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Research Article

Validation of Metallothionein Immunohistochemistry as a Highly Sensitive Screening Test for Wilson Disease

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ABSTRACT

Wilson disease (WD) is a rare autosomal recessive condition with protean clinical manifestations that result from biallelic *ATP7B* mutations. However, nondestructive tissue tests to be applied clinically to tissue specimens are not widely available to effectively assess patients for possible WD. Previously, we showed that metallothionein (MTH) immunohistochemistry (IHC) has a high sensitivity and specificity for WD diagnosis and, thus, represents a potentially powerful diagnostic tool that can be used in routine histologic sections. This study aimed to validate this finding in a large cohort of bona fide patients with WD and to correlate metallothionein expression with other histologic features. We identified 91 cases of WD, which included 28 needle biopsies and 64 explants from 14 centers worldwide. Histologic features were evaluated, and a histopathological pattern was assigned to each case. All cases were evaluated with Masson trichrome and MTH IHC (clone UC1MT,

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Abcam) using a previously published technique. Liver tissues from chronic cholestatic diseases (n = 42) were used as controls. The median age of the cohort was 28.5 years. Of the 91 total cases, 83 were positive for MTH immunostain. In the controls, all 42 cases were negative for MTH immunostain. The sensitivity and specificity of MTH immunostain for WD were 91.20% and 100%, respectively. MTH IHC is a highly sensitive and specific cost-effective screening tool for WD. It can be used for patients across age groups, varied histologic patterns, and fibrosis stages. This marker could prove to be a valuable tool in the evaluation of patients with possible WD.

Introduction

Wilson disease (WD) is an inherited disorder of copper metabolism caused by mutations in the *ATP7B* gene, leading to impaired copper homeostasis and copper overload in the liver, brain, and other organs. Diagnosis of WD is challenging in large part due to its varied and nonspecific clinical manifestations. The reported average times from symptom onset to diagnosis and treatment for WD have ranged from 12 to 14.4 months for hepatic presentation and ~44.4 months for neurologic presentation in various studies.[1,2] This delay is often due to initial misdiagnosis, as the symptoms of WD can mimic more than 100 different entities.[3,4] Progressive liver disease is a common feature.[5]

The degree of liver involvement can vary, ranging from mild elevation in transaminases to acute hepatic failure. Neuropsychiatric symptoms may or may not be present at presentation.[6] Institution of treatment early in the disease course has been shown to improve outcomes. Otherwise, WD may progress and result in complications such as cirrhosis and neurologic degeneration. [2] Diagnostic features such as Kayser–Fleischer (KF) rings, low serum ceruloplasmin level, increased urine copper excretion and liver tissue copper levels, and molecular testing for mutations in the *ATP7B* gene all have significant limitations.[7] Especially, the rarity of KF rings making it a challenging clinical finding to most practitioners, ceruloplasmin being an acute phase reactant making it susceptible to falsely elevated results, 24-hour urine studies being cumbersome to perform, liver histologic findings being nonspecific, and liver tissue copper quantification being available in a limited number of specialty centers, which often require an entire needle biopsy for analysis.[8]

Genetic testing can be helpful, but it is limited by the large number of genetic variants of uncertain significance in the *ATP7B* gene. [9] Unless the genetic testing demonstrates a known variant affecting both alleles, the diagnosis of WD may still remain unclear despite molecular analysis.[10]

Liver biopsy plays an important role in the diagnosis of WD; however, histologic findings vary widely. The liver can show any of several patterns of injury including a near-normal histology, fatty liver disease, panacinar hepatitis, and cirrhosis. It can also show nonspecific features of acute liver failure such as hepatic parenchymal collapse.[9] Given the lack of specificity of laboratory testing, as well as the variability in histologic features, accurate diagnosis of WD still relies on integrating information from clinical examination, liver biopsy, urine and ceruloplasmin assays, and other laboratory investigations.[11]

Metallothioneins (MTHs) are a group of low–molecular weight proteins rich in cysteine residues with various biological functions, including metal binding, heavy metal detoxification, and protection against oxidative stress.[12,13] In the liver, MTH is a cytosolic copper-binding protein that plays a key role in copper homeostasis by binding to copper ions, regulating their intracellular availability, and preventing its toxicity.[14,15] MTH acts as a buffer, sequestering excess copper and releasing it when copper levels are low.[16,17]

In our prior study, we demonstrated that MTH immunohistochemistry (IHC) performed on formalin-fixed, paraffin-embedded (FFPE) liver samples was highly sensitive and specific in a cohort of 20 patients with WD.[18] In this current study, we expanded our evaluation to include patients from 14 institutions in multiple countries to assess MTH IHC as a screening test for WD that can be deployed in liver pathology practice and described an algorithm for its use.

