with Cp between 10 and 20 mg/dL, and 14 of 16 (87.5%) with Cp >20 mg/dL had DBS ATP7B peptide concentrations below diagnostic cut-offs.

ATP7B Analysis and Other Clinical Indications

Where possible, clinical information including age, Leipzig scores, liver copper content, presence of KF rings, initial presentation, and presence of cirrhosis are presented (Supplementary Table 3). The exact number of treated patients and their regimens is unknown. No differences in ATP7B concentration were found among the patients based on any of these factors. Fifty-nine patients had hepatic copper measurements available (Figure 4*G*–*I*). Fifty-one patients (86.4%) had elevated liver copper (>250 μg/g), 46 of them (90.2%) had deficient ATP7B levels. Eight patient had liver copper that ranged from 25 to 248 μg/g, 7 of them with deficient ATP7B levels. Of note, 3 samples were received with suspicion of WD by elevated liver copper, but with no ATP7B variants identified, indicating they are not WD patients. As expected, these samples had normal ATP7B concentrations and were not contained within the final 216 WD patient cohort.

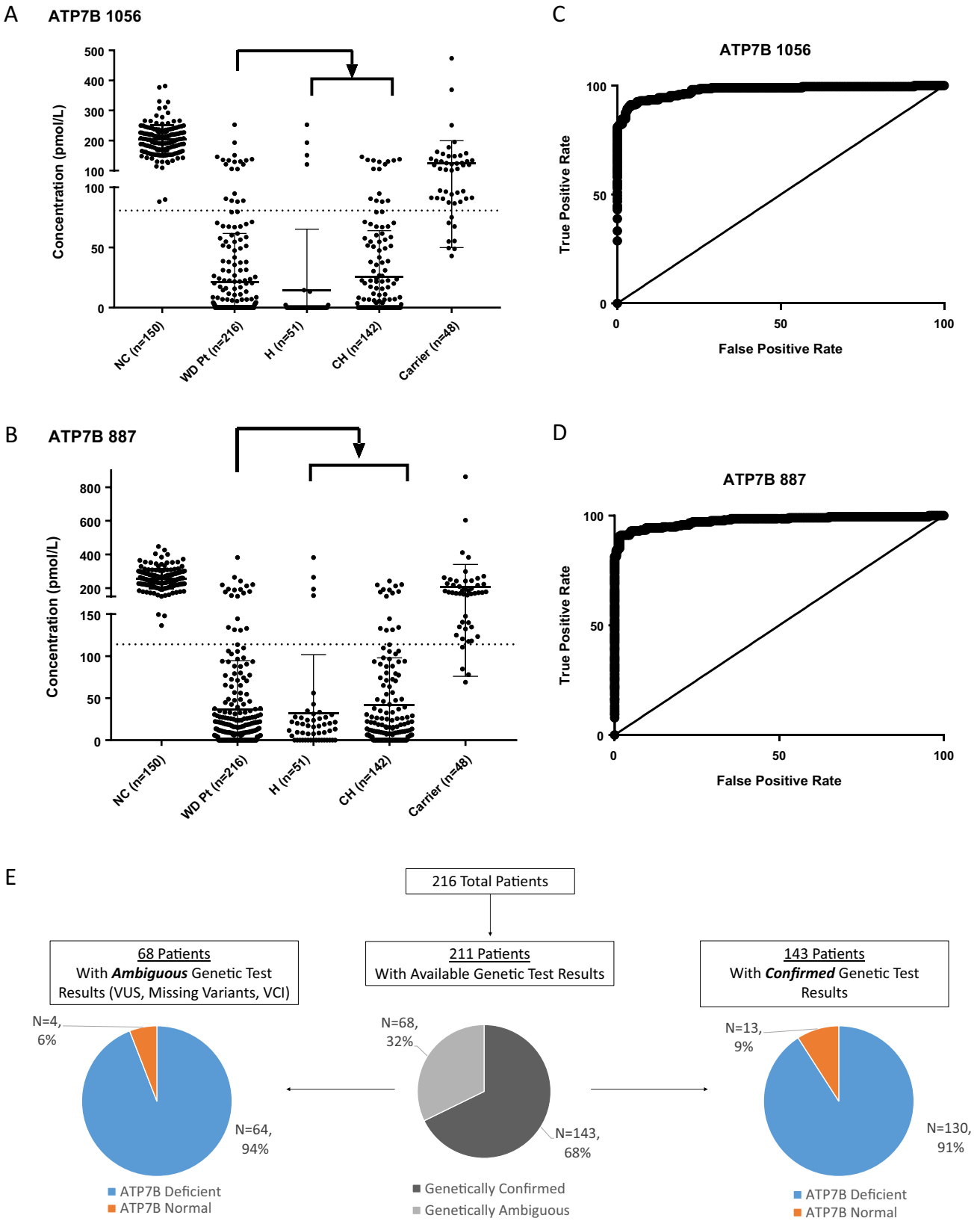


Figure 2. Diagnostic performance of ATP7B peptide analysis. Comparison of ATP7B peptide measurements for ATP7B 1056 (*A*) and ATP7B 887 (*B*) peptides in normal control patients (NC), patients, homozygotes (H), compound heterozygotes (CH), and carriers. *Dotted lines* represent diagnostic cut-offs for each peptide. Receiver operating characteristics curves show the diagnostic performance of ATP7B 1056 (*C*) and ATP7B 887 (*D*). WD patients with genetic test results are readily identified even in subgroups where genetic results are ambiguous (*E*).

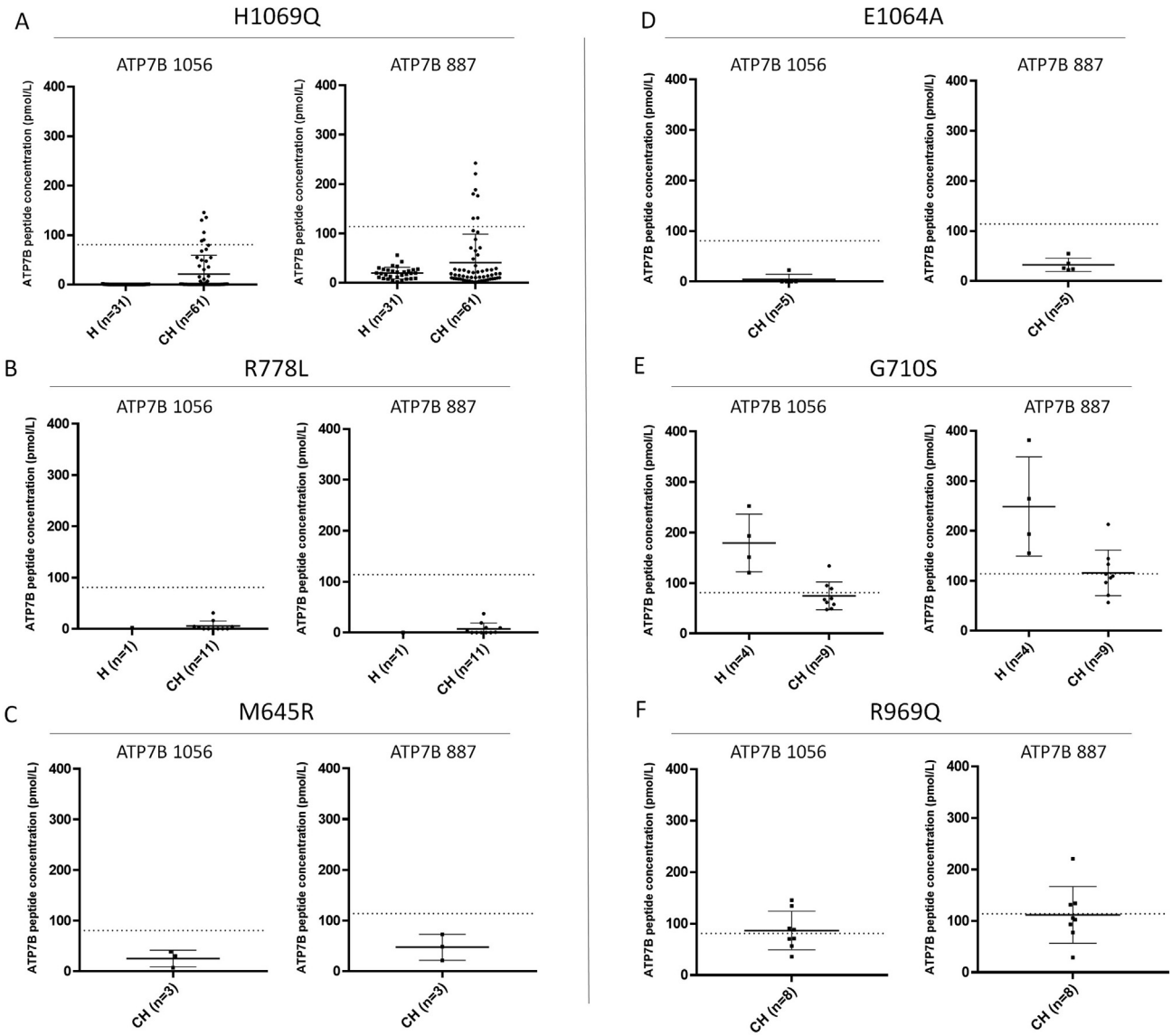


Figure 3. ATP7B peptide concentrations in patients with common variants are often reduced and variants causing false negatives are rare. Patients homozygous and heterozygous for p.H1069Q (*A*), R778L (*B*), M645R (*C*), and E1064A (*D*) are largely reduced. False negatives within the patient cohort are possible with the presence of specific variants, including G710S (*E*) and R969Q (*F*). Patients can have variable peptide concentrations depending on the second variant. CH, compound heterozygote; H, homozygote. *Dotted lines* represent peptide diagnostic cut-offs.

Discussion

This is the first large cohort study directly measuring ATP7B from DBS of WD patients with diverse genetic backgrounds. It showed ATP7B peptide concentrations have a high diagnostic potential. This test is a novel approach to WD testing and screening with a high sensitivity and specificity. It can be applied successfully in patients who do not present with clear clinical and laboratory criteria for WD as a first "second"-line test and expand the clinician's ability to noninvasively diagnose WD by reducing the need for liver biopsy. As the assay measures the ATP7B peptides produced in peripheral blood, liver synthetic dysfunction would have little or no effect on the concentration of ATP7B peptides in DBS, which is another advantage of the assay. In suspected cases, no single test is diagnostic, and a combination of laboratory tests and clinical investigation is required to establish the diagnosis. These include ophthalmologic testing for KF rings, Cp measurement, 24-hour urine copper measurement, liver biopsy to determine copper content, and *ATP7B* gene sequencing. In patients with hepatic WD, KF rings may be absent and Cp in the low to normal range can contribute to diagnostic ambiguity.

Genetic sequencing can give a definitive diagnosis when 2 known pathogenic variants are found. However, many mutations found in the *ATP7B* gene are VUS, VCI, or extremely rare. Now, more than 1300 variants in the ATP7B gene are listed in Varsome (varsome.com)[25]; 649 of them were pathogenic or likely pathogenic, and 692 of them were classified as VUS. Variant interpretation remains a challenge

Table 2. Selected Patients From Cohort

Patient/ gender/age	Variant 1	Annotation 1	Variant 2	Annotation 2	ATP7B 1056 (pmol/L)	ATP7B 887 pmol/L	CPL (mg/dL)	Leipzig score	Liver Copper (ug/g Tissue)	KF Ring	Presentation
False negative (n=17)											
110/F/17	p.G710A	Path	p.G710S	Path	88.9	133.0	14	7	-	NP	-
10/M/34	p.G710S	Path	p.G710S	Path	120.4	155.3	<9	8	-	Y	N
11/M/17	p.G710S	Path	p.G710S	Path	193.2	264.3	12	10	-	Y	B*
12/F/44*	p.G710S	Path	p.G710S	Path	151.1	193.6	9.1	11	164	Y	N
13/M/19	p.G710S	Path	p.G710S	Path	252.4	381.7	20.7	8	1243	Y	B
111/M/47	p.M665I	Conflicting	p.G710S	Path	133.9	213.0	15.5	7	324	NP	H
112/M/NA	p.M665I	Conflicting	p.G710S	Path	94.9	144.4	NA	6	324	NP	H
140/F/29	p.R616Q	Path	p.L1305P	Path	105.5	151.7	2	8	-	Y	B
149/F/17	p.H1069Q	Path	p.M769V	Likely Path	130.2	180.1	10	8	2047	NP	H
150/F/31	p.N41S /p.I1021V	Likely Path	p.M996T	Likely Path	129.1	218.6	4	6	>250	NP	H
153/F/37	p.H1069Q	Path	p.P1273L	Path	105.3	188.5	8	10	-	Y	N
154/F/18	p.M769H-fs	Path	p.P1273L	Path	121.4	170.4	5	10	-	Y	N
170/M/NA	p.H1069Q	Path	p.R969Q	Path	90.5	131.4	NA	4	-	-	-
124/M/17	p.Q7D-fs*14	VUS	p.H1069Q	Path	135.8	242.5	19	5	-	NP	H
171/F/9	p.H1069Q	Path	p.R969Q	Path	145.6	220.9	16	5	-	NP	H
176/M/NA	p.R616W	Path	p.R969Q	Path	134.2	134.2	25	7	-	NP	H
183/F/31	p.T977M	Path	p.T991A	VUS	137.9	191.7	12	3	-	Y	H
Patients with one pathogenic/likely pathogenic variant in combination with one VUS (n=37)											
124/M/17	p.H1069Q	Path	p.Q7D-fs*14	VUS	135.8	242.5	19	5	-	NP	H
125/M/9	p.H1069Q	Path	p.Q7D-fs*14	VUS	79.4	176.3	16	5	-	NP	H
131/F/13	p.H1069Q	Path	p.I1007T-fs	VUS	ND	5.4	14	5	-	Y	H
55/M/59	p.H1069Q	Path	arr[GRCh37] 13q14.3 (52541594_52548863)x1	VUS	ND	16.1	13	5	-	Y	H
87/F/19	p.H1069Q	Path	p.D1447G-fs	VUS	67.5	130.7	13	6	-	NP	H
159/M/40	p.R827W	Likely Path	p.R1320T	VUS	58.7	93.4	13	7	-	NP	N
133/M/18	p.H1069Q	Path	p.K1028S-fs	VUS	ND	3.9	12	7	842	NP	H*
183/F/31	p.T977M	Path	p.T991A	VUS	137.9	191.7	12	3	-	Y	H
164/F/NA	p.H1069Q	Path	p.R778P	VUS	ND	ND	11	9	1332	NP	H

Table 2. Continued

Patient/ gender/age	Variant 1	Annotation 1	Variant 2	Annotation 2	ATP7B 1056 (pmol/L)	ATP7B 887 pmol/L	CPL (mg/dL)	Leipzig score	Liver Copper (ug/g Tissue)	KF Ring	Presentation
118/M/6	p.H1069Q	Path	p.G1011X	VUS	ND	10.0	10	6	-	NP	H
157/F/8	p.R1041W	Likely Path	p.D765Y	VUS	6.8	20.7	10	8	1211	NP	H
163/M/2 mo	p.R778L	Path	p.V1106I	VUS	4.0	9.7	10	-	-	NP	A
182/M/40	p.T850I	Likely Path	p.G515S	VUS	11.6	13.4	10	6	-	Y	B
67/F/17	c.2865+1G>A	Path	p.Ser135*	VUS	ND	ND	<13	9	-	NP	H
167/F/19	p.H1069Q	Path	p.R919L	VUS	3.6	22.1	9	10	-	Y	N
95/M/53	p.H1069Q	Path	p.F1343dup	VUS	ND	ND	9	3	-	Y	H
108/M/15	p.H1069Q	Path	p.G1341E	VUS	ND	10.4	6.7	8	-	Y	H
181/F/41	p.G1266R	Path	p.T807I	VUS	8.3	5.9	6	9	291	NP	H
193/F/15	p.S1365C-fs*12	Path	p.Y743I-fs*19	VUS	ND	ND	<6	7	-	NP	H
80/M/40	p.A874V	Path	c.2299InsC	VUS	6.0	10.5	5.5	10	1575	Y	H*
120/F/13	p.H1069Q	Path	p.Lys269*	VUS	ND	ND	5	5	923	NP	H
142/M/39	p.T977M	Path	p.L1350P	VUS	22.6	32.6	5	10	-	Y	B
76/M/5	p.A1018V	Path	p.E458X	VUS	26.4	47.5	4.1	8	793	NP	H
141/F/46	p.H1069Q	Path	p.L1333P	VUS	ND	6.3	4	7	-	Y	H
184/M/10	p.A874V	Path	p.V1106I	VUS	20.1	15.5	4	-	-	-	-
119/M/38	p.H1069Q	Path	p.K844E-fs*10	VUS	ND	8.7	<4	4	-	NP	N
79/M/59	p.L1088X	Likely Path	p.A1135Q-fs*13	VUS	ND	ND	<4	6	-	Y	H
165/M/15	p.R778W	Path	p.K35N-fs*6	VUS	21.8	25.1	<3	4	-	NP	H
143/F/26	p.R778L	Path	p.L770L	VUS	ND	ND	<3	9	-	Y	H
69/F/16	p.T1029I	Path	c.3060+5G>C	VUS	2.8	ND	<3	8	-	Y	H
74/F/12	p.H1069Q	Path	IVS19-1C>G	VUS	ND	13.5	3	9	-	Y	N
188/F/8	p.M645R	Path	p.V997-fs	VUS	38.7	72.4	2.4	8	>250	NP	H*
102/M/9	p.G1341D	Path	p.F1026F-fs	VUS	ND	ND	2	10	-	Y	N
134/F/10	p.M769H-fs	Path	p.K1028S-fs	VUS	ND	ND	2	10	-	Y	N
72/F/24	p.G710S	Path	c.3400delC	VUS	57.7	96.6	0.6	12	448	Y	B
68/M/11	c.1708-1g>c	Likely Path	c.2866-3c>g	VUS	22.4	37.7	NA	6	-	Y	H
88/F/8	p.M769H-fs	Path	p.D1460Y	VUS	20.3	39.3	low	6	-	NP	H

Table 2. Continued

Patient/ gender/age	Variant 1	Annotation 1	Variant 2	Annotation 2	ATP7B 1056 (pmol/L)	ATP7B 887 pmol/L	CPL (mg/dL)	Leipzig score	Liver Copper (ug/g Tissue)	KF Ring	Presentation
Patients with two VUS's (n=7)											
86/F/68	p.E332K	VUS	p.D1047V	VUS	51.7	79.3	27	6	26	NP	B
3/M/18	p.G1335E	VUS	p.G1335E	VUS	ND	ND	<2	-	-	YP	N
9/F/15	p.G1341E	VUS	p.G1341E	VUS	2.2	ND	1.4	6	-	NP	H
84/M/NA	p.I1336V	VUS	p.C709T	VUS	10.5	15.5	10	8	552	NP	H
85/F/NA	p.I1336V	VUS	p.C709T	VUS	ND	6.3	13	7	900	NP	H
186/M/NA	p.S932L	VUS	p.V1364V-fs	VUS	ND	ND	10	9	-	NP	B
130/M/20	p.T59H-fs*19	VUS	p.H1247Q	VUS	23.6	27.2	<4	9	54	Y	N
Patients with only one variant found (no 2nd variant detected) (n=18)											
208/M/17	c.2299delC	VUS	Unknown	-	ND	ND	<2	-	-	NP	H
209/M/19	c.2299delC	VUS	Unknown	-	ND	ND	<2	-	502	NP	N
195/F/43	c.51+4a>t	Path	Unknown	-	25.6	37.6	4	8	-	Y	N
196/F/28	p.A1049A-fs	VUS	Unknown	-	2.2	9.1	3	7	-	Y	N
194/M/50	p.D765N	Path	Unknown	-	20.4	21.4	<4	8	-	Y	N
197/M/22	p.G1176R	Path	Unknown	-	3.6	7.3	3	5	-	NP	H
199/M/17	p.H1069Q	Path	Unknown	-	8.6	32.9	<10	5	-	NP	N
201/M/14	p.H1069Q	Path	Unknown	-	31.4	29.0	3.4	-	-	NP	N
198/F/NA	p.H1069Q	Path	Unknown	-	ND	5.0	12	6	-	NP	H
200/M/14	p.H1069Q	Path	Unknown	-	ND	6.7	13	4	-	Y	H
211/F/22	p.H1069Q	Path	Unknown	-	ND	7.9	2	5	-	NP	N
210/M/NA	p.L1305P	Path	Unknown	-	ND	6.0	NA	3	-	-	-
202/F/16	p.M769H-fs	Path	Unknown	-	ND	ND	13.8	6	525	Y	H
203/M/37	p.R1319X	Path	Unknown	-	3.0	5.2	<10	9	1042	Y	B
204/M/12	p.R778L	Path	Unknown	-	ND	9.5	<3.0	5	-	NP	H
205/M/25	p.T1220M	Likely Path	Unknown	-	67.7	77.0	<10	4	191	NP	B
207/F/18	p.W779X	Path	Unknown	-	38.0	65.6	15.3	6	258	NP	H
206/M/45	p.W779X	Path	Unknown	-	ND	ND	<-4	6	-	Y	H

*, cirrhosis; A, asymptomatic; B, both hepatic and neurologic; H, hepatic; N, neurologic; NA, not available; ND, not detected; NP, not present; VUS, variant of uncertain significance; Y, present.

