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CLINICAL—LIVER

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

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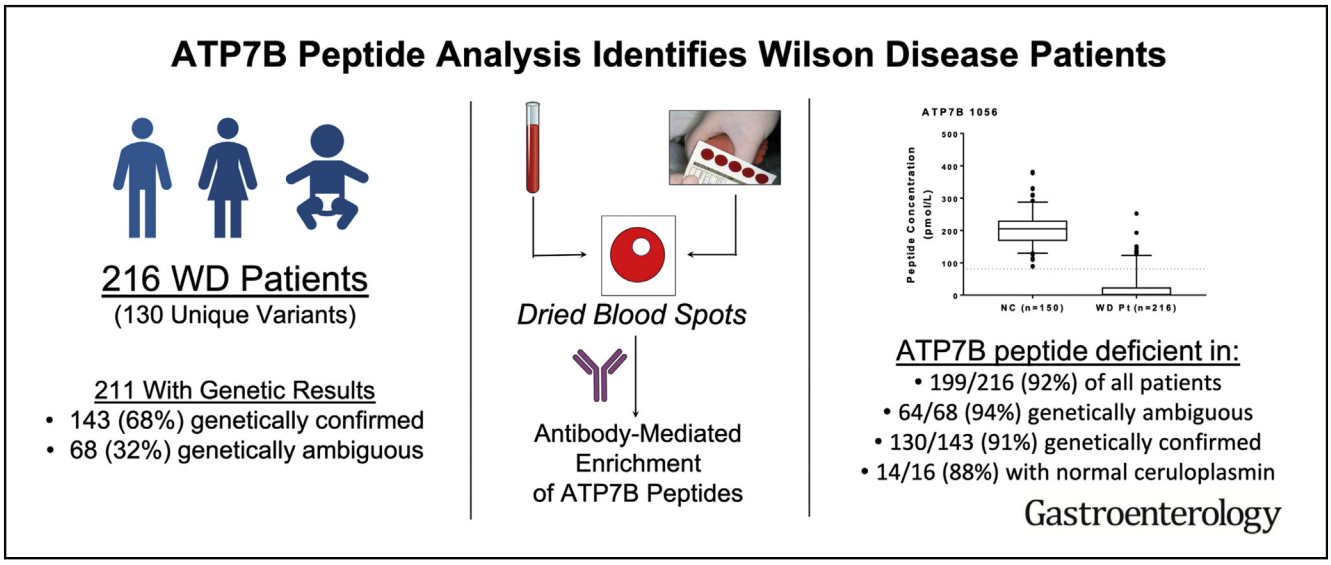

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BACKGROUND & AIMS: Both existing clinical criteria and genetic testing have significant limitations for the diagnosis of Wilson disease (WD), often creating ambiguities in patient identification and leading to delayed diagnosis and ineffective management. 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

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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

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,

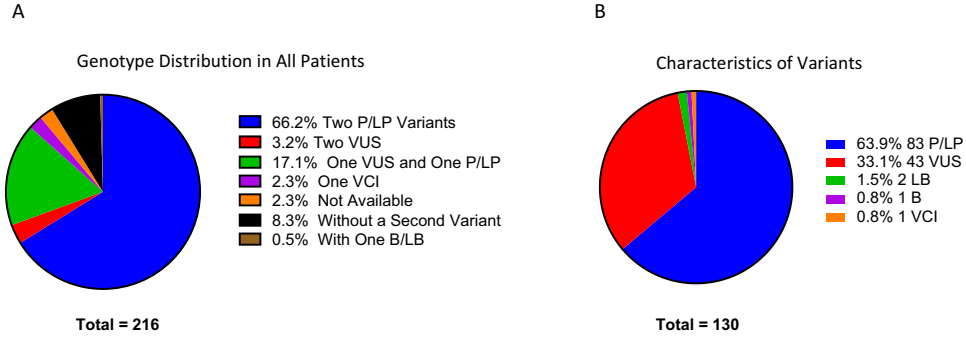


Figure 1. Patient cohort characteristics. Analysis of the genotypes of patients (*A*) and the characteristics of the variants present (*B*) show the diversity of variants and variant combinations present.