Methods

Cases

After the Institutional Review Board approval, cases of WD were retrieved from institutional archives (Table 1). All cases (n = 91) had a clinical diagnosis or were strongly suspected of having WD based on clinical evaluation, serum ceruloplasmin level, 24-hour urine copper level, molecular testing for *ATP7B* mutations, and quantitative tissue copper levels. Liver disease with copper accumulation because of other causes, such as chronic cholestatic diseases, were used as controls (n = 42) for IHC—these included primary sclerosing cholangitis (n = 34) and chronic biliary tract disease (not otherwise specified [n = 8]). In these 8 cases, there was suspicion of primary sclerosing cholangitis but that diagnosis was not confirmed. In our cohort, we have genetic testing results available for a total of 17 cases (13 with pathogenic mutations and 4 with likely pathogenic mutations). In the cases without the genetic testing available (n = 74), supportive clinical signs and/or laboratory findings were used for the

Table 1
Number of cases from each of the above institutions included in the study

Institution name	No. of cases
Assistance Publique-Hospitaux de Paris, Paris	2
Emory University, Atlanta	4
Mass General Hospital, Boston	12
Mayo Clinic, Arizona	8
Northwestern University, Chicago	6
Ochsner Health System, Lafayette	5
Ohio State University, Columbus	1
Princess Alexandra Hospital, Queensland	8
Rela Institute, Chennai	19
Royal Prince Alfred Hospital, Sydney	4
Washington University, St. Louis	10
University of Alabama, Birmingham	2
University of Minnesota, Minneapolis	5
UT Southwestern Medical Center, Dallas	5

diagnosis of WD including the presence of KF rings (n = 20), markedly elevated quantitative copper tissue levels (n = 42), markedly elevated 24-hour urinary copper (n = 51), and low ceruloplasmin levels (n = 46). In 35 patients, 2 or more of these features were present.

Stains

H/E staining (hematoxylin [Richard–Allan Scientific #7211] and eosin [Richard–Allan Scientific #7111]) and Masson's trichrome staining were performed following standard clinical techniques. MTH (clone UC1MT, Abcam) IHC was performed as described previously.[18]

Case Review

All cases and controls were reviewed by the central pathologists (N.S. and R.P.G.). The cases were grouped as follows based on the pattern of injury: hepatitis, fatty liver disease, cirrhosis, and near normal. Fibrosis was staged using the Batts–Ludwig system[19]: 0: no fibrosis (not in the original Batts–Ludwig system), 1: portal fibrous expansion, 2: periportal fibrosis, 3: bridging fibrosis, and 4: cirrhosis. The cases with fibrosis stages 0 to 2 were classified as nonadvanced and stages 3 and 4 were advanced.

We used the threshold for positive MTH staining established in our prior study of at least 50% of hepatocytes showing at least moderate cytoplasmic staining. Briefly, qualitative thresholds were tested and a cutoff was set to diagnose WD with 100% sensitivity and specificity. This was followed by testing a quantitative threshold by calculating the H-score for each case and performing statistical analysis using receiver operator characteristic curve to confirm the marked difference in the staining pattern between cases and controls.

Statistical Analysis

Values are expressed as median or total number (percentage of cases with positive findings).

Results

A total of 91 WD cases were included in this study (Table 2); of the 91 tissue specimens, 28 were biopsies, and the remainder were explants. The mean age of the cohort was 28.5 years ± 15.08 SD. There were 35 women and 53 men, whereas sex information was not available for 3 cases.

Histologic Features

The cases showed a broad spectrum of histologic patterns of injury and fibrosis stages. Specifically, the distribution of histologic patterns of injury was as follows: (1) hepatitis (n = 29); (2) fatty liver disease (n = 25); (3) cirrhosis (n = 32); and (4) near normal (n = 5). As most of the specimens were explanted livers, most cases had advanced fibrosis (68 cases with advanced fibrosis, 21 with nonadvanced fibrosis, and 2 with insufficient tissue for staging.