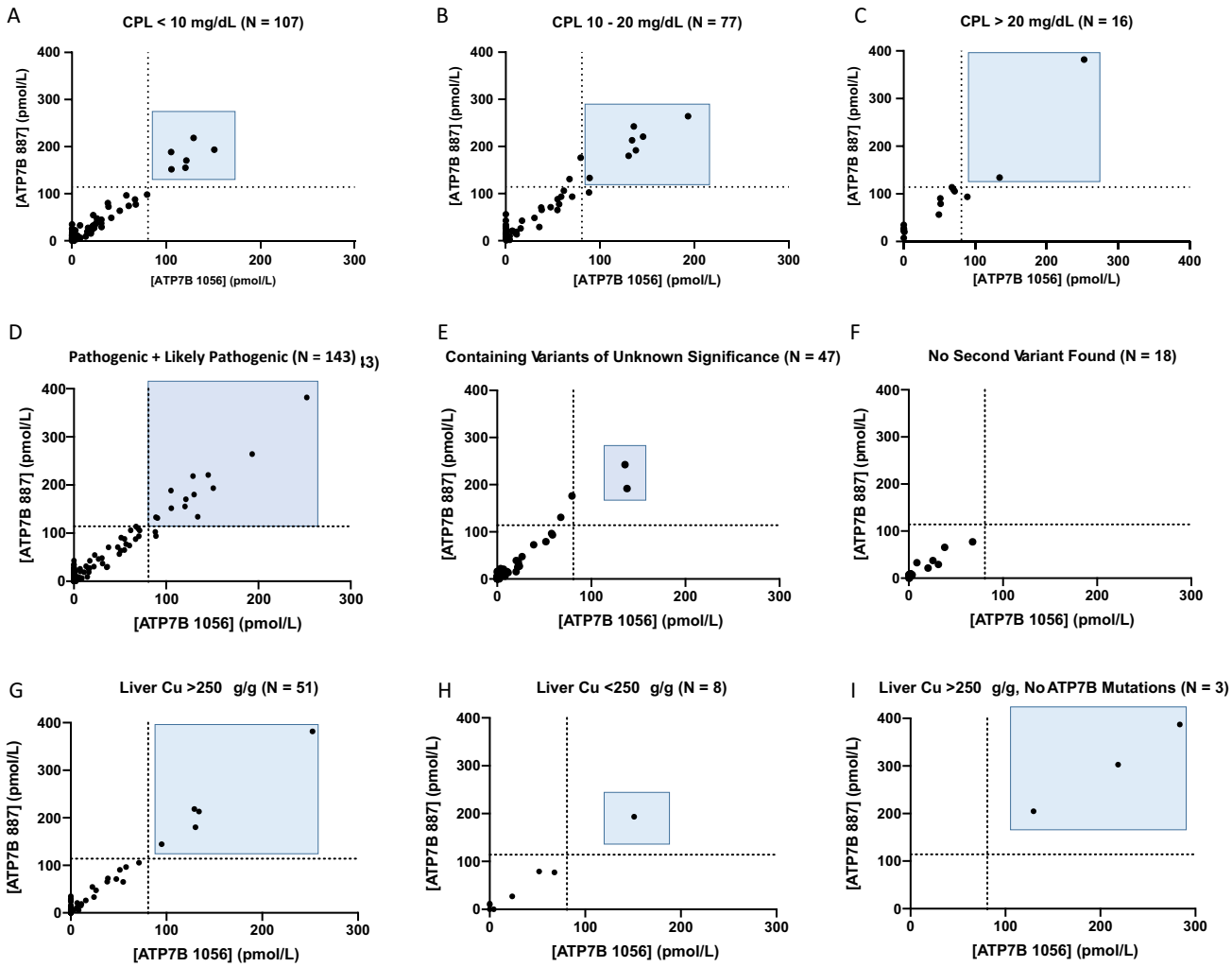
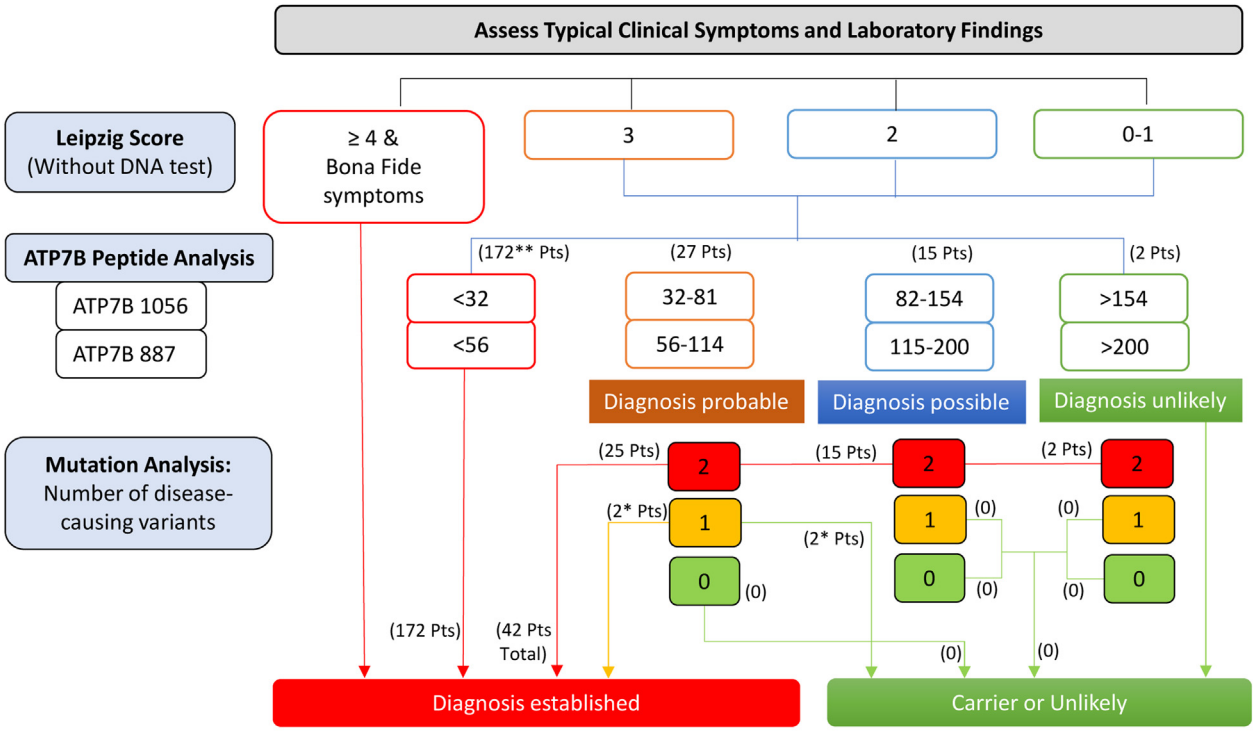


Figure 4. ATP7B peptide concentration analysis can provide clear results where Cp results and genetic analysis are ambiguous. Patients with significantly (*A*), moderately (*B*), and normal (*C*) Cp were readily identified. ATP7B concentrations are reduced regardless of variant status, including in patients with 2 pathogenic or likely pathogenic variants (*D*), at least 1 VUS (*E*), or where no second variant was found (*F*). *Dotted lines* represent peptide diagnostic cut-offs. Patients with liver copper above (*G*) or below 250 μg/g (*H*) are shown. In 3 samples from non-WD patients with elevated liver copper, ATP7B concentrations are normal (*I*).

for clinical laboratories.[26,27] The recommended Leipzig score for diagnosis of WD assigns numerical values to the number of disease-causing mutations to give probable WD diagnoses. The definition of "disease"-causing variant is based on a variety of databases, which can give conflicting answers.[28]

We hypothesized that direct measurement of ATP7B could identify WD patients as a majority of pathogenic mutations often result in protein misfolding, absence of decay of messenger RNA and enhanced degradation. To explore this goal, 2 WT ATP7B peptides were chosen for antipeptide antibody generation. Several factors influence peptide selection, as they must be unique to ATP7B, detectable by mass spectrometry, and elicit specific antibodies for isolation. ATP7B 1056 contains the most common WD-causing mutation, p.H1069Q. If the patient is homozygous for this mutation, WT ATP7B 1056 will not be found because the WT sequence is not present. Having a second peptide, ATP7B 887, builds a redundancy into the assay to ensure accurate performance.

As a primary diagnostic test, quantification of ATP7B from DBS effectively identified WD patients (Figure 2*A* and *B*, Table 1). Reduction of ATP7B concentrations below diagnostic cut-offs for at least 1 ATP7B peptide was evident in 92.1% of WD patients. Because the cut-offs set are based on the number of normal control patients analyzed, receiver operating characteristics curves were constructed showing ATP7B analysis to be highly sensitive and specific for diagnosis. The calculated area under the curve for the dataset is 0.98 regardless of peptide quantified (Figure 2*C* and *D*). Here, 211 patients had available genetic results (Figure 2*E*). WD is genetically confirmed in 143 patients with 2 evident pathogenic or likely pathogenic variants. This leaves 68 patients (32%) without a straightforward genetic diagnosis. Within the genetically confirmed subgroup, 91% were ATP7B-deficient, agreeing with sequencing results.



*# 205 and #207 fall under both outputs: "Diagnosis established" and "Carrier or Unlikely".

**includes 156 patients with 2 variants and 16 patients with 1 variant

ATP7B	Average	SD	-3.5	-2.5	-2	-1
1056.0	203.0	48.9	31.9	80.8	105.2	154.1
887.0	257.7	57.5	56.5	114.0	142.7	200.2

Figure 5. Proposed Wilson disease diagnostic algorithm.

More importantly, 94% of patients without clear genetic results (containing VUS, VCI, or missing variants on sequencing) were ATP7B-deficient. ATP7B peptide concentration analysis can be highly useful in these patients.

ATP7B peptide concentrations were measured in patient samples collected up to 11 years prior, to study whether ATP7B degradation impacts stored samples (Supplementary Table 7). Individuals with reduced ATP7B in freshly or recently collected samples had reduced concentrations across time. This suggests that older samples are not being identified as patients due to ATP7B degradation. No diagnosis in this group changed due to date of sampling. This includes 1 normal control patient with 3 separate samples taken over 6 months. All measured concentrations clearly identified this individual as normal and the CV for the measurements in these samples was approximately 11.4% and 12.4% for ATP7B 1056 and 887, respectively.

Certain variants are highly prevalent in the population and are more commonly seen in clinic. These represent important test cases for the discriminatory ability of ATP7B quantification by immuno-SRM. These variants had predominantly low or undetectable peptide concentrations, supporting the hypothesis that protein levels are reduced in vivo. Four of these high-frequency variants (H1069Q, R778L, E1064A, and M645R) have significantly reduced ATP7B concentrations in both homozygotes and compound heterozygotes (Figure 3*A*–*D*). Patients with these common mutations and, therefore, a significant percentage of patients overall, should be readily discriminated by the use of immuno-SRM as an index test.

Direct measurement of ATP7B peptides means that disease-causing mutations that affect protein activity but not protein concentration will generate false-negative results. In each of the 17 false-negative patients, both ATP7B 887 and ATP7B 1056 were above diagnostic cut-offs, indicating significant production of nonfunctional protein. Identifying these variants and their frequency will be important in interpreting immuno-SRM in the context of other clinical results. If ATP7B concentrations in patient DBS are in the established normal ranges but clinical suspicion for WD is high, continued patient workup is obligatory to confirm the diagnosis. Several such variants were found here and ATP7B levels often depended on the nature of the second variant (Table 2). The variants found in these patients have AFs ranging from unknown to 0.0089% and would therefore represent a small percentage of the overall patient population.

The most common variants with ATP7B levels above the cut-off are p.G710S and p.R969Q (Table 2; Figure 3*E* and *F*). There were 13 WD patients carrying the p.G710S variant and 8 patients with p.R969Q. Seven p.G710S patients had normal concentrations of ATP7B peptides when they were

either homozygous for p.G710S or compound heterozygous with p.G710A and p.M665I. Three p.R969Q patients were false negatives. In 2 of these cases, p.R969Q is in combination with p.H1069Q, which is shown to significantly reduce ATP7B concentrations. p.G710S is a variant associated with severe liver disease, 2 patients required an emergency liver transplantation due to fulminant hepatic failure.[29] These variants are known pathogenic by causing significantly impaired copper transport activity while maintaining normal ATP7B trafficking and phosphorylation in vitro.[24,30,31] Patients with these variants could be missed by immuno-SRM when in combination with variants producing significant ATP7B. When in combination with a variant severely affecting ATP7B concentrations, as in the p.H1069Q cases, ATP7B levels may give false negatives (Table 2). The mechanisms of variant interaction in vivo are currently under study. Diagnostic potential will therefore depend on the specific genotype of these patients. However, knowing that these variants have the potential to generate normal levels of ATP7B will be useful when evaluating genetic analysis after ATP7B measurement.

Like the method-dependent cut-offs used for Cp, 24-hour urinary copper[32] and hepatic copper for diagnosing WD,[33,34] measurement of ATP7B peptides require validation in the population in which it will be used. This will aid in generating clearly defined cut-offs. A true description of the final diagnostic performance of ATP7B concentration measurement will come with conducting a large cohort validation or pilot study, such as newborn screening.

Cp is not useful as a screening test for WD,[9] but levels of Cp <10 mg/dL are regarded as useful for diagnosis (score 2 in Leipzig score) of WD. A recent Chinese study found that Cp levels <12 mg/dL are strongly indicative of a diagnosis of WD.[32] Here, 200 patients had Cp values available, among them 77 (38.5%) had moderate Cp values of 10–20 mg/dL and an additional 16 (8%) had normal Cp levels >20 mg/dL; 92.9% of patients with Cp <10 mg/dL had ATP7B concentrations below diagnostic cut-offs (Figure 4*A*–*C*). When Cp levels were moderately reduced, 91.6% of patients would be identified by ATP7B analysis. Even in those with normal Cp values ATP7B identified 87.5% of cases. Measurement of DBS ATP7B can provide clarity when Cp levels are ambiguous. This is likely because ATP7B is not a measurement of secondary disease effects. Cp and copper measurements are often confounded by external processes or disease influences, including liver cirrhosis and malnutrition. As Cp is an acute-phase reactant possessing ferroxidase activity, the concentration can be elevated by acute inflammation. A prospective study on serum Cp as a screening test for WD in patients referred with liver disease reported a positive predictive value of only 6%.[9] Here, immuno-SRM analysis of ATP7B clearly outperforms Cp measurement.

Clinical information, including age, Leipzig scores, liver copper content, presence of KF rings, initial presentation, and presence of cirrhosis, was obtained when possible (Supplementary Table 3). No significant differences in ATP7B concentration were found based on age (pediatric vs adult), presence of KF ring, presentation (hepatic, neurologic, both hepatic and neurologic, or asymptomatic), or presence of cirrhosis. The mean ± SD ATP7B 887 peptide concentrations in hepatic and neurologic presentation were 33.8 ± 51.7 pmol/L and 38.2 ± 57.1 pmol/L, respectively. This appears aligned with previous observations in a large cohort showing the absence of any phenotype–genotype correlation regarding initial symptomatic manifestation.[35] Fifty-nine patients had hepatic copper measurements and 86.4% of these had elevated liver copper >250 μg/g. This includes 5 with normal ATP7B levels, 3 of them with p.G710S variant, and supports their diagnosis as WD patients despite negative immuno-SRM results (Figure 4*G*–*I*). Of interest, 3 samples were received with suspicion of WD by elevated liver copper but with no ATP7B variants identified. These samples had normal ATP7B concentrations, supporting their status as non-WD patients and providing an example of immuno-SRM analysis in ruling out WD (Figure 4*I*). Finally, it is unknown how many patients were being treated and what their treatment regimens were. Current WD therapies are focused on copper depletion and not restoration of ATP7B protein. As such, treatments would not affect the measured levels of ATP7B.

Examples From Dataset Where Diagnosis Is Simplified

The ability of ATP7B measurement to clarify case results extends to ambiguities in genetic analysis and contributes significantly to advancing noninvasive clinical diagnosis of WD (Figures 2*E* and 4*D*–*F*). There are 7 cases presented in which patients were compound heterozygous or homozygous for 2 VUS, indicating a lack of strong understanding of how these variants will contribute to phenotype (Table 2). All of these patients had significantly reduced ATP7B peptides levels suggesting affected patient status. Three patients in this group had only moderately reduced Cp between 10 and 20 mg/dL. In 1 case (no. 186), sequencing returned 1 VUS computationally predicted to be pathogenic and 1 (p.V1364V-fs) predicted to be benign. This patient had entirely nondetectable DBS ATP7B, indicating the consequence of this genetic combination is disease-causing. One patient (no. 86) presented an interesting clinical situation in which a VUS was found on sequencing and Cp concentrations were clearly within the normal range (27 mg/dL) (Table 2). Here, ATP7B measurement provided a clear indication that these patients severely lacked ATP7B protein and were very likely affected by WD in a way that genetic analysis, prediction, and Cp measurement could not.

Similarly, there were 37 patients in which a known pathogenic or likely pathogenic variant was found in combination with a VUS (Figure 4*E*, Table 2). Thirty-five (94.6%) of these had reduced ATP7B concentrations of at least 1 peptide, indicating positive identification as a WD patient despite a lack of knowledge of the consequences of their specific mutations. In 14 of these patients, Cp was found to be ≥10 mg/dL. Here, a significant reduction in patient ATP7B is particularly valuable for assigning a clinical designation and treatment course.

Of concern are situations where Sanger sequencing and/or next-generation sequencing identifies only 1 variant. Sequencing analysis can be robust for targeting known variants but can return negative results if disease-causing mutations are small or large deletions, duplications, in the deep intronic or promoter regions, or in poly-A tails. These situations can have a significant impact on patient identification and place greater emphasis on Cp measurements and other WD diagnostics while the genetic basis for disease is not known. In 18 patients from our cohort with high suspicion for WD, only 1 *ATP7B* variant was identified (Figure 4*F*, Table 2). All of them had ATP7B measurement with peptide concentrations below established cut-offs. This includes 4 patients with Cp concentrations between 10 and 20 mg/dL, where a single variant and a moderate Cp reduction would be insufficient to establish the diagnosis. In 3 patients, the single variant detected from sequencing was a VUS causing a further complication in genetic interpretation. ATP7B measurement can provide direct evidence of the consequences of existing variants even if the second variant was not detected from sequencing workflows.

It is notable that some carriers with ATP7B peptide concentrations below cut-off create potential false positives. ATP7B 887 analysis showed 4 of 48 carriers had peptide levels below diagnostic cut-offs. The mechanisms by which a single affected allele reduce ATP7B concentrations are unknown and require study. Polymorphisms affecting protein concentration or factors affecting possible co-translated protein interactions may exist but are currently unknown.

Finally, we propose a new algorithm for WD diagnosis incorporating ATP7B measurement findings for each peptide (Figure 5). Here we define 4 possible patient groups based on ATP7B peptide level: directly established WD (<32 pmol/L for ATP7B 1056 and <56 pmol/L for ATP7B 887); probable WD (ATP7B between group 1 and the diagnostic cut-off); possible WD (ATP7B 1056: between cut-off and 154 pmol/L and ATP7B 887: between cut-off and 200 pmol/L); and unlikely WD (>154 pmol/L for ATP7B 1056 and >200 pmol/L for ATP7B 887). In validating the algorithm on the current retrospectively collected patients, we found that 172 of 216 (79.6%) showed ATP7B within the range of patient group 1, making their diagnosis very highly likely. Applying this method to the remaining cases along with mutation analysis show that all are able to reach WD diagnosis, except for 2 cases in which diagnosis or carrier status cannot be determined. Full validation of this proposed algorithm will be necessary through a large cohort prospective study.

Limitations

The study is limited in that data are predominantly for White patients. Patients from the Indian subcontinent, Africa, and South America may have different genotype distributions and possibly differing ATP7B peptide levels. There is also an unavoidable selection bias due to the analysis of only well-described WD cases. A significant limitation comes from the fact that these samples have been collected in a retrospective manner. It will be important to prospectively analyze a large cohort samples before genetic analysis in future applications. Full validation in a large prospective study will allow for more accurate definition of ATP7B variability in healthy normal samples and diagnostic parameters, including sensitivity, specificity, positive predictive value, and negative predictive value. The current statistical measures of predictive significance might change upon conducting a validating study. Furthermore, very few cases of fulminant WD and hemolysis were investigated. Patients presenting with hemolysis might have differing ATP7B peptide concentrations due to red blood cell lysis. In addition, ATP7B analysis is unable to delineate patient groups based on phenotype, that is, hepatic or neurologic types or disease severity. Finally, only 2 signature ATP7B peptides have been analyzed. Alternative peptide sequences may have different discriminating capability such that study of additional candidate peptides may be helpful.

LC-MS/MS is considered highly specialized technology, although it has been used in clinical laboratories in a wide array of fields, such as toxicology, drug monitoring, and newborn screening. Following the standard validation process in the clinical laboratory, we anticipate the assay can be successfully implemented into clinical practice.

ATP7B peptide analysis identified WD patients in a large majority of cases and reduced ambiguities resulting from genetic analysis and Cp levels. This noninvasive assay can serve as an adjunctive test for the diagnosis of WD and is expected to fundamentally advance the use of proteomic technology for a rapid screening tool, an area that holds great promise but is largely untapped.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dxdoi.org/10.1053/j.gastro.2021.02.052.

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Received November 12, 2020. Accepted February 21, 2021.

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Peter Ferenci, MD (Data curation: Equal; Formal analysis: Equal; Resources: Lead; Supervision: Equal; Validation: Equal; Writing – review & editing: Lead).