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.

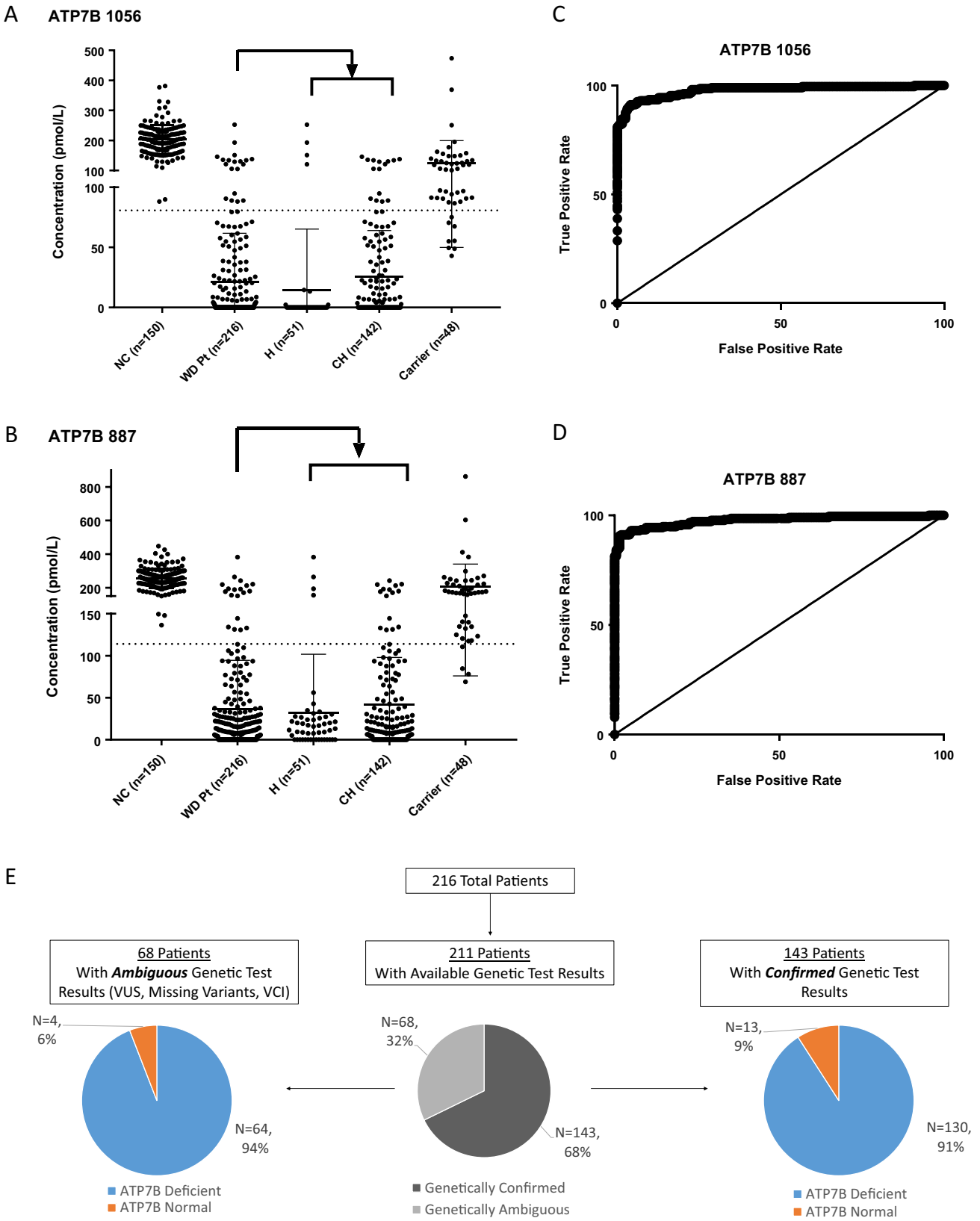


Figure 2. Diagnostic performance of ATP7B peptide analysis. Comparison of ATP7B peptide measurements for ATP7B 1056 (*A*) and ATP7B 887 (*B*) peptides in normal control patients (NC), patients, homozygotes (H), compound heterozygotes (CH), and carriers. *Dotted lines* represent diagnostic cut-offs for 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*).