Table 2
Clinical and pathologic features of study cohort

Study cohort features	Number
N	91
Sex	
Men	53
Women	35
Unknown	3
Age (mean years)	28.5 (SD: 15.080)
Specimen type	
Biopsy	28
Resection	63
Liver histopathology	
Hepatitis	29
Steatohepatitis/steatosis	25
Cryptogenic cirrhosis	32
Near normal	5
Fibrosis	
Advanced	68
Nonadvanced	21
NA	2

Immunohistochemistry

The MTH IHC was sensitive across varied patterns of injury and fibrosis stages. Immunoreactivity was seen in 83 of 91 (91.2%) cases overall. Table 3 demonstrates the distribution of results for cases across the 4 categories of histologic injury patterns. There was no variation in the sensitivity of MTH IHC across the injury pattern. Twenty-eight of 29 cases with hepatitis (96.5%), all 25 cases of fatty liver disease (100%), and all 5 of the near-normal cases (100%) showed staining (see Fig. 1). There was no correlation seen between the histologic patterns and staining. When correlated with fibrosis stage, cases with advanced fibrosis showed a sensitivity of 88.2%, whereas cases without advanced fibrosis showed a sensitivity of 100%. Furthermore, in the cirrhosis group, 25 of 32 (78%) cases showed positive staining. Of the negative cases, 5 were completely negative, whereas 3 had only weak cytoplasmic staining.

All 42 control cases were negative for MTH immunostaining, confirming that chronic cholestatic disorders with copper accumulation do not stain with the MTH immunostaining (Table 4). Figure 2 illustrates select controls and the results of MTH IHC.

Discussion

Approximately, 3 decades ago, hepatic metallothionein liver concentration was studied in patients with WD, primary biliary cholangitis, and hepatic metastases from colorectal cancer, because each of these disease states is associated with altered copper metabolism. Prior to that, our knowledge of the role of

Table 3
Distribution of cases by histopathology and MTH IHC results

Histopathology	MTH negative	MTH positive
Hepatitis (29)	1	28
Fatty liver disease(25)	0	25
Cryptogenic cirrhosis (32)	7	25
Near normal (5)	0	5
Total		83

IHC, immunohistochemistry; MTH, metallothionein.