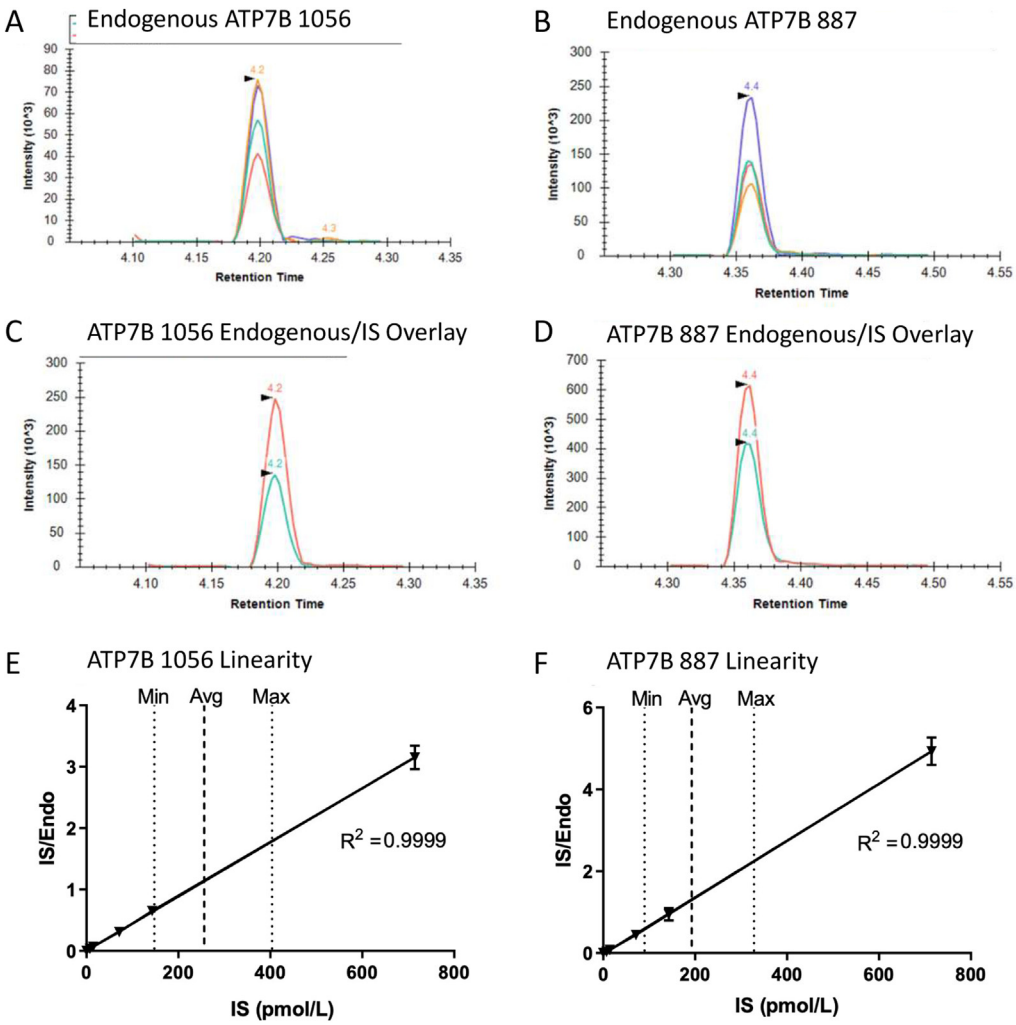
Si Houn Hahn, MD, PhD (Conceptualization: Lead; Data curation: Lead; Formal analysis: Lead; Funding acquisition: Lead; Methodology: Equal; Resources: Supporting; Supervision: Lead; Validation: Lead; Writing – review & editing: Lead).

Conflicts of interest

The authors disclose no conflicts.

Funding

This study was supported by the grants from NIH-R21HD097558, and R01HD098180 and Wilson Disease Association.



Supplementary Figure 1. Representative chromatograms of ATP7B peptides in MS/MS. (*A*) endogenous ATP7B 887; (*B*) endogenous ATP7B 1056; (*C*) overlay of endogenous and IS ATP7B 887; and (*D*) overlay of endogenous and IS ATP7B 1056. Linearity curves of ATP7B 1056 (*A*) and ATP7B 887 (*B*). *Vertical lines* represent the average (avg), minimum (min), and maximum (max) concentration determined in normal controls.

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Conflicts of interest
The authors disclose no conflicts.

Most current article

0016-5085/$36.00
https://doi.org/10.1053/j.gastro.2021.03.030

Expanding the Diagnostic Toolkit of Wilson Disease with ATP7B Peptides

See "Direct measurement of ATP7B peptides is highly effective in the diagnosis of Wilson disease," by Collins CJ, Yi F, Dayuha R, et al, on page 2367.

The diagnosis of Wilson disease (WD) can be determined by a combination of parameters aiming to detect copper accumulation. In this article from Collins et al,[1] a new approach to the diagnosis of WD is proposed. In current clinical practice, low ceruloplasmin levels are used as a minimal screening test, followed by Kayser–Fleischer ring assessment, 24-hour urine collection, liver biopsy for copper quantification, and brain magnetic resonance imaging for demonstration of copper-related changes in the basal ganglia. Although scoring systems can aid in the diagnostic process,[2] the traditional laboratory tools lack sensitivity and specificity for WD. In particular, the major limitation of ceruloplasmin determination is that most clinical laboratories quantify the enzyme levels, but not its oxidase activity, with consequent determination of erroneous elevated levels owing to quantification of both ceruloplasmin and biologically inactive apoceruloplasmin.[3]

Total serum copper is even less valuable as a screening test because it is influenced by ceruloplasmin level and its measurement methods. Genetic testing with *ATP7B* sequencing, whole-exome sequencing[4] and whole-genome sequencing are considered confirmatory tests and their costs are becoming more approachable. Although *ATP7B* variants are associated with various degrees of functional impairment,[5] their clinical correlate is uncertain. Therefore, the major challenge is the lack of genotype–phenotype correlation,[6] with the additional concern that some gene variants may not be associated with clinical manifestation development. Other diagnostic options, including the radioactive copper incorporation test[7] or the exchangeable copper,[8] are interesting but will likely offer several challenges in their access and execution outside selected academic centers. Therefore, although the diagnosis of WD is frequently achievable with available methods, many cases are trapped in a grey area of diagnostic uncertainty characterized by borderline ceruloplasmin levels, gene variants of unknown significance, and ambiguous clinical presentations. These borderline cases often overlap with common conditions, including fatty liver, autoimmune hepatitis, and movement disorders, and require refined clinical judgment and expertise for the final diagnosis.

Striving for a gold standard and improved diagnostic assessment is important because WD is a treatable disease both in the acute and chronic phases and timely diagnosis can prevent, improve, and even resolve many of the clinical manifestations. Particularly concerning are the neuropsychiatric manifestations, which are debilitating and only responsive after years of anticopper treatment and physical therapy. In addition, new treatment options are on the horizon, including next-generation chelating agents[9] and gene therapy approaches.[10] Medical treatment is often limited by drug toxicity, and unsatisfactory improvement, especially of the severe neuropsychiatric symptoms, is frequent. Liver transplantation can be an effective treatment and is often inevitable in cases of acute liver failure, but chronic immunosuppression is not desirable.[11] Therefore, when the phenotypic characterization of WD improves beyond the traditional definitions of prevalent hepatic and neuropsychiatric involvement,[12,13] the goal will be to tailor and optimize medical treatment to the phenotype or predicted disease course.

In this issue of *Gastroenterology*, Collins et al[1] adopt a new approach to the diagnosis of WD based on the quantification of ATP7B protein concentration derived from measurement of 2 surrogate peptides in patient dried blood

spot samples, as direct evidence of WD diagnosis. Two ATP7B peptides, ATP7B 887 and ATP7B 1056, were selected and measured with specific antibodies. The studied samples derived from pre-existing biorepositories originated from European, North American, and South Korean institutions providing samples from patients with WD (n = 216), obligate *ATP7B* variant carriers (n = 48), and healthy patients (n = 150). Patients with WD carried different *ATP7B* variants, mostly highly prevalent in the population of origin. The age range was 2 months to 73 years, which is important because it is now established that the diagnosis of WD should be considered at any age.

The approach was first to identify the analytical and diagnostic performance of the peptides and identify the diagnostic cutoffs. A large proportion of the studied patients had available genetic test results and 92.1% of the patients with genetically confirmed WD had ≥1 of the peptides below the established minimal cutoffs. Among the 67 patients in whom genetic tests could not establish diagnosis of WD, 63 (94%) had their diagnosis clarified by low peptide levels. Of note, ≤16% of individuals heterozygous for WD had peptide levels below the cutoff, but could also have been WD cases carrying unknown variants. ATP7B 887 analysis demonstrated a sensitivity of 91.2% and specificity of 98.1%. In line with the challenges related to varied WD presentation, a small but sizable percentage of genetically confirmed WD cases presented peptide levels above the cutoffs, therefore representing a border line zone of patients that will likely still be difficult to diagnose or rule out as WD.

Owing to the retrospective nature of the sample collection, available clinical data were not extensive and information about current medical treatments were not detailed. Regardless, the possibility of a new diagnostic test to add to the WD toolkit available to practitioners not familiar with WD, is appealing and ultimately beneficial to patients. This study and the significance of the ATP7B peptides is timely as it positions itself in the middle of an ongoing debate about the full penetrance of WD,[14] the role of extra hepatic ATP7B copper transporter on clinical manifestations,[15] and the significance of ATP7B variants on disease phenotype.

Genetic testing and the knowledge to interpret these data remain incomplete. This study on ATP7B peptides opens clinical and research opportunities aiming to correlate peptide levels with disease severity and phenotype on larger populations. As suggested by the authors, integrating peptide levels into a validated diagnostic scoring system which includes copper metabolism parameters and genetic testing should be a major research focus and could lead to improved phenotypic and prognostic patient characterization. Ultimately, the proposed approach may be helpful to diagnose both adult and pediatric cases of WD.

In summary, expanding the diagnostic toolkit of WD is a priority and this study offers an additional option that could have a role as an initial screening or confirmatory test and will ensure that fewer diagnoses are delayed or missed.

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Conflicts of interest
The author has made the following disclosures: Valentina Medici M.D. is a paid consultant for Alexion Pharmaceuticals.

Funding
National Institutes of Health (NIH): R01DK104770 to V.M.

Most current article

0016-5085/$36.00
https://doi.org/10.1053/j.gastro.2021.03.025

COVID-19 Gastrointestinal Symptoms and Attenuation of the Immune Response to SARS-CoV-2

See "Intestinal host response to SARS-CoV-2 infection and COVID-19 outcomes in patients with gastrointestinal symptoms," by Livanos AE, Jha D, Cossarini F, et al, on page 2435.

As we eclipse year 1 of the severe acute respiratory syndrome novel coronavirus-2 (SARS-CoV-2) pandemic, our understanding of this virus is only beginning to unfold despite an exponentially growing body of literature. Early descriptions of the novel respiratory illness coronavirus disease 2019 (COVID-19) were quickly followed by reports of a multisystem disease.[1] Gastrointestinal (GI) symptoms are frequently reported in patients with COVID-19, which raised questions of gut infection and the possibility of fecal–oral transmission. Indeed, viral RNA was detected in stool of infected patients by quantitative reverse transcriptase polymerase chain reaction, which frequently remained positive beyond the duration of positivity of their nasopharyngeal samples.[2–4] Multiple groups have reported GI epithelial coexpression of the host receptor ACE2 and TMPRSS2, a protease enabling cell entry through cleavage of the eponymous spike protein.[5–8] In line with the susceptibility of the GI epithelium to SARS-CoV-2 entry, viral RNA by in situ hybridization[9] and viral nucleocapsid protein (NP) by immunofluorescence have been detected in infected patient intestinal biopsies,[2] whereas viral particles have been demonstrated by transmission electron microscopy.[9] Moreover, recent studies using ex vivo enteroid or organoid models convincingly demonstrated that GI epithelium is permissive to SARS-CoV-2 infection.[6,9–11] Notably, human intestinal enteroids inoculated with the virus or nasopharyngeal aspirates from infected patients showed intracellular NP and increasing viral titers over time, indicating productive viral replication within intestinal epithelium.[9–11] Enteroids also exhibited double-stranded RNA, a requisite intermediate produced during the replication of this positive-sense RNA virus.[11] What happens in vivo remains unclear owing to the inherent logistical challenges of studying large cohorts of patients infected with this novel pathogen. Beyond the possibility of GI infection, viral interactions with GI tract tissues and the immune system, and how this influences clinical outcomes in COVID-19 remain largely unknown.

In this issue of *Gastroenterology*, Livanos et al[12] attempt to correlate clinical observations in patients with COVID-19 with immunologic changes within the GI tract on histologic, transcriptomic, and proteomic levels. The authors analyzed endoscopic biopsy specimens from COVID-19–afflicted patients, finding no gross abnormalities when compared with matched controls.[12] Although they found angiotensin-converting enzyme 2 (ACE2) diffusely expressed throughout the GI tract, viral NP was found more prominently in the ileum versus duodenum in the limited number of specimens studied.[12] They attribute this finding to increased MUC2+ goblet cell prevalence in distal small intestine, which colocalized with NP immunostaining, and their transmission electron microscopy images showing viral particles present primarily in exit vesicles of goblet cells.[12] This finding may corroborate findings from bronchial air–liquid interface cultures demonstrating tropism for goblet cells,[13] but were nevertheless surprising given relatively low ACE2 expression in intestinal goblet cell clusters,[5] evidence of goblet cell depletion in an infected patient's colonic biopsies,[9] and data from human intestinal organoids demonstrating enterocytes were susceptible to infection whereas goblet cells were not.[6,11] Livanos et al[12] also find evidence of NP staining in a nongoblet crypt base cell population but do not discern this population as Paneth, stem cells, or another population.[12] This raises additional questions of cellular tropism and host entry determinants because enterocytes in the villus compartment express the highest levels of ACE2.

The authors also performed a mass cytometry immunophenotypic analysis of endoscopic biopsies and blood samples from these patients.[12] They found that specific dendritic cell populations were diminished in the lamina propria of infected patients, possibly supporting some alteration in antigen presentation.[12] Bulk RNA sequencing of these tissues found decreased expression of several inflammatory pathways in the lamina propria and a trend toward the up-regulation of antiviral pathway signatures within the epithelial compartment.[12]

Notably, Vero E6 cells inoculated with homogenized biopsy tissue obtained from patients did not show evidence of productive infection.[12] This finding may simply result from sampling or technical error, but it may also reflect the biology of SARS-CoV-2 in the gut. There remains a striking lack of evidence of infectious virion isolation from the gut,

Management of Wilson Disease Diagnosed in Infancy: An Appraisal of Available Experience to Generate Discussion

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ABSTRACT

Increased access to molecular genetic testing is changing the demographics for diagnosing inherited disorders and imposing new challenges for medical management. Wilson disease (WD), typically diagnosed in older children and adults, can now be detected in utero and in infants (children younger than 24 months, including neonates) via genetic testing. An evidence-based approach to management of these neonates and extremely young children, who are typically asymptomatic, has been hampered by lack of clinical experience. We present a case of an infantile diagnosis of WD, review available experience, and discuss current trends in antenatal genetic testing of parents and fetus that may lead to a very early diagnosis of WD. Based on physiological and nutritional considerations, we propose an algorithmic approach to management of infantile WD as a starting point for further discussion. Future collaboration amongst specialists is essential to identify evidence-based approaches and best practice for managing treatment of infants with genetically diagnosed WD.

Key Words: *ATP7B*, copper, genetics, screening, zinc salts

(*JPGN* 2020;70: 547–554)

What Is Known

- ATP7B molecular genetic testing can identify homozygotes or compound heterozygotes for pathogenic mutations associated with Wilson disease.
- Screening first-degree relatives of patients with Wilson disease is recommended to identify those affected with Wilson disease.
- Zinc therapy is effective in asymptomatic patients with Wilson disease, but few data are available about optimal treatment for infants with genetically diagnosed Wilson disease.

What Is New

- Testing infants born to parents who are known carriers of *ATP7B* pathogenic mutations can identify infantile Wilson disease.
- Breast milk and formula provide approximately >150% of estimated copper and zinc needs, because copper is critical for normal development.
- Treatment with zinc in infantile Wilson disease may prevent development of significant copper accumulation and end-organ damage; however, timing when to start treatment is uncertain.
- When general newborn screening for Wilson disease becomes feasible, the resultant change in Wilson disease demographics will enhance the need for a coherent approach to management of infants with genetically diagnosed Wilson disease.

Wilson disease (WD) displays toxic accumulation of copper in liver and brain due to dysfunctional or absent ATP7B, the Wilson ATPase, an intracellular copper-transporting P-type ATPase expressed mainly in hepatocytes. ATP7B facilitates production of holoceruloplasmin and, importantly, biliary excretion of copper (1). The average age at diagnosis reported in pediatric WD cohorts is 10 years (2–4). Children older than 24 months with WD, herein referred to as having ''infantile WD,'' are almost invariably asymptomatic (5,6).

With availability of affordable expanded carrier screening-panels, we can detect the carrier status for various genetic diseases in parents. When each parent carries a mutation in *ATP7B*, genetic testing of the infant can lead to an early diagnosis of WD. Alternatively, whole-exome sequencing or sequencing for *ATP7B* mutations independent of the parents' status can also detect WD.

Received August 14, 2019; accepted December 6, 2019.

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Supplemental digital content is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.jpgn.org).

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DOI: 10.1097/MPG.0000000000002608

According to the American College of Obstetricians and Gynecologists Committee on Genetics, criteria for inclusion of a disease in expanded carrier screening-panels include a carrier frequency of ≥1:100, a well-defined phenotype, and onset of disease in childhood (7). Carrier rates for 1 *ATP7B* mutation are estimated at 1:90, except in some isolated populations; a recent British study reported a much higher carrier rate (8). WD is clinically pleomorphic, but its phenotypes are well described: ranging from mild with only liver test abnormalities to severe with hepatic decompensation during childhood, including acute liver failure requiring liver transplantation (2,9). Neurological and psychiatric symptoms are less frequent in childhood (2). With early institution of treatment, disease progression can be prevented and the morbidity associated with neurologic and psychiatric WD can be avoided (10). Thus, WD fits American College of Obstetricians and Gynecologists recommendations for carrier screening inclusion criteria.

Here we present a case of infantile WD as a basis for addressing the implications of genetic diagnosis during infancy. We have collectively reviewed current experience with infantile WD and examined infants' diets for exposure to copper and zinc. Copper is required for normal growth and development in infancy but is potentially toxic in excess. We propose an algorithmic approach for surveillance and management of infantile WD. Our purpose is to stimulate discussion around this emerging issue of how to manage this unique population, and promote multicenter collaborations to gather requisite data.

CASE PRESENTATION

The patient's mother was a healthy 35-year-old woman of Italian-Greek descent who had difficulty with conception due to a uterine septum. Before surgical correction, her fertility evaluation included carrier screening-panel testing. A heterozygous mutation in *ATP7B*, c.3207C>A (p.His1069Gln), was identified, which leads to rapid degradation of ATP7B in the endoplasmic reticulum (11). Her partner, of Italian descent, underwent genetic testing which identified a different heterozygous *ATP7B* mutation: c.845delT (p.Leu282Profs*2) producing a truncated protein (12). These 2 individuals were not known to be consanguineous.

The patient was born following a healthy pregnancy and delivery at term. Birth weight was normal. Genetic testing for *ATP7B* was obtained in the infant at 42 days. Results available by 2 months old revealed that she was compound heterozygote for the 2 parental mutations. Knowledge of the parental genotypes excluded that these 2 mutations were cis, thus affecting both alleles encoding ATP7B. At the initial hepatology evaluation, she was 3 months old, exclusively formula-fed and growing well (weight 76th percentile, length 98th percentile). On examination, she was well-appearing, anicteric, without hepatosplenomegaly. A routine unrestricted pediatric diet was recommended; foods with high copper content were not yet in the diet.

By 8 months of age, the infant was taking 24 oz of formula per day, equating to 329 μg of copper and 3.3 mg of zinc per day. She was ingesting a spectrum of infant purees, all in moderation, and demonstrated appropriate growth.

The infant was evaluated at 12, 15, and 18 months and was consistently asymptomatic with normal development, appropriate growth, and no hepatosplenomegaly. Laboratory results at these visits are included in Table 1. Abdominal ultrasonography at 12 months demonstrated a normal-appearing liver without increased echogenicity or hepatosplenomegaly (Fig. 1).

The parents' utmost concern had been the optimal strategy to avoid any copper overload in their child. At 18 months, she was clinically well and was eating a full diet of table foods, apart from foods high in copper (liver, nuts, mushrooms, chocolate, shellfish). Following discussion of risks and benefits with her parents, at this time treatment was initiated with zinc gluconate 25 mg twice daily, spaced away from meals. Since she was iron deficient before zinc initiation, iron supplementation was administered with meals twice a day. Zinc therapy was well-tolerated. We have not measured basal 24-hour urinary copper (or zinc) excretion as she was not yet toilet trained.

Cases of Infantile Wilson Disease in the Literature

Fourteen cases of infantile WD have been reported plus 2 unpublished cases (J.M.V., R.A.) (Table 2) (4,6,13–21). Genetic testing of infants born to children of known carriers of *ATP7B* mutations will identify patients with WD who are asymptomatic with normal alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Our review of the WD literature revealed that in cases diagnosed during infancy ALT values ranged between normal (12 IU/L) and approximately 10 times the upper limit of normal (556 IU/L). Data are skimpy; however, around the age of 11 to 13 months some had elevated aminotransferases (Table 2). Medical management of these cases was highly variable or else not described.

Physiological Considerations: Copper Metabolism During Fetal Development

Fetal copper trafficking is complicated. Both *ATP7A*, which is defective in Menkes disease, and *ATP7B* encode transporters involved in copper homeostasis in the placenta. ATP7A, the Menkes ATPase, allows copper to be imported across the placenta to the fetus from the maternal circulation (22). ATP7B is also expressed in the placenta and is expected to allow efflux of copper across the placenta back to the maternal circulation (22). In the third trimester this efflux, however, becomes less important as the growing fetus has increased copper needs. In infants whose placental ATP7B is defective, it is unknown if the total body copper is

TABLE 1. Longitudinal serum biochemistry measurements in an infant with Wilson disease before initiation of treatment

Age at testing	8 mo	12 m	15 mo	18 mo
ALT, U/L (normal <33)	21	25	15	20
AST, U/L (normal <32)	54	40	42	39
GGT, U/L (normal <42)	18	14	12	14
Direct bilirubin, mg/dL (normal <0.4)	0.2	0.1	< 0.2	<0.2
Ceruloplasmin, mg/dL (normal > 20)	8	11	11	7
Serum copper, μg/dL (normal 20–70)	25	27	28	25

ALT = alanine aminotransferase; AST = aspartate aminotransferase; GGT = gamma-glutamyltransferase.