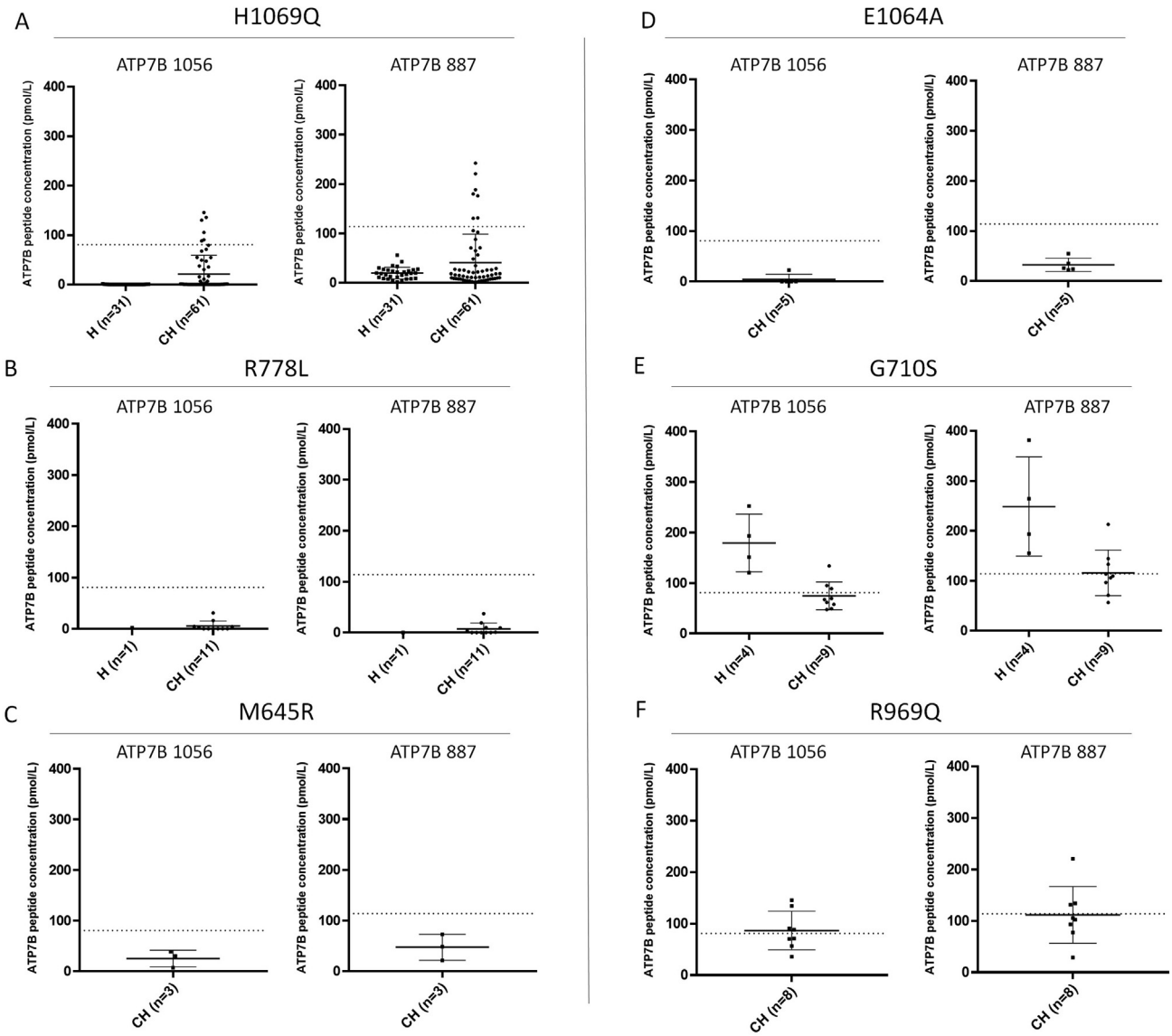


Figure 3. ATP7B peptide concentrations in patients with common variants are often reduced and variants causing false negatives are rare. Patients homozygous and heterozygous for p.H1069Q (*A*), R778L (*B*), M645R (*C*), and E1064A (*D*) are largely reduced. False negatives within the patient cohort are 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.