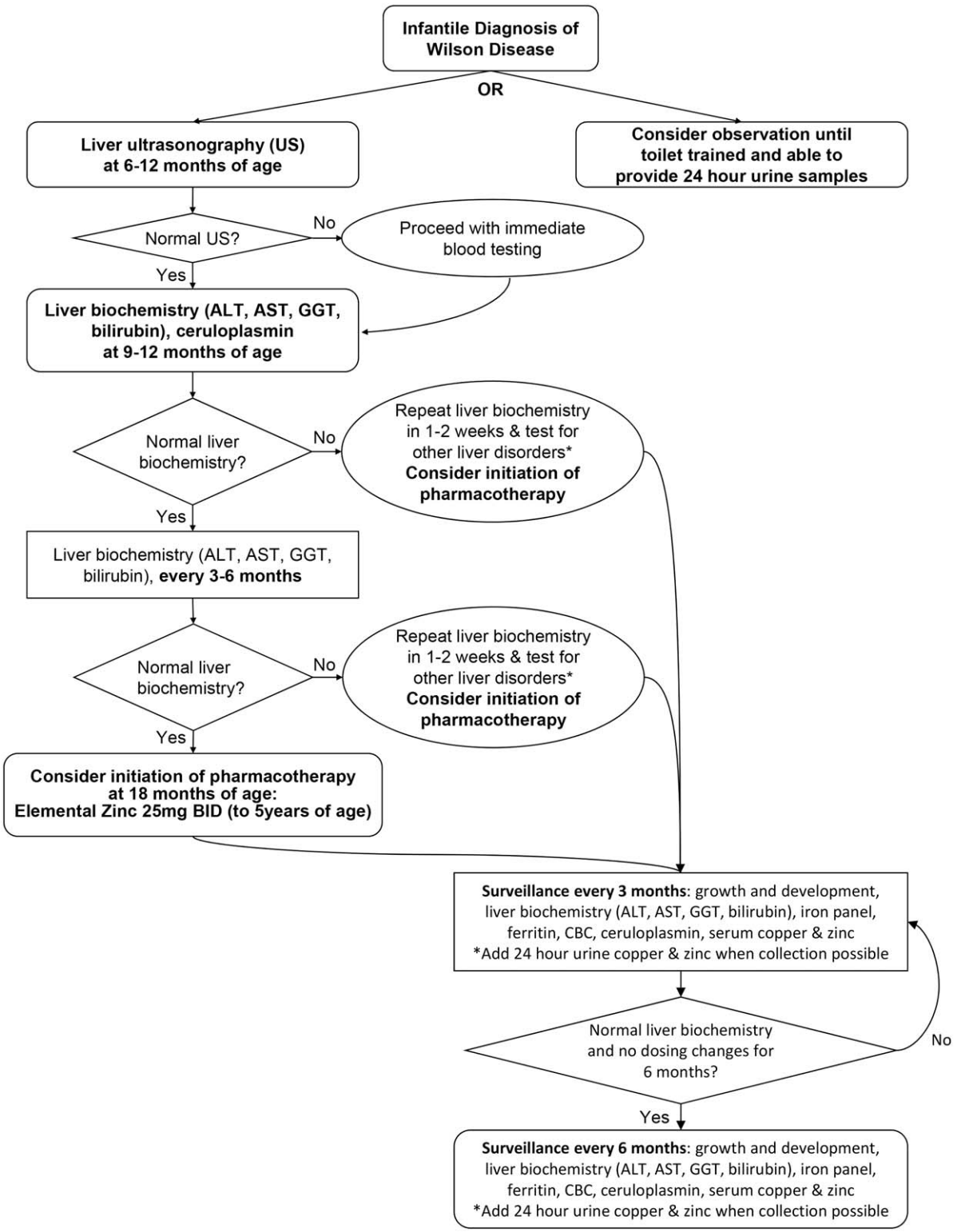

FIGURE 1. Proposed surveillance and treatment algorithm for infants with genetically diagnosed Wilson disease. *Etiologies of other liver disorders include alpha-1-antitrypsin deficiency, viral hepatitis B and C, and autoimmune liver diseases. ALT = alanine aminotransferase; AST = aspartate aminotransferase; GGT = gamma-glutamyltransferase.

TABLE 2. Reported and authors' experience with infantile Wilson disease

Presentation	Origin	ATP7B mutation	Ceruloplasmin, mg/dL	Urinary copper, μg/24 h	Aminotransferases at presentation	Management	Reference
Newborn boy; parents had prenatal genetic testing	USA	p.H1069Q/p.P1379S	26 (at 1 y)	220 (at 1 y)	ALT 110 IU/L AST 57 IU/L (at 1 y)	Low-dose zinc at 16 mo	Bennett et al, 2013 (13)
2-Mo-old girl; parents had prenatal genetic testing	USA (Italian/ Greek ethnicity)	p.H1069Q /c.845delT	8 (at 8 mo)	Not yet performed	ALT 21 IU/L AST 54 IU/L (at 8 mo)	Zinc started at 18 mo of age	Case presented here
3-Mo-old girl; parents had prenatal genetic testing	USA	c.4051C>T / c.2304dupC	<3 (at 6 mo)	Not yet performed	ALT 12 IU/L AST 23 IU/L (at 6 mo)	Dietary counseling	*J. Vittorio, unpublished observations
3-Y-old boy; parents had prenatal genetic testing	USA	p.H1069Q (homozygous)	26 (at 3 y)	23 (at 3 y)	ALT 30 IU/L AST 45 IU/L (at 3 y)	Zinc acetate started at 3 y	*R. Arnon, unpublished observations
4-Mo-old girl; family screening	Greek	Not available	Not available	<100	Not available	Not available	Manolaki et al, 2009 (4)
8-Mo-old boy; unclear reason for ceruloplasmin measurement	Japanese	c.2302insC (homozygous)	9.5 (at 8 mo)	17 (at 8 mo)	Within normal	Not reported	Shimizu et al, 1997 (14)
8-Mo-old boy; ALT measured with an acute diarrheal illness	Chinese	p.G1186S / c.4006delA, and 5 other single nucleotide polymorphisms	7.9 (at 11 mo)	Not reported	ALT 247 IU/L AST 193 IU/L (at 11 mo)	Zinc gluconate 10 mg TID started at 11 mo	Abuduxikuer et al, 2015 (6)
9-Mo-old boy; ALT measured with an acute diarrheal illness	South Korean	p.G1186S / c.4006delA	<9 (at 22 mo)	14.9 (at 23 mo)	ALT 122 IU/L AST 102 IU/L	Zinc 24 mg BID started at ~27 mo. Penicillamine initiated due to increasing aminotransferases	Kim et al, 2013 (15)
10-Mo-old girl; unclear why	Chinese	R776L/none found	Not reported	302	ALT 556 IU/L AST 274 IU/L	Trientine (dose and start date not reported)	Jang et al, 2010 (16)
11-Mo-old boy; family screening	Chinese	G943D/c.2299delC)	16	9 (0.14 μmol)	Not reported	Not reported	Mak et al, 2006 (17)
1-y-old; family screening	Lebanese	c.2299insC / c.2299insC	<2	10	ALT 44 IU/L AST 37 IU/L	Not reported	Usta et al, 2014 (18)
13-Mo-old girl; family screening	Italian	Molecular genetic testing did not reveal a mutation	8	4 (150 After penicillamine challenge)	ALT 210 IU/L AST 168 IU/L	Zinc sulfate ~23 mg BID started at ~16 mo	Iorio et al, 2003 (19)
13-Mo-old girl; unclear why	Italian	c.2299insG ho	7	Not reported	Not reported	Not reported	Nicastro et al, 2010 (20)
15-Mo-old girl; family screening	Chinese	L692P/L1015P	13.4	Not reported	Not reported	Not reported	Zhu et al, 2015 (21)
16-Mo-old girl; unclear why	Italian	P840L/N1270S	3	15	Not reported	Not reported	Nicastro et al, 2010 (20)
19-Mo-old boy; unclear why	Italian	c.2299insG ho	6	15	Not reported	Not reported	Nicastro et al, 2010 (20)
23-Mo-old girl; family screening	Greek	Not available	Not available	Not available	Not reported	Not reported	Manolaki et al, 2009 (4)

ALT = alanine aminotransferase; AST = aspartate aminotransferase.

appropriate or if there is already an over-accumulation present at birth. The experience with the toxic milk mouse models of WD suggest that offspring of an affected dam, who are themselves homozygous for the same *ATP7B* mutation and destined to have murine ''WD,'' are relatively copper deficient: they do not survive unless foster-suckled by a normal mouse (23–25). Even less is known about the contribution to fetal copper homeostasis by a mother who is heterozygous for an *ATP7B* mutation; however, the normal circulating level of copper in these individuals suggests there should be no risk for copper deficiency in infants with breastfeeding. Further study is required to understand the copper balance at birth in infants with WD to guide nutritional and pharmacological recommendations.

With copper restriction or chelation therapy, copper deficiency can potentially arise. Infantile copper deficiency is rarely severe unless the child has Menkes disease. Copper deficiency may, however, develop in infants with malnourishment, premature birth, severe gastrointestinal disorders, intractable diarrhea, and parenteral/enteral nutrition dependence (26). Signs and symptoms of copper deficiency include anemia, neutropenia, bone or vascular lesions, and central nervous system disorders (26). Although copper deficiency is rare overall, the consequences to neurological development can be severe and irreversible (26).

Nutritional Considerations: Sources of Copper and Zinc in Infant Diet

Dietary counseling for patients with WD typically includes a recommendation to avoid foods with high copper content (27). Some pediatric practitioners attach little importance to these restrictions (28). Supplemental Table 1 (Supplemental Digital Content, *http://links.lww.com/MPG/B762*) includes a list of ''high'' copper foods, defined as copper content of >0.2 mg per portion. Foods that are most typically discussed in a restrictive diet are organ meat (liver), shellfish, mushrooms, chocolate, and nuts. These foods are not commonly consumed by infants; thus, their dietary exclusion is not a challenge. The concern for infants is the amount of copper contained in infant formula and baby foods, which tend to be heavy on copper (Supplemental Table 2, Supplemental Digital Content, *http://links.lww.com/MPG/B762*).

Another consideration is the amount of zinc in infants' diets, because dietary zinc may blunt the effect of copper intake by inhibiting gut absorption. The bioavailability of zinc in breast milk is higher than that in infant formulas (29). After birth, the concentration of zinc in breast milk is initially abundant, but by the age of 6 months, like other nutrients, it decreases quite rapidly, potentially putting an infant at risk for zinc deficiency (30). A vegetarian diet of plant-based foods, such as whole grains and legumes, contains less bioavailable zinc (29).

During the first 6 months of life the primary source of nutrition is almost exclusively breast milk or formula, which provides approximately >150% estimated copper and zinc needs. The more hydrolyzed protein formulas provide higher amounts of both copper and zinc. Copper and zinc content of these formulas must be balanced against the presence of gastrointestinal disease.

In infants 6 to 12 months old, breast milk and formula remain a primary source of nutrition, whereas complementary baby foods are added (Supplemental Table 2B, Supplemental Digital Content, *http://links.lww.com/MPG/B762*). Although many families continue to offer pureed baby foods, there has been a recent movement toward the practice of ''baby-led weaning'' where solid foods are offered in their whole form (31). Notably, many of the foods provided are high in copper (>>0.2 mg copper per portion [Supplemental Table 1, Supplemental Digital Content, *http://links.lww.com/MPG/B762*]), including beans/lentils, liver (as an alternative source of iron), avocado, and sweet potatoes.

In the second year of life, infants eat a more varied diet. An average balanced diet in children 12 to 24 months old, with the associated copper and zinc content, is presented in Supplemental Table 2C (Supplemental Digital Content, *http://links.lww.com/MPG/B762*). Many infants demonstrate picky eating behaviors, which may affect copper intake.

Management of Infantile Wilson Disease: Surveillance

Monitoring WD in infancy cannot simply be a modification of how we monitor adults. Infancy is a time of rapid growth and development and obtaining blood samples and timed urine collections is difficult. Reported clinical experience suggests that serum aminotransferases are seldom abnormal before the infant is 9 to 12 months old. This is a reasonable age at which to begin monitoring liver biochemistry: ALT, AST, gamma-glutamyl transferase, total and conjugated bilirubin, albumin, and serum ceruloplasmin. Liver biochemistry can be repeated every 3 to 6 months until starting pharmacotherapy. Detection of elevated liver biochemistry (>1.5 times the upper limit of normal) requires further investigation for other etiologies of liver disease including alpha-1-antitrypsin deficiency, viral hepatitis B and C, and autoimmune liver diseases. In the absence of other infantile liver disease, abnormal liver biochemistries may warrant initiation of therapy. The identification of an additional liver disorder may make it reasonable to delay commencing WD therapy until the effect of copper overload can be confirmed as the cause of elevated liver biochemistry. Such delays should not be unduly protracted.

Liver ultrasonography provides a noninvasive examination of the liver. It can provide pertinent information about hepatic steatosis (32). Obtaining a liver ultrasound around 6 to 12 months old in a healthy infant with genetically diagnosed WD, or earlier if symptomatic, may allay parental anxiety or provide a justification for earlier intervention.

The role of liver biopsy for surveillance is unclear. In general, it should be reserved for patients with liver test abnormalities in whom the diagnosis of WD is uncertain. The role of noninvasive surveillance markers, such as transient elastography, is indeterminate as few infants have been included in studies, and no studies have been reported for infants with WD.

Importantly, this strategy is also suitable for infants whose genetic testing reveals *ATP7B* alterations characterized as variants of unknown significance. Diagnosis in this situation is perplexing. Biochemical confirmation of copper overload is necessary in patients with genetic variants of unknown significance before starting life-long treatment; consultation with a geneticist may be indicated (27).

Management of Infantile Wilson Disease: Treatment

Little published data or experience are available to determine the optimal timing for starting pharmacotherapy in infants. We require data on copper status in this young population to help guide management. A 24-hour urine collection for copper and zinc quantification is not feasible in infants who are not yet toilet trained, unless a urinary catheter is placed (20). The risks of infection or trauma from 24-hour urinary catheterization are disproportionate. Because treatment monitoring is challenging during infancy, a conservative strategy may be most appropriate. The challenge is

to balance achieving disease control against placing the infant at risk of copper deficiency during this critical phase of development.

Oral zinc is a well-established pharmacotherapy for the treatment of WD (10,33–36). Zinc, once absorbed, leads to increased synthesis of metallothionein (MT) in the enterocyte. MT binds copper preferentially over zinc, leading to sequestration of the MT-bound copper in the enterocyte and preventing copper uptake into the portal system (33). Copper is then removed via fecal excretion during regular enterocyte senescence. Zinc salts have fewer adverse effects than oral chelators; the main problem is adherence due to frequent dosing (ie, 2–3 times daily) spaced away from meals. The actual salt is not important for efficacy but may affect tolerability. In pediatric experience, zinc sulfate has been associated with notable gastric distress (33,37). A few zinc preparations exist and may theoretically differ in their tolerability and effect on appetite, nausea, vomiting, and diarrhea (38–40). Zinc as primary treatment for children with asymptomatic WD appears efficacious and well-tolerated (6,36,41,42) with the occasional dissenting report (43). Importantly, chronic treatment with zinc does not decrease hepatic parenchymal copper.

The dose of elemental zinc for treating young children (2–5 years old) is deduced from adult dosing regimens: 25 mg twice daily. This is a relatively high, truly pharmacological daily intake of zinc. Over the long term in adults with WD, zinc treatment may lead to actual copper deficiency, usually evident as anemia with neutropenia or leukopenia, although neurological deficits can develop. Little is known about any potential increased sensitivity of infants to the adverse effects of zinc treatment.

Thus, it seems reasonable to recommend zinc as initial therapy for infantile WD, based on available literature regarding management of presymptomatic children with WD diagnosed through first-degree relative screening (10). Admittedly, treatment options are limited since liquid preparations of chelators are not currently available. Based on review of previous pediatric WD case series, we would consider initial elemental zinc dosing of 25 mg per dose, twice daily, spaced 1 hour before, or 2 hours after meals, as suitable for the infant at the age of 18 months (34,44). If a suspension is unavailable, zinc should be dissolved in water. A more aggressive regimen would be smaller doses of elemental zinc (12.5 mg BID) starting at 12 months or earlier. Patients need to be monitored for copper deficiency with clinical examinations and complete blood count testing (45). Should evidence of copper deficiency develop, zinc can be held for approximately 2 to 3 months while monitoring neurological development and resolution of gastrointestinal symptoms or cytopenias. Further research is needed to determine the actual risk of copper deficiency with zinc treatment in infants with WD: it appears uncommon. Some infants may not benefit from zinc if they have advanced liver damage or persistently abnormal serum aminotransferases while on zinc therapy. They may actually require an oral chelator.

Another reasonable approach could be delaying treatment until liver biochemistry becomes abnormal or until the child is toilet-trained and can provide samples for 24-hour urinary copper measurement (46). Reported ages for successful toilet training vary in the literature, although many children are continent by 22 to 29 months.

Proposed Approach to Management of Infantile Wilson Disease: A Basis for Discussion

The broad outline of our proposal is confirmation of WD, preferably by molecular genetic testing identifying 2 trans mutations; close surveillance in the first 18 months of life; attention to dietary copper and zinc intake; initiation of zinc treatment at the age of 18 months; flexibility to alter this approach if the infant develops symptoms or biochemical evidence of liver disease earlier; and reassurance and support to parents throughout. The possibility that an infant could have WD plus some other more common liver disease cannot be disregarded. The difficulties of treatment monitoring need to be acknowledged upfront.

Special considerations relate to diet during infancy, warranting consultation with a dietician. Breast milk and baby formulas are surprisingly high in copper. Foods introduced at time of formula weaning are often high in copper. As the older infant transitions to an ordinary diet, exposure to most foods in moderation seems like a good plan. Parents may be fearful to allow their infant any intake of high copper foods, but relevant literature should be shared with parents to let them weigh risks and benefits of food choices. Addition of a chewable multivitamin (not containing copper) may be appropriate. Infants with WD should receive all routine vaccinations, including those against hepatitis A and B.

Arguably, given the variability of when WD becomes symptomatic, early treatment of asymptomatic, genetically diagnosed WD may be inappropriate. Further experience is required to address this issue. One possible strategy is that the nature of the mutation needs to be considered. Mutations predicted to result in absent ATP7B may be associated with earlier disease onset. The 3-year-old child presenting with cirrhosis due to WD was homozygous for a mutation predicted to interfere completely with production of the ATP7B (47). In a study of 59 genetically confirmed WD patients, those with 2 ''severe mutations'' (frameshift, nonsense, splice site mutations) had an earlier diagnosis of WD (median 13 years, interquartile range 9–13), whereas ''mild mutations'' were in patients diagnosed later (median 22 years, interquartile range 14–27) (48). An understanding of the infant's specific genetic mutations may influence when to start treatment, but this needs to be confirmed in formal study.

Future Directions

Family screening of first-degree relatives is already recognized as necessary following the identification of an index case. Infants born subsequent to such screening may be found to have WD (27). The scope of the problem of how to manage infants identified as having WD will increase substantially with the implementation of newborn screening. Early detection of WD via newborn screening may be feasible by quantifying ATP7B protein on dry blood cards (49). This approach is still in development.

Likewise, new treatments for WD may affect management of affected infants. Alternative therapies may include agents which ameliorate the effects of an ATP7B mutational folding defect (50). Recent findings show that inhibition of p38 and JNK pathways rescues ATP7B protein with a H1069Q mutation (51). Gene therapy, mediated by the adeno-associated virus, may become an option to correct mutations (52). Infantile WD may be the optimal setting for utilizing some of these treatments to prevent future organ damage.

CONCLUSION

Few data are available in the current literature to guide management of infants diagnosed with WD. Monitored clinical practice and multicenter collaboration will allow for an analysis of aggregate data and help guide future best management. Increased awareness of this issue and critical discussion are needed. Given a

coherent management strategy, newborn screening for WD becomes more practical. Early detection and effective management of WD hold promise of avoiding severe WD in children.

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Wilson Disease

Synonym: Hepatolenticular Degeneration

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Initial Posting: October 22, 1999; Last Update: January 12, 2023.

Estimated reading time: 36 minutes

Summary

Clinical characteristics. Wilson disease is a disorder of copper metabolism that, when untreated, can present with hepatic, neurologic, or psychiatric disturbances – or a combination of these – in individuals ages three years to older than 70 years. Manifestations in untreated individuals vary among and within families.

- Liver disease can include recurrent jaundice, simple acute self-limited hepatitis-like illness, autoimmune-type hepatitis, fulminant hepatic failure, or chronic liver disease.
- Neurologic presentations can include dysarthria, movement disorders (tremors, involuntary movements, chorea, choreoathetosis), dystonia (mask-like facies, rigidity, gait disturbance, pseudobulbar involvement), dysautonomia, seizures, sleep disorders, or insomnia.
- Psychiatric disturbances can include depression, bipolar disorder / bipolar spectrum disorder, neurotic behaviors, personality changes, or psychosis.
- Other multisystem involvement can include the eye (Kayser-Fleischer rings), hemolytic anemia, the kidneys, the endocrine glands, and the heart.

Diagnosis/testing. The diagnosis of Wilson disease is established in most instances by a combination of biochemical findings (low serum ceruloplasmin concentration, low serum concentration of total copper, and increased urinary copper excretion) and/or detection of biallelic pathogenic (or likely pathogenic) variants in *ATP7B* identified by molecular genetic testing, based on the diagnostic scoring system developed at the 8th International Meeting on Wilson Disease.

Management. *Treatment of manifestations:* Lifelong medical interventions to prevent/treat copper accumulation need to be instituted as soon as possible in all individuals with Wilson disease whether they are asymptomatic (i.e., individuals with biallelic *ATP7B* pathogenic variants who have no clinical manifestations or tissue damage related to Wilson disease), clinically asymptomatic (i.e., individuals with biallelic *ATP7B* pathogenic variants who have no clinical manifestations of Wilson disease, but have Wilson disease-related tissue damage), or symptomatic (i.e., individuals with clinical manifestations of Wilson disease and Wilson disease-related tissue damage), regardless of age and including pregnant women. As Wilson disease treatment decisions might be complex, the consultation of disease experts (primarily hepatologists and neurologists) or Wilson disease centers of excellence is advised. The first-line therapy is copper chelating agents (D-penicillamine and trientine). Zinc salts (which interfere with absorption of copper from the gastrointestinal tract) cannot be used with a copper chelating agent and are most effective after initial decoppering with a chelating agent; however, in some individuals zinc salts can be used as an initial treatment. Orthotopic liver transplantation is used for individuals who fail to respond to medical therapy or present with fulminant acute liver failure.

The goals of supportive treatment for extrahepatic manifestations of individuals with symptomatic Wilson disease are individualized to maximize function and reduce complications. Depending on their clinical manifestations, symptomatic individuals may require specialists in neurology, occupational therapy, physical therapy, physiatry, orthopedics, nutrition, speech-language pathology, social work, and psychology/psychiatry.

Surveillance: To assess treatment effectiveness and adherence to medical interventions that prevent/treat copper accumulation, the following are recommended:

- At least twice annually: assessment of serum copper and ceruloplasmin levels, liver biochemistries, international normalized ratio, complete blood count, urinalysis, and physical examination including neurologic assessment
- At least once annually: measurement of 24-hour urinary excretion of copper

Monitoring the individual's response to supportive treatment for extrahepatic manifestations and the emergence of new manifestations is per the recommendations of the treating clinical specialists.

Agents/circumstances to avoid: Foods very high in copper (liver, brain, chocolate, mushrooms, shellfish, and nuts) should be avoided, especially at the beginning of treatment.

In case of biochemical abnormalities in liver function tests or transaminases, alcohol consumption is strongly discouraged.

Evaluation of relatives at risk: It is appropriate to clarify the genetic status of asymptomatic older and younger at-risk relatives of an affected individual in order to identify as early as possible those who would benefit from prompt initiation of medical interventions to prevent/treat copper accumulation.

Pregnancy management: Treatment must be continued during pregnancy because of the risk for fulminant hepatic failure or irreversible neurologic deterioration. Because of possible adverse effects on the fetus from chelating agents, the dose should be kept as low as possible.

Genetic counseling. Wilson disease is inherited in an autosomal recessive manner. If both parents are known to be heterozygous for an *ATP7B* pathogenic variant, each sib of an affected individual has at conception a 25% chance of being affected, a 50% chance of being heterozygous, and a 25% chance of inheriting neither of the familial pathogenic variants. Once both *ATP7B* pathogenic variants have been identified in an affected family member, carrier and predictive genetic testing for at-risk relatives, prenatal testing for a pregnancy at increased risk, and preimplantation genetic testing for Wilson disease are possible.

Diagnosis

The diagnostic algorithm for Wilson disease in the European Association for Study of Liver (EASL) Clinical Practice Guidelines [European Association for Study of Liver 2012] is based on a diagnostic index ("Leipzig" score) proposed by an expert panel [Ferenci et al 2003]. This score includes clinical, biochemical, and molecular findings, but has not been validated in large patient series. The most recent diagnostic pathway of the American Association for Study of the Liver Diseases (AASLD) highlights diagnostic approaches when clinical and biochemical evaluations are ambiguous [Schilsky et al 2022b].

Suggestive Findings

Wilson disease **should be suspected** in individuals ages three to 45 years, but age alone should not exclude consideration of the diagnosis, as affected individuals have been diagnosed in their early 70s. At diagnosis, individuals with Wilson disease may have varying combinations of the following clinical findings, brain MRI findings (in those with neurologic manifestations), biochemical findings, and family history [Schilsky et al 2022b].

Clinical Findings

Children under age 18 years often present with hepatic disease exclusively.

Adults often present with hepatic disease with or without concurrent neuropsychiatric disease.

- **Liver disease** can range from recurrent jaundice, persistently elevated serum aminotransferase activity (AST, ALT), fatty liver, acute hepatitis (varying in severity, including acute liver injury), autoimmune-type hepatitis, and cirrhosis (compensated or decompensated) to acute liver failure (ALF).

  Note: Specific instances when Wilson disease should be considered is ALF with nonimmune hemolytic anemia or autoimmune hepatitis.
- **Neurologic manifestations,** resulting from central nervous system damage as a result of copper storage, can include the following:
  - Dysarthria
  - Movement disorders (tremors, involuntary movements, chorea, choreoathetosis)

- Dystonia (mask-like facies, rigidity, gait disturbance, pseudobulbar involvement)
- Dysautonomia
- Seizures
- Sleep disorders / insomnia

- **Psychiatric disturbances** can include depression, bipolar disorder / bipolar spectrum disorder, neurotic behaviors, personality changes, and psychosis.
- **Other extrahepatic involvement** can include the following:
  - Eye: Kayser-Fleisher rings, copper deposits in the periphery of the cornea, are observed by slit lamp examination and anterior segment optical coherence tomography (see Członkowska et al [2018], Figure 8). Sunflower cataracts and corneal nerve alterations can also occur.
  - Self-limited hemolytic anemia, with or without acute liver failure
  - Kidney abnormalities: aminoaciduria and nephrolithiasis
  - Hypoparathyroidism, pancreatitis
  - Cardiomyopathy, arrhythmias
  - Premature osteoporosis and arthritis
  - Infertility, recurrent miscarriages

Brain Imaging

Modalities such as magnetic resonance imaging (MRI) are of limited value in determining the extent of clinical neurologic disease but may help initially in supporting a diagnosis of Wilson disease and excluding other neurologic disorders.

Brain MRI findings consistent with Wilson disease include signal changes in the basal ganglia, thalami, pons, and white matter, as well as atrophy. Although the "face of the giant panda" sign (see Schilsky et al [2022b], Figure 1; full text), which consists of increased T_2 signal in the midbrain, has been considered pathognomonic for Wilson disease, several other findings are more commonly seen.

Biochemical Findings

Suggestive biochemical findings in a symptomatic individual relies on a combination of the following findings:

- Low serum ceruloplasmin concentration
  - **In children,** interpretation of test results requires age correction or age-specific reference ranges.

    Note: Healthy newborns have low serum ceruloplasmin concentrations. The concentrations increase during the first six months of life and peak by age two to three years at a concentration that may exceed the healthy adult reference range.
  - **In adults** with Wilson disease, serum ceruloplasmin concentration is often below the normal range (<0.2 g/L) and typically very low (<0.1 g/L).

    Note: A normal serum ceruloplasmin concentration is found in at least 5% of individuals with Wilson disease with neurologic manifestations and up to 40% of individuals with hepatic findings [Steindl et al 1997]. Serum ceruloplasmin concentration is, therefore, not a reliable screening test for Wilson disease.
- **Low serum concentration of total copper.** Most individuals with Wilson disease have a subnormal serum copper concentration that is proportional to the serum ceruloplasmin concentration (as ceruloplasmin is the main copper transporter in blood). The copper bound to ceruloplasmin (i.e., ceruloplasmin-bound copper) is considered nontoxic.

Note: Serum copper is low in healthy newborns. The concentrations increase during the first six months of life and peak by age two to three years at a concentration that may exceed the healthy adult reference range.

- **High urinary copper.** Measurement of copper in three 24-hour urine collections, free from contamination by external sources of copper, is advised. The testing laboratory should be consulted regarding its trace element urine collection protocol prior to initiating urine specimen collection.
  - **Basal urinary copper excretion** (without the use of chelating agents) is almost invariably elevated above 40 µg or ~0.6 µmol/24 hours in most individuals with Wilson disease, and above 100 µg or ~1.6 µmol/24 hours in symptomatic individuals.
  - **A provocative test of urinary copper excretion** following oral administration of D-penicillamine has been validated only in pediatric cohorts, but has proven useful in some adults [Martins da Costa et al 1992]; however, levels in affected individuals can overlap with those of heterozygotes. Note: The use of a lower value for basal urinary copper excretion of 40 µg or ~0.6 µmol/24 hours increases diagnostic sensitivity and may obviate the need for the D-penicillamine provocation test.
- **Hepatic copper quantification.** Although liver biopsy is an invasive procedure, it can be helpful when clinical findings, biochemical findings, and/or molecular genetic test results are ambiguous. Hepatic copper concentration in Wilson disease is usually greater than 250 µg/g dry weight (normal: <55 µg/g dry weight [Nuttall et al 2003]); however, such levels may be seen in other chronic liver disorders as well as cholestatic conditions [Schilsky et al 2022b].

  Note: (1) In later stages of Wilson disease, copper is distributed unevenly in the liver and measurement of hepatic copper concentration is less reliable. (2) Some individuals have only a moderately elevated hepatic copper concentration (100-250 µg/g dry weight), which overlaps with values occasionally found in heterozygotes. Thus, hepatic copper concentration in this range does not exclude the diagnosis of Wilson disease.

Family History

Family history is consistent with autosomal recessive inheritance. The family history may include affected sibs (e.g., sibs with liver disease, neurologic manifestations, and/or psychiatric disturbance) and/or parental consanguinity. Absence of a known family history does not preclude the diagnosis.

Establishing the Diagnosis

The diagnosis of Wilson disease, using clinical, biochemical, and molecular genetic findings, is based on the diagnostic scoring system developed at the 8th International Meeting on Wilson Disease, Leipzig 2001 [Ferenci et al 2003, Członkowska et al 2018] (see Table 1).

Table 1.

Diagnostic Scoring System for Wilson disease

Test	Parameter	Score
Typical clinical symptoms & signs		
Kayser-Fleischer rings	Present	2
	Absent	0
Neurologic manifestations [1]	Severe	2
	Mild	1
	Absent	0
Serum ceruloplasmin	Normal (>0.2 g/L)	0
	0.1-0.2 g/L	1
	<0.1 g/L	2

Test	Parameter	Score
Coombs-negative hemolytic anemia	Present	1
	Absent	0
Other tests		
Liver copper (in the absence of cholestasis)	>250 µg (>4 µmol)/g dry weight	2
	50-249 µg (0.8–4 µmol)/g dry weight	1
	Normal: <50 µg (<0.8 µmol)/g dry weight	-1
	Rhodanine-positive granules [2]	1
Urinary copper (in the absence of acute hepatitis)	Normal	0
	1-2x ULN	1
	>2x ULN	2
	Normal but >5x ULN after D-penicillamine	2
ATP7B molecular genetic testing	Biallelic pathogenic variants detected	4
	One pathogenic variant detected	1
	No pathogenic variants detected	0
Evaluation	**Total score**	
	Diagnosis established	≥4
	Diagnosis possible, more tests needed	3
	Diagnosis very unlikely	≤2

Adapted with permission from Ferenci et al [2003]

ULN = upper limit of normal

1. Or typical abnormalities on brain MRI
2. If no quantitative liver copper available

Per the diagnostic scoring system (see Table 1), the diagnosis of Wilson disease **can be established** in a proband with suggestive findings and biallelic pathogenic (or likely pathogenic) variants in *ATP7B* identified by molecular genetic testing (see Table 2).

Note: (1) Per ACMG/AMP variant interpretation guidelines, the terms "pathogenic variants" and "likely pathogenic variants" are synonymous in a clinical setting, meaning that both are considered diagnostic and both can be used for clinical decision making [Richards et al 2015]. Reference to "pathogenic variants" in this section is understood to include any likely pathogenic variants. (2) Identification of biallelic *ATP7B* variants of uncertain significance (or of one known *ATP7B* pathogenic variant and one *ATP7B* variant of uncertain significance) does not establish or rule out a diagnosis.

Molecular genetic testing approaches can include a combination of **gene-targeted testing** (single-gene testing, multigene panel) (see Option 1) and **comprehensive genomic testing** (exome sequencing, genome sequencing) (see Option 2). Gene-targeted testing requires that the clinician determine which gene(s) are likely involved, whereas genomic testing does not.

Option 1

Single-gene testing. When clinical and biochemical findings strongly suggest the diagnosis of Wilson disease, sequence analysis of *ATP7B* is performed first to detect small intragenic deletions/insertions and missense, nonsense, and splice site variants. Note: Depending on the sequencing method used, single-exon, multiexon, or whole-gene deletions/duplications may not be

detected. If only one or no variant is detected by the sequencing method used, the next step is to perform gene-targeted deletion/duplication analysis to detect exon and whole-gene deletions or duplications.

Note: Targeted analysis can be performed first in individuals from populations with known founder variants (e.g., Ashkenazi Jewish, Canary Islands, Druze, Sardinia; see Table 7).

A multigene panel that includes *ATP7B* and other genes of interest (see Differential Diagnosis) is most likely to identify the genetic cause of the condition while limiting identification of variants of uncertain significance and pathogenic variants in genes that do not explain the underlying phenotype. Note: (1) The genes included in the panel and the diagnostic sensitivity of the testing used for each gene vary by laboratory and are likely to change over time. (2) Some multigene panels may include genes not associated with the condition discussed in this *GeneReview*. (3) In some laboratories, panel options may include a custom laboratory-designed panel and/or custom phenotype-focused exome analysis that includes genes specified by the clinician. (4) Methods used in a panel may include sequence analysis, deletion/duplication analysis, and/or other non-sequencing-based tests.

For an introduction to multigene panels click here. More detailed information for clinicians ordering genetic tests can be found here.

Option 2

Comprehensive genomic testing does not require the clinician to determine which gene is likely involved. **Exome sequencing** is most commonly used; **genome sequencing** is also possible.

For an introduction to comprehensive genomic testing click here. More detailed information for clinicians ordering genomic testing can be found here.

Table 2.

Molecular Genetic Testing Used in Wilson Disease

Gene [1]	Method	Proportion of Probands with Pathogenic Variants [2] Detectable by Method
ATP7B	Sequence analysis [3]	98% [4]
	Gene-targeted deletion/duplication analysis [5]	Rare [6]

1. See Table A. Genes and Databases for chromosome locus and protein.
2. See Molecular Genetics for information on variants detected in this gene.
3. Sequence analysis detects variants that are benign, likely benign, of uncertain significance, likely pathogenic, or pathogenic. Variants may include small intragenic deletions/insertions and missense, nonsense, and splice site variants; typically, exon or whole-gene deletions/duplications are not detected. For issues to consider in interpretation of sequence analysis results, click here.
4. Data derived from the subscription-based professional view of Human Gene Mutation Database [Stenson et al 2020]
5. Gene-targeted deletion/duplication analysis detects intragenic deletions or duplications. Methods used may include a range of techniques such as quantitative PCR, long-range PCR, multiplex ligation-dependent probe amplification (MLPA), and a gene-targeted microarray designed to detect single-exon deletions or duplications.
6. Large deletions and duplications, encompassing one or more exons, are rare. Exon and multiexon deletions have been reported (see, e.g., Møller et al [2005], Incollu et al [2011], Møller et al [2011], Tatsumi et al [2011]).

Clinical Characteristics

Clinical Description

Untreated symptomatic Wilson disease can manifest in individuals ages three years to older than 70 years as hepatic, neurologic, psychiatric, or hematologic disturbances, or a combination of these. Phenotypic expression varies even within families. The understanding of the phenotypic spectrum has further expanded through the widespread use of molecular genetic testing, which has confirmed the diagnosis in individuals with atypical clinical and biochemical findings.

Table 3 outlines the typical presenting clinical findings of untreated Wilson disease. Of note, the "classic triad" of liver disease, movement disorder, and Kayser-Fleischer ring is uncommon.

Table 3.

Clinical Findings in Individuals with Untreated Symptomatic Wilson Disease by Presenting Finding

Presenting Finding	% of Persons	Typical Age of Presentation (Range)	Liver Disease	Neurologic Disease	Psychiatric Disturbance	Kayser-Fleischer Rings
Liver disease	~40%	6-45 yrs (3-70 yrs)	+	+/–	+/–	~50%
Neurologic disease	~40%	Mid-teen to mid-adult (6-50 yrs)	–/mild	+	+/–	~90%
Psychiatric disturbance	~20%	Adolescent to young adult	–/mild	+/–	+	~90%
Hemolytic anemia	Few		+	–	–	+

Bruha et al [2011], Weiss et al [2011], Hofer et al [2012], Weiss et al [2013b]

Untreated Symptomatic Wilson Disease

Liver disease. Untreated Wilson disease manifests as liver disease more commonly in children and younger adults, typically between ages six and 45 years; however, severe liver disease can be the initial finding in preschool-aged children [Wilson et al 2000] and in older adults. The clinical manifestations vary and can include the following findings:

- **Recurrent jaundice,** possibly caused by hemolysis
- **Simple, acute, self-limited hepatitis-like illness** with fatigue, anorexia, and/or abdominal pain
- **Autoimmune hepatitis,** often manifesting acutely with fatigue, malaise, arthropathy, and rashes. This form of liver disease responds well to chelation therapy even if cirrhosis is present (see Management).
- **Fulminant hepatic failure** with severe coagulopathy, encephalopathy, acute Coombs-negative intravascular hemolysis, and often rapidly progressive renal failure. Serum activity of aminotransferases is only moderately increased, and serum concentration of alkaline phosphatase is normal or extremely low. These individuals do not respond to chelation treatment and require urgent liver transplantation (see Management).
- **Chronic liver disease** with portal hypertension, hepatosplenomegaly, ascites, low serum albumin concentration, and coagulopathy
- **Fatty liver** of mild-to-moderate degree with abnormal liver function

Neurologic involvement follows two general patterns: movement disorders or rigid dystonia.

- Movement disorders tend to occur earlier and include tremors, poor coordination, loss of fine motor control, micrographia (abnormally small, cramped handwriting), chorea, and/or choreoathetosis.
- Spastic dystonia disorders manifest as mask-like facies, rigidity, and gait disturbance [Svetel et al 2001].

Pseudobulbar involvement such as dysarthria, drooling, and difficulty swallowing is more common in older individuals, but also occurs in children and adolescents.

In contrast to the neurologic findings in individuals with a frank neurologic presentation, the neurologic findings in individuals with a hepatic presentation may be subtle. Mood disturbance (mainly depression; occasionally poor impulse control), changes in school performance, and/or difficulty with fine motor skills (especially handwriting) or gross motor skills may be observed.

In individuals with a neurologic presentation, extensive changes on brain imaging (such as evidence of tissue cavitation) suggest structural, irreversible brain damage. These individuals are less likely to improve with treatment [Sinha et al 2007].

Psychiatric manifestations are variable. Depression is common. Neurotic behavior includes phobias, compulsive behaviors, aggression, or antisocial behavior. Older individuals may have subtle psychopathology (e.g., progressive disorganization of personality with anxiety) and affective changes (e.g., labile mood and disinhibition). Pure psychotic disorders are uncommon.

Intellectual deterioration may also occur with poor memory, difficulty in abstract thinking, and shortened attention span.

Hemolytic anemia, with either acute or chronic hemolysis, indicates a high serum concentration of non-ceruloplasmin-bound copper, which leads to destruction of erythrocytes. Liver disease is likely to be present in such individuals, as are Kayser-Fleischer rings. Recurrent hemolysis predisposes to cholelithiasis, even in children.

Other extrahepatic involvement

- Kayser-Fleischer rings result from copper deposition in Descemet's membrane of the cornea and reflect a high degree of copper storage in the body. They do not affect vision and are reduced or disappear with effective decoppering treatment (see Management).
- Kidney involvement: low molecular weight proteinuria, microscopic hematuria, Fanconi syndrome, aminoaciduria, and nephrolithiasis
- Arthritis: involvement of large joints from synovial copper accumulation
- Reduced bone mineral density with an increased prevalence of osteoporosis (in approximately 10% of affected individuals)
- Pancreatitis, cardiomyopathy, cardiac arrhythmias, rhabdomyolysis of skeletal muscle, and various endocrine disorders
- Sunflower cataracts: observed occasionally on slit lamp examination

Hepatocellular carcinoma rarely develops in Wilson disease; the estimated incidence is below 1% [Devarbhavi et al 2012].

Fertility and pregnancy. Most individuals with Wilson disease are fertile.

Successful pregnancies of women with Wilson disease who received treatment have been reported [Brewer et al 2000, Tarnacka et al 2000, Furman et al 2001]. Prior to diagnosis and treatment of Wilson disease, affected women may experience amenorrhea, infertility, or recurrent miscarriage [Członkowska et al 2018].

Treated Wilson Disease

The mainstay of treatment for Wilson disease remains lifelong oral pharmacotherapy and dietary copper restriction [Schilsky et al 2022b] (see Management, Medical Interventions to Prevent/Treat Copper Accumulation). Liver transplantation, which corrects the underlying hepatic defect in Wilson disease, is reserved for individuals with chronic or acute liver failure and those resistant to pharmacotherapy.

- **"Asymptomatic individuals with Wilson disease"** are those who have biallelic *ATP7B* pathogenic variants who are clinically asymptomatic and **do not have any Wilson disease-related tissue damage**. Typically these individuals are young infants, born to parents known to be carriers, identified by genetic testing during family screening. These children should remain asymptomatic on treatment, even if they have biochemical abnormalities but not Wilson disease-related tissue damage. (See Management, Evaluation of Relatives at Risk and Medical Interventions to Prevent/Treat Copper Accumulation.)
- **"Clinically asymptomatic individuals with Wilson disease"** are those who have biallelic *ATP7B* pathogenic variants who are clinically asymptomatic but **have Wilson disease-related tissue damage**. Treatment at this stage of disease is highly successful and is focused on stabilizing and reversing tissue injury and preventing the progression of symptoms.
- **Individuals with Wilson disease with symptomatic liver disease.** Improvement in synthetic function and clinical signs such as jaundice and ascites begins during the first two to six months of treatment, with further recovery possible over time.
- **Individuals with Wilson disease with neurologic or psychiatric manifestations.** Most stabilize within six to 18 months after initiation of consistent therapy. However, neurologic findings may not respond to medical treatment, and in a few instances individuals with preexisting neurologic findings might show a paradoxical worsening, with acceleration of neurologic involvement or development of new manifestations.

Genotype-Phenotype Correlations

No genotype-phenotype correlations for *ATP7B* have been identified [Członkowska et al 2018, Ferenci et al 2019].

Nomenclature

The neurologic form of Wilson disease has also been known as Westphal-Strumpell pseudosclerosis.

Prevalence

The prevalence of Wilson disease is estimated at one in 30,000 in most populations, with a corresponding carrier frequency in the general population of one in 90 [Sandahl et al 2020].

In some population-based studies, the genetic prevalence was three to four times higher than clinically based estimates [Olivarez et al 2001, Coffey et al 2013], pointing to the complexity when classifying variants regarding its disease-causing potential and raising the question of whether penetrance is really 100%, as generally assumed.

Recent studies suggest a prevalence as high as one in 10,000, especially in isolated populations such as Sardinia [Gialluisi et al 2013].

Founder variants have been identified in persons of Ashkenazi Jewish and Druze heritage, as well as individuals from the Canary Islands and Sardinia (see Table 7).

Genetically Related Disorders

No phenotypes other than those discussed in this *GeneReview* are known to be associated with germline pathogenic variants in *ATP7B*.