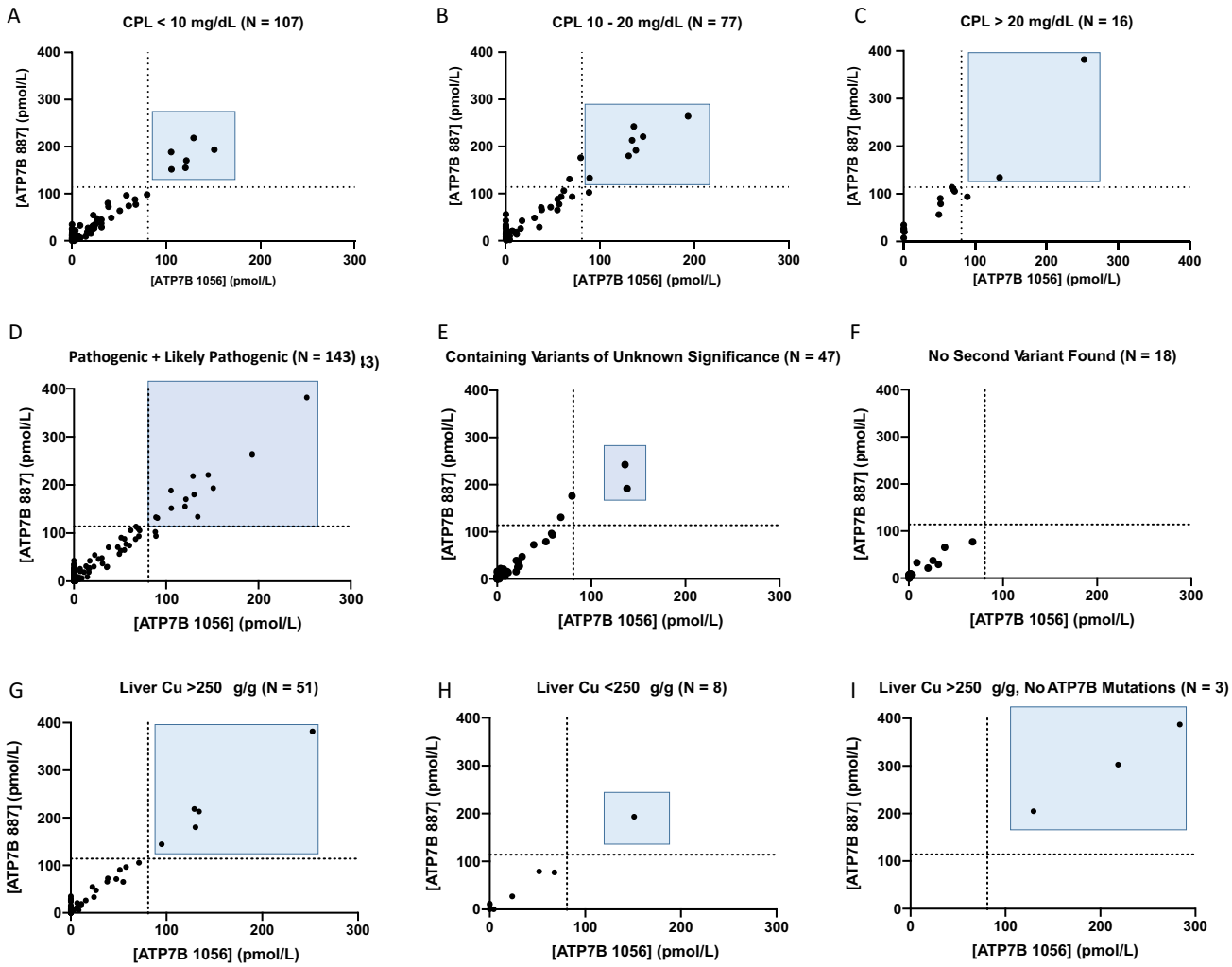
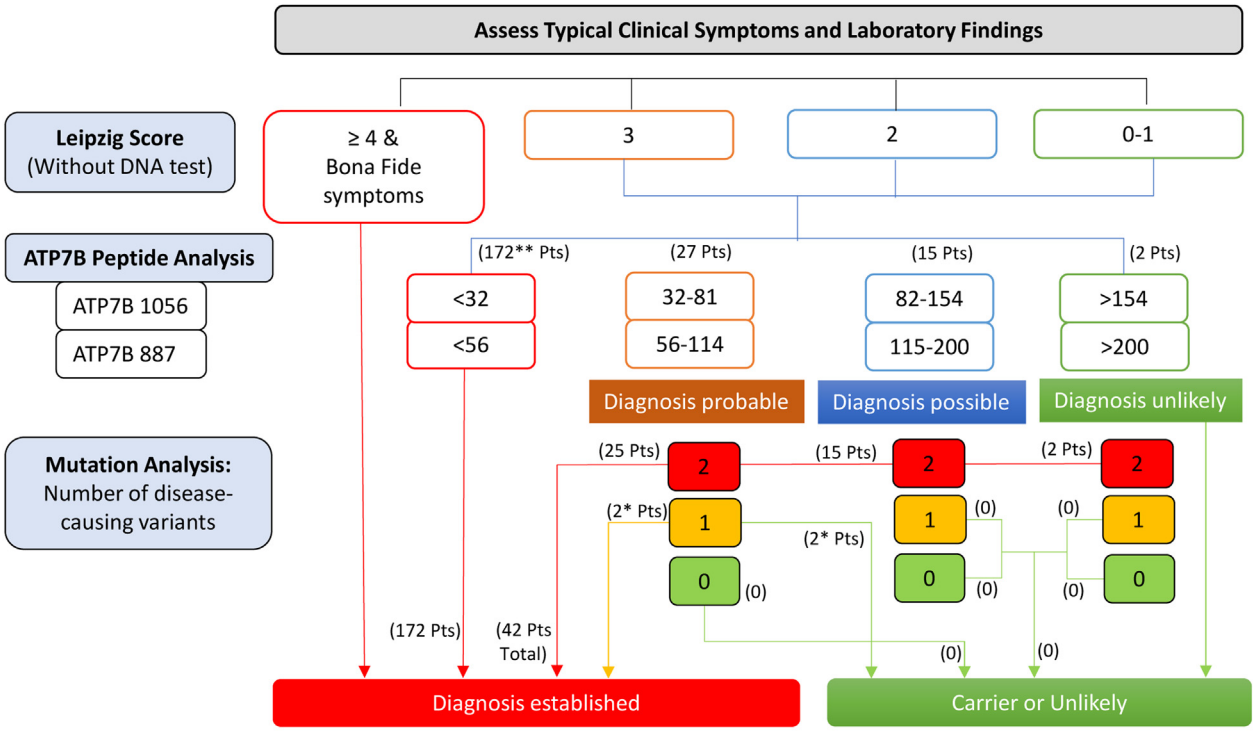


Figure 4. ATP7B peptide concentration analysis can provide clear results where Cp results and genetic analysis are ambiguous. Patients with significantly (*A*), moderately (*B*), and normal (*C*) Cp were readily identified. ATP7B concentrations are reduced regardless of variant status, including in patients 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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Conflicts of interest

The authors disclose no conflicts.