Differential Diagnosis

The complete differential diagnosis of Wilson disease is extensive and includes:

- Copper metabolism disorders;
- Hereditary disorders involving the liver;
- Hereditary disorders involving the nervous system; and
- Acquired conditions such as viral hepatitis, severe drug toxicity, and nonalcoholic steatohepatitis (NASH).

Note: Wilson disease must be specifically excluded in individuals thought to have NASH, or the opportunity for life-saving treatment will be missed.

Table 4 lists selected genetic disorders of interest in the differential diagnosis of Wilson disease (see also Schilsky et al [2022b], Table 5).

Table 4.

Hereditary Disorders of Known Genetic Cause in the Differential Diagnosis of Wilson Disease

Gene(s)	Disorder	MOI	Copper Metabolism
Copper metabolism disorders			
AP1S1	MEDNIK syndrome (OMIM 609313)	AR	Low ceruloplasmin
ATP7A	Menkes disease (See *ATP7A*-Related Copper Transport Disorders.) [1]	XL	Low serum copper & low ceruloplasmin
	Occipital horn syndrome (See *ATP7A*-Related Copper Transport Disorders.)	XL	
	ATP7A-related distal motor neuropathy (See *ATP7A*-Related Copper Transport Disorders.)	XL	Normal
CP	Aceruloplasminemia [2]	AR	Low ceruloplasmin
SLC33A1	Huppke-Brendel syndrome [3]	AR	Low serum copper & low ceruloplasmin
Liver diseases [4]			
ABCB4	MDR3 deficiency (PFIC3) (See Pediatric Genetic Cholestatic Liver Disease Overview.)	AR	Hepatic copper retention due to cholestasis

Gene(s)	Disorder	MOI	Copper Metabolism
HFE	*HFE* hemochromatosis [5]	AR	Hepatic copper retention due to cholestasis is possible.
SERPINA1	Alpha-1 antitrypsin deficiency [5]	AD [6]	
Neurologic disorders			
ATN1	DRPLA	AD	Normal
DNAJC6 *FBXO7* *PARK7* *PINK1* *PRKN* *SYNJ1* *VPS13C*	Early-onset Parkinson disease (See Parkinson Disease Overview.)	AR	
GCH1 *TOR1A*	Inherited forms of dystonia incl DYT1 early-onset isolated dystonia & GTPCH1-deficient dopa-responsive dystonia	AD	
HTT	Huntington disease	AD	
NPC1 *NPC2*	Niemann-Pick disease type C	AR	
Many genes [7]	Hereditary ataxia	AD AR XL Mat [8]	

AD = autosomal dominant; AR = autosomal recessive; Mat = maternal; MOI = mode of inheritance; PFIC = progressive familial intrahepatic cholestasis; XL = X-linked

1. Onset during infancy
2. Iron overload due to lack of oxidase activity of ceruloplasmin
3. Characterized by cataract, sensorineural deafness, and severe developmental delay
4. Primary sclerosing cholangitis (OMIM 613806) and primary biliary cirrhosis (OMIM 109720) also present with abnormal liver biochemistries with or without hepatomegaly. The genetic basis of these disorders is unknown.
5. Presents with abnormal liver biochemistries with or without hepatomegaly
6. Alpha-1 antitrypsin deficiency is inherited in an autosomal codominant manner.
7. See Hereditary Ataxia Overview, Causes.
8. The mode of inheritance depends on the genetic etiology of ataxia.

Management

Evaluations Following Initial Diagnosis

To establish the extent of disease and needs in an individual with symptomatic untreated Wilson disease, the evaluations summarized in Table 5 (if not performed as part of the evaluation that led to the diagnosis) are recommended.

Table 5.

Recommended Evaluations Following Initial Diagnosis in Individuals with Symptomatic Untreated Wilson Disease

System/Concern		Evaluation	Comment
Primary manifestations			
Liver disease		Liver biopsy or biochemical testing & imaging of liver	• Establish baseline copper studies (serum ceruloplasmin & serum copper & 24-hr urinary copper excretion). • Consider additional upper GI endoscopy to exclude or confirm esophageal varices.
Neurologic		Neurologist assess for: • Movement disorders • Gait & balance disturbance	Using validated neurologic rating scale (neurologic subscale of Unified Wilson's Disease Rating Scale) [1]
Speech		For those w/dysarthria: eval by speech-language pathologist	
Musculoskeletal/ADL		Eval by physiatrist/OT/PT	To assess gross motor & fine motor skills, gait, ambulation, need for adaptive devices
Cognitive		Assess for cognitive dysfunction.	
Psychiatric		Eval by psychiatrist, psychologist, neuropsychologist if needed	For personality & mood disorders
Eyes		Complete eye exam	To incl assessment for Kayser-Fleischer rings, sunflower cataracts
Possible secondary manifestations			
Endocrine disorders	Glucose intolerance	Basic biochemical profile	Underlying liver disease might affect hormone metabolism.
	Parathyroid insufficiency		
	Disordered growth		
	Males: gynecomastia		
	Females: menstrual irregularity / amenorrhea		
	Frequent miscarriage		
Cardiac involvement	Cardiac arrhythmia	By cardiolologist	
	Cardiomyopathy		
Renal involvement		By nephrologist	Assess for: • Tubular dysfunction (e.g., aminoaciduria, hypercalcuria, hyperphosphaturia) • Nephrolithiasis, nephrocalcinosis
Genetic counseling		By genetics professionals [2]	To inform affected persons & their families re nature, MOI, & implications of Wilson disease to facilitate medical & personal decision making

System/Concern	Evaluation	Comment
Family support & resources	By treating clinicians, social workers	Assess need for: • Community or online resources; • Social work involvement for parental/caregiver support; • Home nursing referral.

ADL = activities of daily living; GI = gastrointestinal; OT = occupational therapist; PT = physical therapist

Adapted from Schilsky et al [2022b]

1. Członkowska et al [2007], Leinweber et al [2008]
2. Medical geneticist, certified genetic counselor, certified advanced genetic nurse

Treatment of Manifestations

Medical Interventions to Prevent/Treat Copper Accumulation in Individuals with Wilson Disease Who Are Asymptomatic, Clinically Asymptomatic, or Symptomatic

See extensive review by the American Association for the Study of Liver Diseases [Schilsky et al 2022b] (full text) and EASL Clinical Practice Guidelines: Wilson's disease [European Association for Study of Liver 2012] (full text).

Individuals with Wilson disease can be clinically categorized as:

- "Asymptomatic" (individuals who have no clinical manifestations or tissue damage related to Wilson disease);
- "Clinically asymptomatic" (individuals who have no clinical manifestations of Wilson disease but have Wilson disease-related tissue damage); or
- "Symptomatic" (individuals who have clinical manifestations of Wilson disease and Wilson disease-related tissue damage).

The goal of therapy is to institute treatment with chelating agents as soon as possible in individuals with Wilson disease who are asymptomatic, clinically asymptomatic, or symptomatic.

- Treatment is lifelong, including during pregnancy.
- If one treatment is discontinued, an alternative modality must be substituted to prevent disease progression.
- Discontinuation of all treatment leads to hepatic and neurologic decompensation that is usually refractory to further medical intervention.
- During lifelong treatment, failure of any medication used to treat Wilson disease may occur, either at initiation of treatment or during maintenance therapy. Once concurrent disease and nonadherence are excluded, pharmacologic therapy should be re-evaluated and likely altered. For individuals who have more advanced liver disease or develop liver failure, evaluation for liver transplantation should be considered. Currently, no surrogate markers are established for evaluating treatment failure.

Asymptomatic individuals should be treated either with lower dosages (10-15 mg/kg) of a copper chelating agent (D-penicillamine or trientine) or zinc salts.

Clinically asymptomatic individuals should be treated with 15-20 mg/kg of a copper chelating agent (D-penicillamine or trientine).

Symptomatic individuals should be treated with 15-20 mg/kg of a copper chelating agent (D-penicillamine or trientine). However, some individuals with advanced liver disease may require more intensive therapy, and temporally separated combination therapy may be utilized.

Copper chelating agents that increase urinary excretion of copper are the first-line treatment for persons with symptomatic Wilson disease. Note: Routine institution of chelation therapy before age three years has not been adequately assessed and may have adverse effects on growth.

- **D-penicillamine (chelator).** Used since the 1950s as first-line therapy for Wilson disease [Durand et al 2001, Walshe 2003], D-penicillamine is given as tablets by mouth two or three times daily. Pyridoxine must be given along with D-penicillamine. Twenty-four-hour urine copper excretion is used to confirm chelation and increased excretion of copper. Urinary copper values should be five to ten times normal; if the values are lower, noncompliance may be an issue, or body copper stores may have been adequately depleted.
    - Complete blood count and urinalysis must be monitored regularly during D-penicillamine therapy. Serious side effects can occur in up to 30% of individuals, and include severe thrombocytopenia, leukopenia, aplastic anemia, proteinuria, nephrotic syndrome, polyserositis, Goodpasture syndrome, and severe skin reactions. An early allergic reaction with fever, rash, and proteinuria may occur. Evidence of any such side effects may require discontinuation of D-penicillamine and substitution of an alternate treatment. If such alternate therapies are unavailable, D-penicillamine-induced adverse events may be manageable by coadministration of steroids.
    - D-penicillamine inhibits collagen cross-linking and has some immunosuppressant properties. After decades of treatment, individuals may have abnormal skin and connective tissue collagen, and possible chronic depletion of copper and (possibly) other trace metals.
    - D-penicillamine should NOT be used simultaneously with zinc, pending adequate clinical assessment of this treatment strategy.
- **Trientine (chelator),** also known as triethylene tetramine dihydrochloride (2,2,2-tetramine) or trien, has been the usual second-line treatment for individuals who cannot tolerate D-penicillamine. However, a clinical trial of an alternative formulation, triethylene tetramine tetrahydrochloride, revealed good efficacy and better tolerance than D-penicillamine, supporting the concept of its use as first-line therapy [Schilsky et al 2022a].
    - Complete blood count and urinalysis must be monitored regularly in all individuals on trientine.
    - Rare side effects include gastritis with nausea and, in cases of overtreatment, iron deficiency anemia.
    - Trientine should NOT be used simultaneously with zinc pending adequate assessment of this combination. Current reports suggest that the combination of trientine and zinc, temporally dispersed throughout the day such that each drug is administered five to six hours apart from the other, may be effective in severely decompensated hepatic Wilson disease [Santos Silva et al 1996, Askari et al 2003].

Zinc (metallothionein inducer). High-dose oral zinc interferes with absorption of copper from the gastrointestinal tract, presumably by inducing enterocyte metallothionein, which preferentially binds copper from the intestinal contents and is lost in the feces as enterocytes are shed in normal turnover. Zinc therapy is most effective after initial decoppering with a chelating agent [Brewer 2001, Brewer et al 2001]. In selected individuals, it can be used as an initial treatment [Milanino et al 1992, Linn et al 2009].

Zinc is taken as tablets by mouth at least twice (usually 3 times) daily before meals. The dose is based on the elemental zinc in the tablet.

Twenty-four-hour urine copper excretion is used to monitor total body copper stores, which should decrease. Increase of urinary copper excretion under zinc therapy may indicate insufficient treatment efficacy [Weiss et al 2011]. Serum or urinary zinc concentration can be measured to monitor compliance in individuals taking zinc.

Note: (1) Gastritis, a common side effect, can be reduced with the use of zinc acetate or zinc gluconate. (2) Zinc should NOT be used simultaneously with any chelator, pending further clinical investigation.

Restriction of foods very high in copper (liver, brain, chocolate, mushrooms, shellfish, and nuts) is likely prudent, especially at the beginning of treatment. It is recommended that individuals with special dietary needs (e.g., vegetarians) consult with a trained dietitian [Schilsky et al 2022b].

Orthotopic Liver Transplantation

Orthotopic liver transplantation (OLT) is reserved for individuals who fail to respond to medical therapy or cannot tolerate it because of serious adverse side effects [Schilsky et al 2022b].

It remains controversial whether orthotopic liver transplantation should be a primary treatment for individuals with Wilson disease who have severe neurologic disease [Medici et al 2005, Weiss et al 2013a, Litwin et al 2022].

Supportive Treatment for Extrahepatic Manifestations

The goals of supportive treatment for extrahepatic manifestations of individuals with symptomatic Wilson disease are individualized to maximize function and reduce complications. Ideally each individual consults with multidisciplinary specialists in fields such as neurology, occupational therapy, physical therapy, physiatry, orthopedics, nutrition, speech-language pathology, social work, and psychology/psychiatry, depending on the clinical manifestations.

Surveillance

Assessment of Treatment Effectiveness and Adherence to Medical Interventions to Prevent/Treat Copper Accumulation

Monitoring of individuals under therapy should include routine assessments of treatment efficacy by biochemical testing and clinical evaluation.

- Insufficient therapy, underdosage, or poor compliance could lead to reaccumulation of copper and development of new symptoms.
- Adverse events related to medical treatment (especially under D-penicillamine treatment) should be evaluated.
- Excessive long-term treatment could result in copper deficiency, leading to immobilization of iron (as observed in aceruloplasminemia) and neurologic symptoms of copper deficiency [Horvath et al 2010, da Silva-Júnior et al 2011].

According to current guidelines (AASLD [Schilsky et al 2022b] and EASL Clinical Practice Guidelines [European Association for Study of Liver 2012]), routine monitoring should include the following examinations:

- At least twice annually: serum copper and ceruloplasmin, liver biochemistries, international normalized ratio, complete blood count, urinalysis, and physical examination including neurologic assessment

  Note: Individuals receiving chelation therapy require a complete blood count and urinalysis regularly, no matter how long they have been on treatment.
- At least once annually: 24-hour urinary excretion of copper

  Note: Measurements are recommended more frequently if there are questions on compliance or if dosage of medications is adjusted.

Supportive Care

To monitor the individual's response to supportive care and the emergence of new manifestations, the evaluations in Table 6 are recommended based on the supportive treatment required by the individual.

Table 6.

Recommended Surveillance of Extrahepatic Manifestations for Individuals with Symptomatic Wilson Disease

System/Concern	Evaluation	Frequency
Neurologic	Assess for new manifestations such as seizures, changes in tone, & movement disorders.	At each visit
	Consider neuroimaging if new manifestations occur.	Per treating neurologist
Cognitive	Monitor educational needs.	At each visit
Speech & language	By speech-language pathologist & consideration of alternative means of communication	When clinically evident
Feeding	Eval of nutritional status & safety of oral intake	At each visit
Psychiatric/Behavioral	Behavioral assessment for depression, bipolar disorder, personality changes, & aggressive or self-injurious behavior	

System/Concern	Evaluation	Frequency
Musculoskeletal	Physical medicine, OT/PT assessment of mobility, self-help skills	
Family/Community	Assess family need for social work support (e.g., palliative/respite care, home nursing, other local resources), care coordination, or follow-up genetic counseling if new questions arise (e.g., family planning).	

OT = occupational therapy; PT = physical therapy

Agents/Circumstances to Avoid

Foods very high in copper (liver, brain, chocolate, mushrooms, shellfish, and nuts) should be avoided, especially at the beginning of treatment.

In case of biochemical abnormalities in liver function tests or transaminases, alcohol consumption is strongly discouraged.

Evaluation of Relatives at Risk

It is appropriate to clarify the genetic status of apparently asymptomatic older and younger at-risk relatives of an affected individual in order to identify as early as possible those who would benefit from prompt initiation of medical interventions to prevent/treat copper accumulation (see Medical Interventions to Prevent/Treat Copper Accumulation). Asymptomatic and clinically asymptomatic individuals with Wilson disease should remain asymptomatic on treatment, even if they have biochemical abnormalities, histologic findings, or imaging evidence of organ damage. Evaluations can include:

- Molecular genetic testing if the *ATP7B* pathogenic variants in the family are known;
- If the *ATP7B* pathogenic variants in an affected family member are not known, biochemical assessment of parameters of copper metabolism (serum copper, urinary copper, ceruloplasmin) and liver function tests as well as ultrasound imaging of the liver (the finding of a "fatty liver" is common, even in young or asymptomatic individuals) and slit lamp examination for the presence of Kayser-Fleischer rings.

  Note: Asymptomatic individuals with Wilson disease generally have a low serum concentration of ceruloplasmin and mildly increased basal 24-hour urinary copper excretion; however, sometimes asymptomatic individuals with Wilson disease cannot be easily distinguished from heterozygotes.

Although Wilson disease is an autosomal recessive disorder and the risk to the parents and offspring of a proband is low, screening of all first-degree relatives is recommended in order to ascertain clinically asymptomatic family members in whom treatment may prevent liver disease and other manifestations of Wilson disease [Schilsky et al 2022b].

See Genetic Counseling for issues related to testing of at-risk relatives for genetic counseling purposes.

Pregnancy and Lactation Concerns

Pregnancy. Treatment must be continued during pregnancy because of the risk of fulminant hepatic failure and/or neurologic decline in the affected pregnant woman. Baseline biochemical and clinical assessment as soon as a pregnancy is recognized is important. This includes evaluation for portal hypertension in those who have cirrhosis because of the risk of peripartum variceal hemorrhage [Członkowska et al 2018].

- D-penicillamine has been used in many pregnancies with no adverse outcomes; however, congenital connective tissue disorders encompassing inguinal hernias and skin laxity have been reported in some exposed infants. Such adverse outcomes may depend on dose, which should be kept as low as possible while still preventing copper deficiency in the pregnant woman and accounting for the need for fetal copper during development [Członkowska et al 2018]. The dose of D-penicillamine should be maintained at the lowest effective dose during the first and second trimesters of pregnancy. Further reduction in dose may be considered in the third trimester – based on acceptable results of maternal biochemical liver function tests – to account for the increasing copper utilization by the growing fetus.
- Trientine has been used successfully during pregnancy, but the total number of reported individuals is small. Reduction of the dose to the lowest effective dose is recommended using a comparable approach to that for D-penicillamine.

- Zinc has been used effectively in pregnant women and typically does not require a decreased dose during pregnancy. However, changing medical therapy to zinc during pregnancy does not appear to decrease the risk of either miscarriage or adverse fetal outcomes [Członkowska et al 2018].

Lactation. All anti-copper medications appear to pass into breast milk, which can lead to copper deficiency in infants. Therefore, breastfeeding or using expressed maternal breast milk from a mother taking an anti-copper medication is not generally recommended [Członkowska et al 2018].

See MotherToBaby for further information on medication use during pregnancy and lactation.

Therapies Under Investigation

Tetrathiomolybdate (TTM) is an orally administered chelating agent proposed to work by multiple mechanisms [Plitz & Boyling 2019] including:

- Detoxifying non-ceruloplasmin-bound copper by creating a nonreactive tripartite complex with albumin and copper;
- Extracting copper from the endogenous cellular chelator metallothionein (based on its high affinity for copper); and
- Interfering with the intestinal uptake of copper when administered with food.

In a Phase II study [Weiss et al 2017], TTM effectively reduced non-ceruloplasmin-bound copper (corrected for copper-TTM-albumin complex) and improved clinical neurologic findings, without paradoxical neurologic worsening, as demonstrated by an overall improvement in Unified Wilson's Disease Rating Scale scores [Leinweber et al 2008]. Early elevation of serum aminotransferases in approximately 30% of individuals resolved with dose discontinuation or reduction; none developed evidence of drug-induced liver injury. Suitability for treating advanced hepatic Wilson disease requires further investigation. A Phase III trial of bis-choline TTM for Wilson disease is under way.

Search ClinicalTrials.gov in the US and EU Clinical Trials Register in Europe for access to information on clinical studies for a wide range of diseases and conditions.

Genetic Counseling

Genetic counseling is the process of providing individuals and families with information on the nature, mode(s) of inheritance, and implications of genetic disorders to help them make informed medical and personal decisions. The following section deals with genetic risk assessment and the use of family history and genetic testing to clarify genetic status for family members; it is not meant to address all personal, cultural, or ethical issues that may arise or to substitute for consultation with a genetics professional. —ED.

Mode of Inheritance

Wilson disease is inherited in an autosomal recessive manner.

Risk to Family Members

Parents of a proband

- The parents of an affected individual are presumed to be heterozygous for an *ATP7B* pathogenic variant.
- If a molecular diagnosis has been established in the proband, genetic testing is recommended for the parents of a proband to confirm that both parents are heterozygous for an *ATP7B* pathogenic variant and to allow reliable recurrence risk assessment.
- If a pathogenic variant is detected in only one parent and parental identity testing has confirmed biological maternity and paternity, it is possible that one of the pathogenic variants identified in the proband occurred as a *de novo* event in the proband or as a postzygotic *de novo* event in a mosaic parent [Jónsson et al 2017]. If the proband appears to have homozygous pathogenic variants (i.e., the same two pathogenic variants), additional possibilities to consider include:
    - A single- or multiexon deletion in the proband that was not detected by sequence analysis and that resulted in the artifactual appearance of homozygosity;
    - Uniparental isodisomy for the parental chromosome with the pathogenic variant that resulted in homozygosity for the pathogenic variant in the proband.

- Clinical disease is not known to occur in heterozygotes (carriers), although the possibility has not been adequately excluded at older ages. Note: Heterozygotes may have subclinical biochemical findings including low serum ceruloplasmin concentrations, borderline normal urinary copper, elevated urinary copper on provocative testing with D-penicillamine, and/or moderate elevation of hepatic copper (100-250 mg/g dry weight).

Sibs of a proband

- If both parents are known to be heterozygous for an *ATP7B* pathogenic variant, each sib of an affected individual has at conception a 25% chance of being affected, a 50% chance of being heterozygous, and a 25% chance of inheriting neither of the familial pathogenic variants.
- Clinical symptoms may vary between sibs (including monozygotic twins) with untreated Wilson disease. The range of clinical variability observed between sibs with the same biallelic *ATP7B* pathogenic variants and treated Wilson disease depends primarily on the age of diagnosis and treatment initiation, reflecting the period of exposure to copper overload conditions.
- Clinical disease is not known to occur in heterozygotes (carriers), although the possibility has not been adequately excluded at older ages. Note: Heterozygotes may have subclinical biochemical findings including low serum ceruloplasmin concentrations, borderline normal urinary copper, elevated urinary copper on provocative testing with D-penicillamine, and/or moderate elevation of hepatic copper (100-250 mg/g dry weight).

Offspring of a proband

- Unless an affected individual's reproductive partner also has Wilson disease or is a carrier, offspring will be obligate heterozygotes (carriers) for a pathogenic variant in *ATP7B*.
- Given the carrier rate of one in 90 in the general population, the likelihood that an affected individual will have an affected child is one in 180. A higher carrier frequency is observed in some population groups due to founder variants (see Prevalence).
- Because the risk that an individual with Wilson disease will have an affected child is low, testing of serum ceruloplasmin concentration after age one year should be an adequate screening in offspring of a proband, except in populations with a high incidence of Wilson disease and/or a high incidence of consanguinity. In these populations, molecular testing may be useful. If molecular testing is not performed, repeat biochemical testing (including ceruloplasmin and urinary copper excretion) of offspring is strongly encouraged if initial biochemical testing was performed before age three years.

Other family members. Each sib of the proband's parents is at a 50% risk of being a carrier of an *ATP7B* pathogenic variant.

Carrier Detection

Molecular genetic carrier testing for at-risk relatives requires prior identification of the *ATP7B* pathogenic variants in the family.

Heterozygotes may have low serum ceruloplasmin concentrations, borderline normal urinary copper, elevated urinary copper on provocative testing with D-penicillamine, and/or moderate elevation of hepatic copper (100-250 mg/g dry weight), which make these tests unreliable in clarifying carrier status.

Related Genetic Counseling Issues

Predictive testing of adults and children. Because Wilson disease is a treatable condition, it is appropriate to offer predictive testing to asymptomatic at-risk adults and children (see Management, Evaluation of Relatives at Risk).

Family planning

- The optimal time for determination of genetic risk and discussion of the availability of prenatal/preimplantation genetic testing is before pregnancy.
- It is appropriate to offer genetic counseling (including discussion of potential risks to offspring and reproductive options) to young adults who are affected, are carriers, or are at risk of being carriers.
- Carrier testing for the reproductive partners of affected individuals and known carriers should be considered, particularly if consanguinity is likely and/or if both partners are of the same ethnic background. Founder variants have been identified in some populations (see Table 7).

DNA banking. Because it is likely that testing methodology and our understanding of genes, pathogenic mechanisms, and diseases will improve in the future, consideration should be given to banking DNA from probands in whom a molecular diagnosis has not been confirmed (i.e., the causative pathogenic mechanism is unknown). For more information, see Huang et al [2022].

Prenatal Testing and Preimplantation Genetic Testing

Once the *ATP7B* pathogenic variants have been identified in an affected family member, prenatal and preimplantation genetic testing for Wilson disease are possible.

Differences in perspective may exist among medical professionals and within families regarding the use of prenatal testing. While most centers would consider use of prenatal testing to be a personal decision, discussion of these issues may be helpful.

Resources

GeneReviews staff has selected the following disease-specific and/or umbrella support organizations and/or registries for the benefit of individuals with this disorder and their families. GeneReviews is not responsible for the information provided by other organizations. For information on selection criteria, click here.

- **Association Bernard Pépin pour la Maladie de Wilson (ABPWilson)**
  France
  www.abpmaladiewilson.fr
- **Associazone Nazionale Malattida di Wilson**
  Italy
  www.malattiadiwilson.org
- **Deutsche Leberhilfe e.V.**
  Germany
  www.leberhilfe.org/lebererkrankungen/morbus-wilson
- **Morbus Wilson e.V.**
  Germany
  www.morbus-wilson.de/de
- **Wilson Disease Association**
  Phone: 866-961-0533; 414-961-0533
  Email: info@wilsonsdisease.org
  www.wilsonsdisease.org
- **Wilson's Disease Support Group - UK**
  United Kingdom
  www.wilsonsdisease.org.uk
- **American Liver Foundation**
  Phone: 800-465-4837 (HelpLine)
  www.liverfoundation.org
- **Canadian Liver Foundation**
  Canada
  Phone: 800-563-5483
  Email: clf@liver.ca
  www.liver.ca

- **Eurodis**
  Rare Disease Europe
  www.eurordis.org
- **Medline Plus**
  Wilson disease

Molecular Genetics

Information in the Molecular Genetics and OMIM tables may differ from that elsewhere in the GeneReview: tables may contain more recent information. —ED.

Table A.

Wilson Disease: Genes and Databases

Gene	Chromosome Locus	Protein	Locus-Specific Databases	HGMD	ClinVar
ATP7B	13q14.3	Copper-transporting ATPase 2	ATP7B @ LOVD WilsonGen	ATP7B	ATP7B

Data are compiled from the following standard references: gene from HGNC; chromosome locus from OMIM; protein from UniProt. For a description of databases (Locus Specific, HGMD, ClinVar) to which links are provided, click here.

Table B.

OMIM Entries for Wilson Disease (View All in OMIM)

277900	WILSON DISEASE; WND
606882	ATPase, Cu(2+)-TRANSPORTING, BETA POLYPEPTIDE; ATP7B

Molecular Pathogenesis

The product of *ATP7B* is copper-transporting ATPase 2, an intracellular transmembrane copper transporter that is key in incorporating copper into ceruloplasmin and in moving copper out of the hepatocyte into bile. The protein is a P-type ATPase, characterized by cation channel and phosphorylation domains containing a highly conserved Asp-Lys-Thr-Gly-Thr (DKTGT) motif, in which the aspartate residue forms a phosphorylated intermediate during the transport cycle. The gene is expressed mainly in the liver and kidneys.

Tissue damage occurs after excessive copper accumulation resulting from lack of copper transport from the liver. Even when no transporter function is present, accumulation of copper occurs over several years.

Mechanism of disease causation. Various pathogenic variants lead to different impairments in *ATP7B* function.

***ATP7B*-specific laboratory technical considerations.** Comprehensive *ATP7B* testing should include promotor variants, as assessment of exonic sequences only does not rule out the diagnosis of Wilson disease when biochemical and/or clinical features are consistent with the diagnosis.

Table 7.

Notable *ATP7B* Pathogenic Variants

Reference Sequences	DNA Nucleotide Change	Predicted Protein Change	Comment [Reference]
NM_000053.4	c.-436_-422delTGGCCGAGACCGCGG	--	Founder variant common in Sardinia [Loudianos et al 1999]
NM_000053.4 NP_000044.2	c.1934T>G	p.Met645Arg	Founder variants comprising 85% of pathogenic variants in persons of Ashkenazi Jewish ancestry [Shi et al 2017]
	c.3191A>C	p.Glu1064Ala	
	c.3207C>A	p.His1069Gln	
	c.2123T>C	p.Leu708Pro	Founder variant common in Gran Canaria, Canary Islands, Spain [García-Villarreal et al 2000]
	c.3649_3654 delGTTCTG	p.Val1217_ Leu1218del	Founder variant in persons of Druze ancestry [Kalinsky et al 1998]

Variants listed in the table have been provided by the authors. *GeneReviews* staff have not independently verified the classification of variants.

GeneReviews follows the standard naming conventions of the Human Genome Variation Society (varnomen.hgvs.org). See Quick Reference for an explanation of nomenclature.

Chapter Notes

Author Notes

Dr Michael Schilsky's clinical and research interests include transplant hepatology, acute liver failure, and inherited metabolic disorders of the liver, in particular Wilson disease and hemochromatosis. Dr Schilsky co-wrote the AASLD and EASL practice guidelines for Wilson disease and chaired the writing group for the newly released 2022 AASLD practice guidance on Wilson disease. He is author of numerous original manuscripts and reviews on the subject. He is the Principal Investigator on clinical trials of pharmacotherapy and gene therapy for Wilson disease. Dr Schilsky is the organizer and Principal Investigator for the multicenter, multinational registry trial for Wilson disease sponsored by the Wilson Disease Association with data coordinating center at Yale University. He is a member of the NIH-sponsored Acute Liver Failure Study Group. He currently serves as Chair of the Medical Advisory Committee for the Wilson Disease Association.

Dr Karl Heinz Weiss's clinical and research interests include transplant hepatology, Wilson disease, and liver tumors. Dr Weiss co-wrote the 2022 AASLD practice guidance on Wilson disease. He is author of numerous original manuscripts and reviews on the subject.

Drs Weiss and Schilsky are actively involved in clinical research regarding individuals with Wilson disease. They would be happy to communicate with persons who have any questions regarding diagnosis of Wilson disease or other considerations.

Contact Drs Weiss and Schilsky to inquire about review of *ATP7B* variants of uncertain significance. Both authors are also interested in hearing from clinicians treating families affected by Wilson disease in whom no causative variant has been identified.

Acknowledgments

MLS receives research support from the Wilson Disease Association (USA), Alexion, Orphalan, and Vivet Therapeutics. KHW receives research support from Morbus Wilson e.V., Alexion, Orphalan, Univar, and Vivet Therapeutics.

We would like to acknowledge the Wilson Disease Association for their support of patient care, research, and educational activities.

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Revision History

- 12 January 2023 (bp) Comprehensive update posted live
- 29 July 2016 (bp) Comprehensive update posted live
- 16 May 2013 (me) Comprehensive update posted live
- 24 January 2006 (me) Comprehensive update posted live
- 24 April 2003 (me) Comprehensive update posted live
- 22 October 1999 (me) Review posted live
- 12 May 1999 (dc) Original submission

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Bookshelf ID: NBK1512 PMID: 20301685

WILSON DISEASE ASSOCIATION
Unmasking Strength. Unleashing Promise.

July 23, 2024

Board of Directors
Washington State Department of Health

On behalf of the Wilson Disease Association (WDA), a patient organization dedicated to supporting and educating individuals affected by Wilson disease (WD), I am writing in support of your efforts to include Key Proteo's newborn screening test for newborn screening test (NBS) for Wilson disease in your state's screening panel.

WD is a genetic disorder that is fatal unless detected and treated before serious illness from copper toxicity develops. It affects approximately one in 30,000 people worldwide. The genetic defect causes excess dietary copper to accumulate in the liver or brain. Copper begins to accumulate immediately after birth. Over time, the excess copper harms the liver or brain, resulting in liver, neurologic or psychiatric symptoms. The symptoms usually appear in late adolescence to early adulthood but can occur at any age. WD is fatal if not diagnosed and treated.

Diagnosing WD is challenging for even the most experienced doctors since it can masquerade like many other disorders and is often misdiagnosed, sometimes for many years. Many tests are usually necessary, and sometimes the test results are inconclusive. Early diagnosis and proper treatment are essential to prevent progression of the disease.

We believe an effective newborn screening test would provide better outcomes for patients with Wilson disease so that treatment can be initiated as early as possible. The global Wilson disease community is very excited about the potential for NBS for WD.

We applaud your department for showing leadership in your efforts to have NBS for Wilson disease added to your state's screening panel. We hope that Washington state will be the first jurisdiction to adopt this test, setting the standard for other states to follow.

Thank you for all of your efforts in this area.

Best regards,

Rhonda Rowland

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Spain

To whom it may concern,

Wilson disease is caused by the accumulation of copper in organs such as the liver and brain. The longer it takes to diagnose and treat, the more copper will accumulate, increasing the probability of symptoms such as liver cirrhosis, speech and walking difficulties, psychiatric symptoms, and even death.

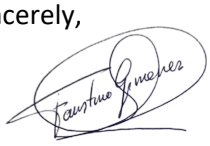
Early diagnosis and treatment of Wilson disease are essential for leading a normal life and avoiding serious complications. Many people remain undiagnosed and suffer from severe neurological symptoms or die from copper accumulation without ever knowing they had Wilson disease or receiving proper treatment.

Implementing newborn screening will improve the quality of life for the boys and girls diagnosed. It will lead to earlier detection, thereby enhancing our knowledge and treatment of the disease. This will also benefit those who are already diagnosed, providing them with more timely and effective care.

The Spanish Association of Relatives and Patients with Wilson Disease (Asociación Española de Familiares y Enfermos de Wilson) wholeheartedly supports the Wilson Disease Association and recognizes the critical importance of implementing newborn screening for Wilson disease in Washington. This initiative will serve as a model, encouraging its adoption in as many states and countries as possible worldwide.

Life has incalculable value, and newborn screening will save countless lives of those affected by Wilson disease.

Sincerely,



Faustino Gimenez Felices
President of
Spanish Association of Relatives and Patients with Wilson Disease
Asociación Española de Familiares y Enfermos de Wilson

UniversitätsKlinikum Heidelberg

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WA State Department of Health

Board of Directors

Heidelberg, 20th July 2024

Dear Washington State Board of Health,

I am writing this letter in support of Dr. Hahn and Key Proteo, Inc. and their petition to include Wilson Disease (WD) on the list of conditions in the WA state newborn screening program.

As a physician and expert in Wilson disease, I have had the privilege of working with Dr. Sihoun Hahn, founder of Key Proteo, Inc., and can attest to their expertise in Wilson Disease and newborn screening.

Wilson Disease (WD) is an autosomal recessive disease of copper metabolism caused by pathogenic mutations in the ATP7B gene, a copper-transporting ATPase. About 1 out of 30,000 individuals are affected by WD. The symptoms typically appear between the ages of 5 and 45 years, and most affected individuals are clinically diagnosed often after they develop significant life-threatening complications, including liver cirrhosis, acute kidney failure, and brain and nerve damage. Early diagnosis and treatment of WD is highly effective in preventing these irreversible damages and can greatly improve the patients' quality of life. Currently there are no efficient and cost-effective screening methods available for early detection of WD. However, the technology developed by Dr. Hahn's laboratory and Key Proteo, Inc. allows early detection of WD prior to clinical manifestation.

Dr. Hahn has over 30 years of experience with WD and has been a pioneer in WD diagnosis. He found that WD patients have very low levels of ATP7B protein, making it a useful biomarker for WD screening. The Immuno-SRM technology developed at Key Proteo, Inc. allows highly precise quantification of ATP7B protein from dried blood spots and can successfully identify WD patients from healthy unaffected individuals. This assay can be readily performed by the WA newborn screening laboratory, since the laboratory already utilizes tandem mass spectrometry on the dried blood spot samples collected from newborn babies. Adopting Key Proteo's technology to screen newborns and detect WD before onset of clinical symptoms can prevent newborns from developing serious permanent complications that require long-term medical care, reduce the financial burden of WD treatment, and greatly improve the long-term clinical outcomes of affected patients.

I wholeheartedly believe that including WD in the WA state newborn screening program can make a lasting impact on thousands of newborns and their families. By testing newborns for WD and providing early intervention to those with WD, we can ensure these children are saved from life-threatening consequences and treated in a timely manner to enjoy healthy lives. Thank you for your consideration.

Sincerely,

Prof. Karl Heinz Weiss
MedicaL Director

Yale School of Medicine

MICHAEL L. SCHILSKY, M.D.
Professor of Medicine

Medical Director, Adult Liver Transplant
Yale-New Haven Transplantation Center

PO Box 208019 New Haven, CT 06510-8019
T 203-737-1592 F 203-785-6645
michael.schilsky@yale.edu

To:
WA State Department of Health
Board of Directors

From:
Michael L. Schilsky MD
Professor of Medicine and Surgery
Director, Center of Excellence for Wilson disease at Yale

Re: Newborn screening for Wilson disease

Dear Board members,

I am Professor of Medicine and Surgery and Director of the Wilson Disease Center of Excellence at Yale, and Chair of the Medical Advisory Committee for the Wilson Disease Association, and currently direct our clinical activities and research program on Wilson disease at Yale. I have over 35 years of experience in working in the field of copper metabolism and Wilson disease with clinical and research expertise in this area. I currently treat approximately over 320 patients with this disorder and direct the Wilson disease patient registry project and an NIH sponsored grant on biomarkers for copper metabolism.

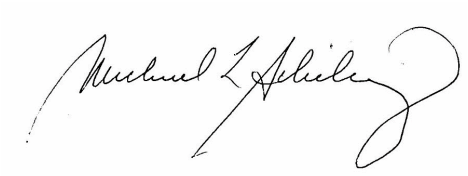
While we are fortunate to have treatments for Wilson disease, there are serious limitations of the available treatment options and disease caused disability due to the late diagnosis of this disorder. For these reasons, the work of Professor Hahn and his colleagues on identifying newborns with Wilson disease is so critically important. When the disorder is identified at an early stage before there is significant liver disease or extrahepatic disease, the patient can then be managed more easily with outcomes for survival comparable to those without the disease. Additionally, the medical and financial burden imposed by disease caused disability due to liver or neuropsychiatric disease can be avoided altogether. This is most desirable.

For these reasons I support the ongoing efforts to utilize the novel testing developed by Professor Hahn to detect this disorder in the earliest stages. We look forward to the successful implementation of the newborn screening program and to the challenge of working with younger patients to prevent disease throughout their lifetime, including the development of new therapeutics that may provide a cure for the disorder. However, all therapeutic efforts are dependent on the

ability to accurately detect the disease. Therefore, the incredible work that has been done by Dr. Hahn in advancing the diagnostics for Wilson disease to where it can be used in newborn screening is extremely important.

I most strongly support the ongoing work of Dr. Hahn and colleagues for the newborn screening project for Wilson disease, and very much appreciate your efforts to date. I look forward to the future universal use of this diagnostic test in clinical practice.

Sincerely,



Michael L. Schilsky MD FAASLD
Professor of Medicine and Surgery
Yale University Medical Center

July 18, 2024

Board of Directors
Washington State Department of Health

I am writing in support of Key Proteo and Dr. Sihoun's Hahn's newborn screening test for Wilson disease. I urge the Washington Department of Health to add Wilson disease to your state's newborn screening panel.

Please indulge me as I share my family's story with Wilson disease.

In December 2022 I had just retired from a 30+ year career in corporate communications and was looking forward to retirement. Our two children (son 23 and daughter 20) were successfully launched. My son had finished university and had a good job. My daughter was in her second year university studying economics and doing well. My husband and I congratulated ourselves on what a great job we had done raising our kids.

Then the walls caved in.

My daughter came home from university feeling very tired, bloated, nauseous and weak. I thought maybe she has irritable bowel syndrome, burnout from studying for exams, the freshman 15 weight gain, her appendix, gall bladder? I thought anything but Wilson disease.

Why would I? I have never heard of Wilson disease.

She suffered through the holidays and slept a ridiculous amount. The fluid was getting more pronounced in her abdomen and she started vomiting at random. A visit to our family doctor who took some blood work and an ultrasound showed fluid in her abdomen and elevated liver enzymes. "Let's monitor and test again in a month," our doctor advised. I realize now that if we had waited, she might not be here.

After the holiday break, my daughter insisted on going back to university to start winter semester. I suggested she stay home so we could arrange for more tests but she was eager to get back to school. She did agree that if the symptoms worsened or didn't improve, she would go to emergency. Two days later, she did just that. Little did we know that she was at end stage liver disease, due to Wilson disease, a condition that she had battled since birth, unbeknownst to any of us.

Fast forward a month later and she was back at home in Toronto Ontario with her new, transplanted liver.

To say we were blindsided is an understatement.

At emergency, doctors quickly diagnosed her with Wilson disease, within a few days. Her liver was so damaged that it couldn't be saved. She needed an emergency liver transplant.

I will never forget the doctor's words. "It's the only lifesaving option at this point." I was frozen and numb. How could this happen to an otherwise healthy young woman who didn't do drugs, didn't drink much, worked out and ate well?

We were lucky. My daughter was put at the top of the provincial transplant list in Ontario and received her new liver within 10 days of her Wilson disease diagnosis. I am forever grateful to her anonymous, deceased donor and their family who made the decision in their unimaginable grief to give back and do some- thing compassionate.

They saved my daughter's life.

While I am thankful for her new liver, I am still haunted with the knowledge that my daughter had a disease since birth that I had no idea about. If we had known that my daughter had Wilson disease as a baby, we could have potentially saved her from needing a liver transplant. She could have been monitored and treated so much earlier before the disease had progressed.

Sadly, my family's story is like many others. A difficult disease to diagnose because it can make other symptoms, people often endure significant liver, neurological and psychiatric symptoms, often irreversible. Left untreated, Wilson idea is fatal.

When I learned about Dr. Sihoun and Key Proteo's work with NBS for Wilson disease, I was very excited. It gives me great hope. If we could diagnose Wilson disease in infants, this could be a game changer and end so many years of suffering and anguish for patients and their families, not to mention significant health savings for the system.

Thank you for your consideration. I hope that Washington State can show leadership and courage and proudly stand as the first jurisdiction to add NBs for Wilson disease to its newborn screening panel.

Sincerely,

Alice Williams

Toronto, Ontario, Canada

To the Washington Board of Medicine

Our family's journey is one that is far too common with Wilson's Disease. Our son Rowdy was 17 years old and preparing to go serve an LDS mission for two years somewhere in the world. As a part of that process to get ready, he went in for his routine physical with our family doctor and friend. Two days after that physical I received an urgent phone call from our family doctor indicating that I needed to get Rowdy to the hospital lab to do additional work to figure out what was going on and that it was a mystery, urgently! As we were discussing things with the doctor, we learned that Rowdy's Platelet blood count was approximately 38 instead of the normal 200, also blood cell shapes and other things were mysteriously wrong. We did as requested and instructed by our doctor and urgently rushed him to the hospital lab to try to find out what was going on with his blood.

Over the course of the next six months with two different oncology specialist doctors we chased every leukemia, bone cancer, and blood cancer that are known. He underwent two separate bone marrow biopsies and was pulled from every physical activity that a normal 17-year-old boy does in fear of bleeding out and possible death. His world was turned upside down. Six months of tests, procedures, countless office visits to doctors and we had no answers. Following his second bone marrow biopsy, under the suggestion of two wonderful doctors at the Anchorage Providence Hospital, we started seeking an internalist to help coordinate all of the care for our son.

We reached out to our family doctor, and inquired who we should be seeing for internal medicine and he made some recommendations, one of the providers I knew. So we made our appointment and began yet another journey of unknowns, additional tests and more doctor visits. Our provider Karen Clements had remembered studying something about these types of mysteries while she was in school, and elected to do a larger blood panel to include many different enzymes and other such things in my son's body. When results came back, there were many red flags of concern as to the balance and health of my son's liver. A few more doctor visits, and our new provider had confirmed that our son had Wilson's Disease.