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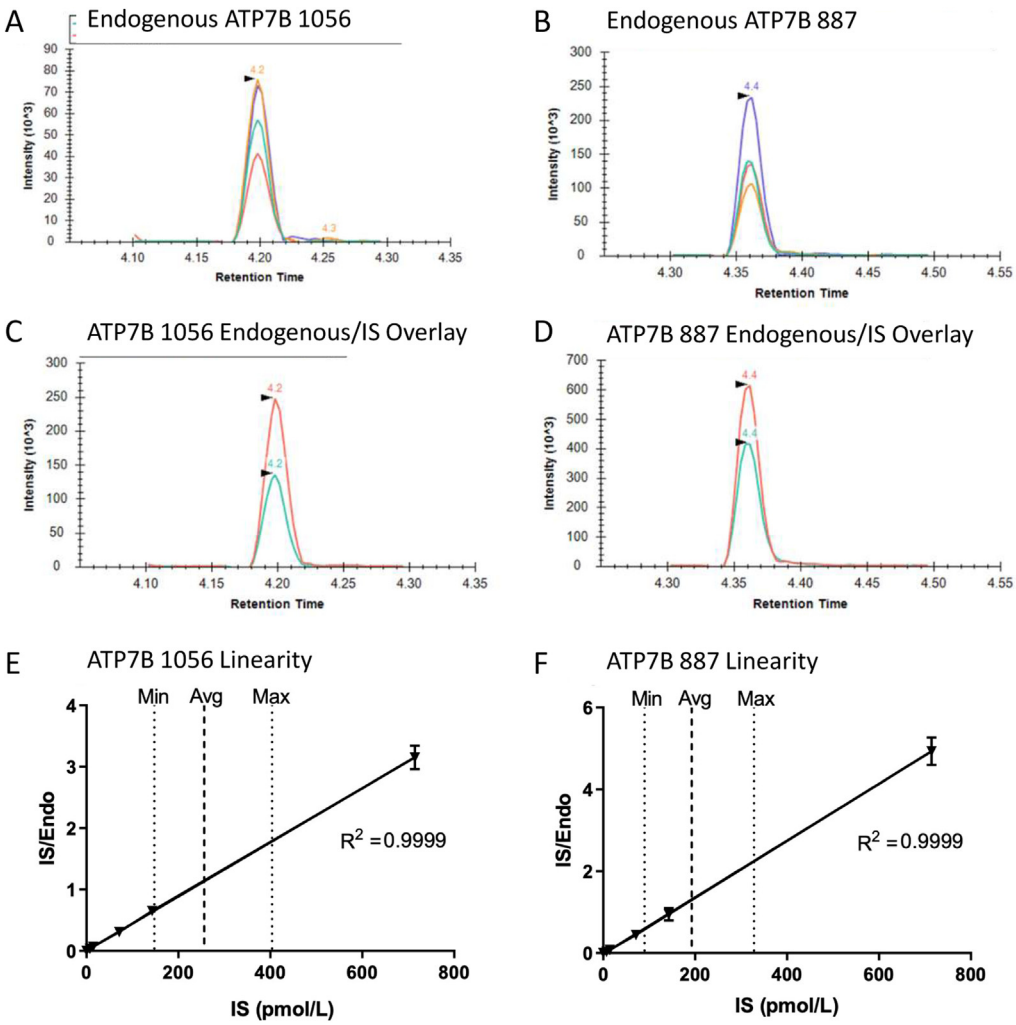

Supplementary Figure 1. Representative chromatograms of ATP7B peptides in MS/MS. (*A*) endogenous ATP7B 887; (*B*) endogenous ATP7B 1056; (*C*) overlay of endogenous and IS ATP7B 887; and (*D*) overlay of endogenous and IS ATP7B 1056. Linearity curves of ATP7B 1056 (*A*) and ATP7B 887 (*B*). *Vertical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