We had one answer and a whole New World of mysteries. We were referred to Biomedical Genetics at Seattle Primary Children's to begin coordinating care for my son. Additional tests were ordered again and more visits to doctors and hospitals. Ultrasounds, MRIs and CAT scans were ordered to deter,iome the extent of damage caused by the prolonged nature of undiagnosed Wilson's Disease. Our son, now 18-years-old has stage 4 cirrhosis of the liver that will never heal. The cirrhosis of the liver is a direct result of the disease and complications with the copper in his body. The required medication for an individual at this stage of Wilson's Disease is literally $40,000 a month without insurance. He will be on these medications for the next 3 to 5 years, possibly longer. We have now been working with Dr. Hahn and other specialists, geneticists, nutritionists and many others, in our wonderful care team, to treat my son's condition, to prolong the life of his liver, and give him the highest quality of life that he can have at this stage of cirrhosis.

We have a large family and as this is a genetic disorder, genetic tests were ordered for all of our children. This is how we discovered that his younger brother also has the disease. Teancum's journey will be very different from that of Rowdy because his disease was found 5 years earlier in his life. The cirrhosis in his liver is approximately 1.5 on the scale of 5. The

financial burden of the medication is not the same for him as he does not require the mining medication that Rowdy is on. He is able to treat his condition with diet and a therapeutic zinc taken daily, the cost of which is only about $40 a month. His life journey will be very different from Rowdy's because of an earlier diagnosis.

Due to the extent of the damage of Wilson's disease being undiagnosed in his body, there's a very high likelihood that Rowdy will require a liver transplant that could have been avoided with some form of early detection in place. Our family fully supports Dr. Hahn, his team, their research and the desire to make Wilson's Disease testing a functional and easy test at the time of birth. We already conduct many tests through a simple pin prick on the heel of every newborn child in every hospital across the United States to ensure the health safety and quality of life of those children. Wilson's Disease is uncommon and rare. It is debilitating and life altering, and a burden to be born by those who have it. If the State Board will embrace, endorse and approve the testing for newborn children in the hospital, the families of those children who test positive for Wilson's Disease will be able to begin their level of care at birth with low copper diets with high zinc intake. This prevents any damage, liver or the neurological damage, for those children, thus providing them a fullest possible life from beginning to end. Had this testing been available to our family, there's a high likelihood that Rowdy would not have level 4 cirrhosis. He would not be looking at the possibility of a liver transplant; he would be able to engage in regular activities as an 18-year-old young man, all of which is currently being denied to him because of the lack of early testing. If a simple pinprick could've changed my son's life. I would do it 1000 times over. Children should not have to worry about liver failure, Splenomegaly, and the possibility of bleeding to death because they play with their siblings.

A simple test could provide that peace to children. A test that they will never remember, a test that they will never have a memory of feeling,a test that they will never have the memory of conducting and changing their lives for the better. My family supports Dr. Hahn's proposal, and we would hope under the pleading of many many families who face this situation, the State Medical Board will also support this test and support his team in providing this to the newborn children. Once proven in Washington State, this test can be made available in other states, to other children, to other families. We talk about the potential elimination of diseases like polio, mumps, measles and rubella in the United States through simple testing and vaccines. Similarly the long-term debilitating effects of Wilson's Disease is virtually eliminated if care could begin on day one and patients would never feel the effects of the disease that they carry in their genetics, through no fault of their own. Please approve the institution of the heel prick testing of infants for Wilson's Disease.

Sincerely
Christopher and Rachel Johnson
Rowdy and Teancum Johnson
And family

Nora Closser
70 Barons Road
Rochester, NY 14617

July 25, 2024

Washington Department of Health
Board of Directors

To Whom it May Concern,

We are writing this in support of Dr. Sihoun Hahn's newborn screening assay for Wilson's Disease. Our story is like so many others that are diagnosed with a rare disease, it took a very long time to get a diagnosis. The caveat to Wilson's Disease is that unlike many other rare genetic diseases-it is treatable and in most cases manageable. Early diagnosis would have prevented so much disability and heartache that we have endured in the last 23 months of our lives. Our hope is that no family has to go through what we have gone through.

Our 13 year old daughter, Brinley started with abrupt muscle spasms one day at school in October, 2022. These muscle spasms and myoclonus progressed over the course of the next 2 months to balance issues, swallowing issues, extreme fatigue and weakness. I cannot describe the look of terror on her face when she could not control her own body. When we could not get in to see a neurologist or an outpatient MRI of her brain, our former pediatrician recommended taking her to the Emergency Department. The attending neurologist told us that he believed this was a functional disorder and that a brain MRI was not necessary unless we needed it to get on board because in order to treat a functional disorder-formerly known as a conversion disorder, all parties need to be on board. I have been a nurse for over 25 years and in pediatrics for the last 15. I told them we needed the MRI. It was unremarkable. They did not do any additional labs or testing at that 24 hour admission. She was placed on Gabapentin and continued the Hydroxyzine that she was taking. Two months later they added Amantadine because she couldn't focus. She could not focus because she could not control the myoclonus or tremors she was experiencing.

She missed her entire year of 6th grade.
We did home tutoring. The diagnosis of Functional/Conversion disorder followed her until diagnosis.

Her symptoms progressed. She started with severe leg pain in March of 2023. No medications helped her. In May of 2023, she started with severe back pain. Her former pediatrician told me that the only things that would help her would be to get her to sleep, get her to school, get her in a pool and refused to write a continuation letter for home tutoring. The school nurse knew my daughter well and let me call her into school every day. She knew that something was really wrong and urged me to go to a bigger city like Boston and present to their ER. She had been seen by Infectious Disease, Neurology, and Rheumatology. Symptoms progressed. She had

mottling of her hands, Mee's lines on her nails, and looked sickly. I vowed that I would find an answer for her. This was not my daughter. I knew something was very wrong. I researched for hours many nights trying to find an answer. Brinley researched with me. In May of 2023, we changed pediatricians. I had researched all of her new symptoms and Wilson's Disease came up as a differential diagnosis. Her liver function and brain MRI was normal. It wasn't on the radar because of these factors. I needed to talk to a provider who while she didn't know me on a personal level, knew me on a professional level and knew my clinical assessment skills. She immediately wrote the note to continue home tutoring. I asked about Wilson's Disease and she ordered a ceruloplasmin test, which had not been done on this journey. It was low. There was finally something that could explain all of her symptoms. I told Brinley that we may be on the right path to a diagnosis and she said, "Mom, I know this is what it is."

Referral to GI was the next step. She had suffered from unexplained abdominal pain since the age of 10 so we already had a relationship there. The pediatrician sent in the referral the evening the ceruloplasmin level was low. I messaged the GI provider the next morning and she ordered the initial liver screening work up. Everything was still normal except for the ceruloplasmin level. The 24 hour urine screen for copper was normal. We met with the liver team who ordered a referral to Genetics. All of this took time. The positive genetic test did not come back until August 2023. When I told Brinley about the positive test, her response was "I knew it."
No medications helped her symptoms. She tried and failed so many times during this course. She was immediately referred to a Pediatric Hepatologist at Columbia as there were none in our area. We had one in Rochester that left the area 2 days prior to the genetic test results. She was immediately started on Zinc and then did a Penicillamine challenge. She had a seizure with the first dose. When we presented to the ER, we were told by the attending Neurologist that it was likely functional. With one dose, she still had excessive copper in the 24 hour urine. We needed to quantify how much copper was present given the normal liver labs and MRI's. A liver biopsy confirmed high copper content. Columbia wanted us to see specialists at Yale as they are a Center of Excellence. We were seen by Dr. Bamfort at Yale in early December 2023 and then by Dr. Schilsky on December 22, 2024. She started Trientine on December 23, 2023.

Brinley missed her entire year of 7th grade at school.

As a result of her positive test, our 10 year old daughter, Emma was found to be positive for Wilson's disease. She is currently taking trientine. She is asymptomatic and will hopefully remain that way since she was started on treatment earlier. She is diligent about taking her medication and adhering to the dietary restrictions because she saw the hell her sister went through. She never wants to get to that point.

Family testing revealed my nephew was positive and his sisters were carriers.

Brinley took every medication they tried, did all tests ordered, every MRI, every lab draw, every 24 hour urine without a complaint. Never did she say "Why me?". She knew there was an answer, we needed to find it and was willing to do anything to get there.

The impact of a delayed diagnosis took its toll on every aspect of our lives. Physically, mentally, financially….all of it was impacted. No one should have to go through that when a newborn screening tool exists. It would have prevented so much.

In May of 2024, she wrote that she was happy vs. neutral for the first time in over a year. The medication is working.

The retail cost of a 90 day supply of Trientine is $22,700 per child. Thank God we have good insurance. I shudder to think of those that have to choose between paying their electric bill and getting life saving medication.

It has been 7 months since she started chelation. She still has a ways to go, improvements are there. Small things are huge. The hypomimia has improved, I can hear her sing in the car to the radio. I tear up every time. As I write this, we are on our first vacation in 2 years. She is able to participate in so much more these days. She will hopefully get to participate in her last year of Junior High with support.

Carrier status is 1:90. I suspect the incidence of Wilson's Disease is much higher than it is diagnosed. The reality is that it is treatable in most instances. A newborn screening tool would have prevented so much for all of us.

My daughters have exhibited a strength that some adults may struggle with. I cannot express how proud I am of them. We will be advocates for this disease and try to help those navigating it as we navigate it ourselves. Brinley often tells me that she wants her story told.

I thank you for your time and consideration.

Sincerely,

Nora Closser
noratoole@hotmail.com
585-737-5323

Erin Brooks
4907 N Wolcott Avenue, Unit 2B
Chicago, IL 60640

July 25, 2024

Board of Directors
Washington Department of Health
Town Center 2
111 Israel Rd. SE
Tumwater, WA 98501

To Whom It May Concern:

My name is Erin Brooks, and I am writing to urge you to add to add Wilson Disease (WD) to the Washington State newborn screening panel.

My father passed away from Wilson Disease in 2000. At the time, Wilson Disease was a highly unknown disease with minimal resources and options for treatment. It took over a year for my dad to be diagnosed and although there were wonderful doctors overseeing his care, the disease was too much for my dad's body to handle.

Over the past 24 years there have been so many advances made in the diagnosis and treatment for Wilson Disease and if caught early, WD patients and families can live normal and healthy lives. I am so grateful for Dr. Sihoun Hahn's efforts at Seattle Children's Hospital to develop this screening test for WD and lead the pilot project to test this assay on 25,000 newborns in Washington. If this test had been available for my father, his Wilson's would have been discovered long before it took such a serious toll on his body, and he may still be with us today.

So once again, please seriously consider adding Wilson Disease to your newborn screening panel. This is a life-or-death screening for many families.

With deep gratitude,
Erin K Brooks

Dear Dr. Han,

I wasn't diagnosed with Wilson's season 226 mate feeling my second year of medical school at Texas Tech health science Center. I had a Trevor and a grin. Trevor was getting worse and I thought God was punishing me for various events that I thought I was guilty of

It was missed by three expert Neurologist over prior 15 years! It is rarely seen by any neurologist or hepatologist and both kinds of doctors just overlook it. It's inevitable that they will overlook it because I just made you see one case and they're alive If That many?

Hence, with modern medical testing of a newborn DNA would easily diagnose the Wilson's disease gene along with all the other genetic disorders that are diagnosed Postpartum.
Like hemochromatosis ,hemophilia, et al.

Ok ? I feel just Adding Another Fault in a babies DNA the Cost would be in saving and making a life hell of a lot better as well as saving the cost of a liver transplant, which is in the hundreds of thousands of dollars!

In my case it was the cost of the college education and medical school education expenses, while living. Giving up of my dreams of practice medicine and field psychiatry where we know psychiatrist are all over the country. I took disability insurance while I had it. Dammit, That was when I was 40.

Thank You,

Dr Kirk Vestal MD

Date: July 25, 2024

To: WA State Department of Health, Board of Directors

re: My personal story with Wilson Disease

I first started experiencing symptoms of Wilson Disease when I was 20 years old while attending the University of Washington. My symptoms included tremors in my hands and arms, difficulty walking, and slowed speech. My symptoms became so bad that I had to drop out of the University and move back in with my parents.

My parents and I went to see a doctor so that we could find out what was causing my symptoms. After undergoing several medical tests, the doctor misdiagnosed me as having multiple sclerosis. For approximately five years, I lived with this misdiagnosis, while my symptoms kept getting worse, especially the tremors. During this five year period, in order to help correct the tremors, I underwent a type of brain surgery, called a thalamotomy. The surgery was a success, as it definitely helped lessen the tremors in my arms and hands. After the surgery, I did have to go through lengthy physical and occupational therapy to regain my body's full motor skills, in what I referred to at the time as "learning how to walk again".

After the five years of my misdiagnosis of multiple sclerosis, and while undergoing a routine eye exam, I found that I had a brown colored ring around my cornea. The eye doctor informed me that this was unusual and suggested I get it checked out by my doctor. With this new information, I went to see my neurologist. After undergoing some medical tests, I was correctly diagnosed as having Wilson Disease. I then was prescribed the correct medication, cuprimine, to help manage the excess amount of copper in my body. Within about six months of treatment most of my original symptoms of Wilson Disease greatly improved.

I was not correctly diagnosed with Wilson Disease until I was 25 years old (I am currently 61), so my life could have benefited from an earlier detection of the disease. My story shows how important it is to have Wilson Disease as part of mandatory newborn screenings.

Sincerely,

Thomas Sandall

Marilee & Gary Wolter
7324 Lantana Way
Naples, FL 34119

To: Washington Department of Health, Board of Directors,

My name is Marilee Wolter, my husband and I are writing to you to join in the advocating of newborn screening for Wilson disease (WD).

Our daughter Rhonda Rowland was diagnosed at the age of 21 with Wilson disease in Madison, WI by a new gastroenterologist. Looking back, we know she was incredibly lucky that this young physician thought of WD.

Six months prior to her diagnosis, she developed sudden, severe abdominal pain that kept her in bed so she couldn't attend her college classes. We brought her to our family internist in Milwaukee who attributed her abdominal pain to drinking bad water during a recent trip to Florida. Because of abdominal pain and fatigue, she ended her college junior year on academic probation. That summer she had extensive fatigue, experienced moodiness and made careless mistakes at her summer job. By September she developed jaundice, swelling, ascites and cirrhosis of the liver. Fortunately after receiving the correct diagnosis, she responded well to WD medication and has lived a normal life.

In 2016, my husband and I joined Rhonda at a Wilson Disease Association conference. We were shocked and saddened to see the devastating effects of WD. There were people in wheelchairs or walked with difficulty; many people couldn't speak clearly or control their verbal outbursts. Some needed assistance with eating. I couldn't believe they had the same disease as our daughter and we realized just how lucky we were.

Fortunately her younger sister and brother do not have Wilsons. Rhonda is in good health, and has dedicated the last 41 years of her life reporting medical information and raising awareness of this rare disease. We are proud of her work as vice president of the Wilsons Disease Association.

We strongly encourage the Washington Dept. of Health, Board of Directors, to consider and include WD in its newborn screening panel. While life-threatening if not detected, it's no longer a "rare" disease to those who experience it!

Thank-you for your support.

Best Regards,
Marilee & Gary Wolter

Sirs:

I read today that you have developed a screening test for WD. You plan to present letters from patients, caregivers and supporters reinforcing the importance of adding WD to Washington's newborn screening panel.

We live in Highland Park, IL. Our daughter, Hilary, was diagnosed with WD at age 25.

Before she was diagnosed, since she was little, she suffered from stomach pains, illnesses that could not be explained, and general discomfort. WD was not suggested by any doctor. We are not surprised; it is so rare. Her doctors attributed it all to stomach and intestinal issues. Hilary managed to do everything (school, activities, summer camp, college, graduate school) but too frequently with great discomfort. After graduating with a master's degree from NYU, she stayed in New York.

She became extremely sick, exhausted and incapacitated while living in NYC. Doctors gave her disparate diagnoses; one even thought she could be cured with marijuana. Unable to work, she was terrified at the unknown nature of her problems. We dropped everything to join her to try to find out what was going on.

We were led to the wonderful Dr. Dietrich and his PA Helen Adams at Mt Sinai Liver Institute. They diagnosed WD and saved her life. We learned to our shock that we are carriers of recessive genes for a disease we had never heard of. Much damage had already been done to Hilary's liver as a result of copper she could not eliminate. Thankfully, she did not need a liver transplant, but it was discussed. This all could have been avoided had there been a screening test at birth.

Hilary has been successfully treated for 5 years now and we have nothing but optimism for her future.

How much easier her life would have been had she been diagnosed at birth. Please add WD to the newborn screening panel.

Maxine and Michael Bonn

350 North Deere Park Drive West

Highland Park, IL. 60035

mmbonn@gmail.com

To: Washington Department of Health, Board of Directors

I am writing in support of adding the screening test for Wilson's Disease to the newborn screening. Our son was diagnosed with Wilson's Disease on his 24th birthday. He is now 26 and is fully chelated and healthier than ever. That is the good news. The bad news is all of the years that he (and we) suffered with his symptoms, mostly psychological and behavioral. Again, those symptoms are greatly improved now that he is chelated.

When he was a teenager, he had elevated liver enzymes in his bloodwork results. Physicians assumed that it was caused by the medicines he was on because of his psychiatric issues, and they did not assess for other possible reasons. No one considered Wilson's Disease, and we had never heard of it. Just think how different his life (and ours) would have been if he had been diagnosed (and treated) beginning at birth. It would have saved years of heartache for all of us, and he would not have to deal with cirrhosis of the liver for the rest of his life.

Please begin testing all children at birth for this serious disease so that no one else has to experience the sometimes permanent damage caused by Wilson's disease. Thank you for your consideration.

Janet Laubgross, PhD

Clinical Psychologist

July 24, 2024

Board of Director
Washington State Department of Health

I'm writing in support of Key Proteo and Dr. Sihoun's Hahn's newborn screening test for Wilson disease. I urge the Washington Department of Health to add Wilson disease to your state's newborn screening panel.

Please indulge me as I share situation of people with Wilson disease in the place where I live.

My family's journey with Wilson's disease began in 2011. My older daughter, then 9 years old, often complained of abdominal pains. At the beginning we thought this was related to the stress of starting primary school in 2009 and the arrival of her younger sister at the same time. The recurring complaints prompted our physician to extend the diagnostics, which showed high levels of liver tests. For the next six months, doctors observed and monitored my child. Unfortunately, there was liver damage during this period, so we decided to transfer our daughter to another hospital in December 2010. Fortunately, experts in Wilson's disease were working there and in January 2010 we had a diagnosis and treatment began. In February 2011, my younger daughter, who was then 2.5 years old, was also diagnosed.

I run a support group on Facebook for people with Wilson's disease, which currently has 270 patients or relatives as members. A year ago, a dad of a 16-year-old girl who had been diagnosed with Wilson's disease four days earlier wrote in the Facebook support group. The girl required an urgent liver transplant. She had brain swelling and was put into a pharmacological coma. Her liver was transplanted but unfortunately this child's life could not be saved. There is also another student. She is her mother's second child, the first died undiagnosed. There are several more similar stories...

I would like to emphasize that in Wilson's disease, the key issue is the earliest possible diagnosis. Unfortunately, knowledge of the disease among people and physicians is still very low. The introduction of screening could save many lives and certainly dramatically improve diagnosis and treatment and thus prevent side effects and improve the quality of life of those suffering from this disease.

I am in the process of registering a patients' association, of which I will be president. I am in contact with patients from many countries and I intend to take any action that improves the quality of life of people affected by this disease.

As patients and their families, we look forward to organized early diagnosis, to screening. We look to the future with hope. We have high hopes that Washington State will show leadership and courage and stand the first jurisdiction to add NBs for Wilson disease to its newborn screening panel and become in the future an example for other states and countries.

Best regards,
Anna Aniol
Wroclaw, Poland

Board of Directors

Washington State Department of Health

P.O. Box 47890

Olympia, WA 98504-7890

29th July 2024

Dear Board Members,

I am writing to express my strong support for Dr. Sihoun Hahn's newborn screening test for Wilson Disease (WD) and to urge you to consider adding this critical test to your screening panel. Including Wilson Disease in the newborn screening programme could spare many families the devastating consequences of a late WD diagnosis, as my family has endured.

Please allow me to share my daughter Sophie's story, which underscores the urgent need for early detection. Sophie was a happy, (seemingly) healthy child and teenager who excelled in her studies, and she was a talented pianist.

At the age of 15, Sophie experienced a sudden and dramatic personality change accompanied by severe psychiatric symptoms. Like many WD patients, her diagnosis was delayed by about a year from the onset of symptoms and she deteriorated significantly during this time. Sophie is now 19 years old and has spent the last four years in mental health institutions or acute healthcare settings. She hasn't spent a Christmas day at home since she was 14. Sophie's condition has worsened over time. In addition to her initial psychiatric symptoms, she now suffers from hepatic and neurological problems.

Sadly, she has also developed cognitive deficits due to the WD brain damage she has suffered, and it is nothing short of a tragedy that she was unable to complete her education, receiving no qualifications despite being a straight A student. She had always dreamed of becoming a paediatric nurse. Albeit minor in the scheme of things but of significant magnitude to Sophie, sadly, she can no longer play the piano.

Sophie now requires 24-hour care and is under a Deprivation of Liberty order. The once incredibly witty, smart, vibrant, and talented girl I knew has been stripped of everything that brought her joy or function in life. She now has an extremely poor quality of life, and despite four years of treatment with penicillamine, there has been no improvement in her condition. I fear she may never recover, but I will never lose hope.

In Sophie's words: "Mum, it just isn't living."

During a recent visit to our local emergency department, a physician greeted her with, "*I don't know how you are still alive!"* This stark comment underscores the severity of her condition. This is our reality now - a reality I am forced to accept, but if she had been diagnosed as a baby, *oh, how different things would be*!

I would give anything to have known she had WD as a baby. This profound loss and suffering could have been prevented. There are other children out there right now who have WD but don't know it yet, and devastating consequences are looming. This thought keeps me awake at night.

You have the power to prevent other children in your state from suffering the profound, relentless, enduring suffering and loss that we do, every single day. You have the power to save lives.

Thank you for kindly reading about Sophie and for your consideration of this critical matter.

Yours sincerely,

Claire Stapleton

Mother of Sophie, WD Patient

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