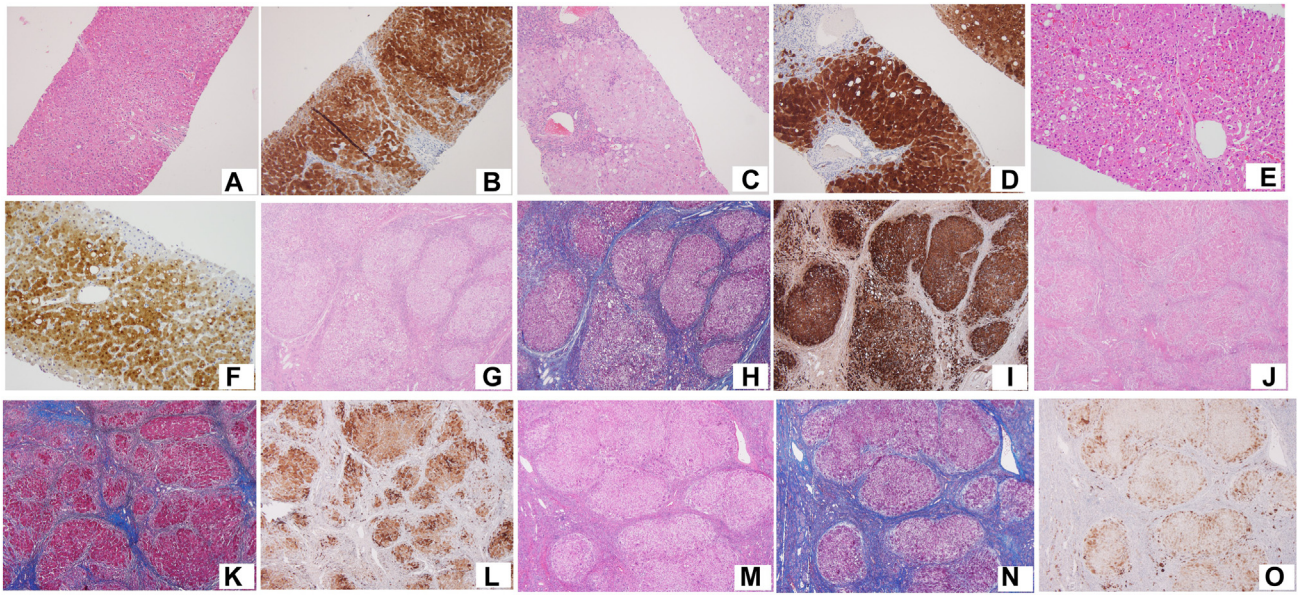

Figure 1.
Different histopathological patterns with H/E stain, in some cases trichrome stain, and corresponding MTH IHC in Wilson's disease cases. (A) Near-normal pattern (×10). (B) Strong diffuse cytoplasmic stain (×10). (C) Hepatitic pattern with mild steatosis (×10). (D) Strong diffuse cytoplasmic staining (×10). (E) Steatohepatitic pattern (×20). (F) Strong diffuse staining (×20). (G, H) Cirrhotic pattern (×4). (I) Strong diffuse cytoplasmic stain (×4). (J, K) Cirrhotic pattern (×4). (L) Moderate diffuse cytoplasmic stain (×4). (M, N) Cirrhotic pattern (×4). (O) Weak cytoplasmic stain (×4). IHC, immunohistochemistry; H/E, hematoxylin and eosin; MTH, metallothionein.

MTH in copper metabolism was entirely derived from animal studies. Using tissue homogenization, Mulder et al[20] evaluated these 3 liver diseases in humans and compared them with normal liver controls. They found that tissue MTH was increased in WD compared with the other diseases.

Using a mass spectrometry-based proteomics approach, we recently confirmed that increased MTH expression is seen in the FFPE liver biopsies from patients with WD. In addition, we showed that the levels of MTH were significantly increased compared with other liver diseases, including autoimmune hepatitis, chronic biliary tract disease, and viral hepatitis.[18]

Next, we validated that observation using IHC on 20 cases of WD and 203 controls (steatohepatitis [n = 51], chronic viral hepatitis [n = 40], autoimmune hepatitis [n = 50], chronic biliary tract disease [n = 42], and normal liver [n = 20]). Those initial data supported the utility of MTH IHC as a potential screening test in patients with WD. In this current study, we expanded testing to a larger cohort of institutions and patients to better represent the diversity of patients affected with WD. Our data confirmed that MTH IHC is a highly sensitive and specific marker of WD, using the threshold of 50% of hepatocytes with at least moderate staining. When combined with data from our prior study, including 111 patients with WD and 245 control cases, there were no false positives. This biomarker worked well on both biopsies and resections and across various stages of fibrosis and histologic patterns of injury. In this current large cohort of cases, the sensitivity was 92%, confirming that this immunohistochemical tool is a sensitive tool for clinical practice.

Table 4
Distribution of controls by histopathology and MTH IHC results

Histopathology	MTH negative
Primary sclerosing cholangitis	34
Biliary tract disease, NOS	8
Total	42

IHC, immunohistochemistry; MTH, metallothionein.

Only a few prior studies have evaluated the use of MTH expression as a diagnostic tool in WD. A recent study performed by Wiethoff et al[21] included a cohort of 69 patients with WD from Germany. They correlated MTH expression by MTH IHC on liver specimens with clinical parameters and found a significant association between MTH expression and 24-hour urine copper levels, the presence of KF rings, and neurologic symptoms. They also showed an overall sensitivity of 85.7%, specificity of 96.9%, and accuracy of 94.9% for MTH IHC in patients with a suspicion of WD, similar to our data. Their data further supports the utility of the MTH stain as a marker to support the diagnosis of WD.

As noted above, challenges in the clinical diagnosis of WD stem from its varied clinical manifestations and the limitations of available diagnostic tests. Consequently, it is not surprising that WD diagnosis is often delayed.[3,4,22,23] The availability of an easy-to-use, inexpensive biomarker such as this one fills a significant gap in diagnostic testing for patients suspected of WD.[9,10]

Given the variability of fibrosis stage and histologic features in patients with WD,[8] any test that is useful with liver biopsy would need to be effective across all stages and a wide variety of patterns of injury. We demonstrated that this is indeed the case with MTH IHC. In cases without advanced fibrosis, MTH was 100% sensitive, and the sensitivity was 88.2% for WD cases with advanced fibrosis (all cases with cirrhosis). One possible explanation for this mildly reduced sensitivity is that cirrhosis has been shown to result in reduced hepatic synthesis of MTHs using both serum and tissue measurements.[24,25] Reduced hepatic synthetic function in cirrhosis[26] therefore may explain the reduced expression by IHC.

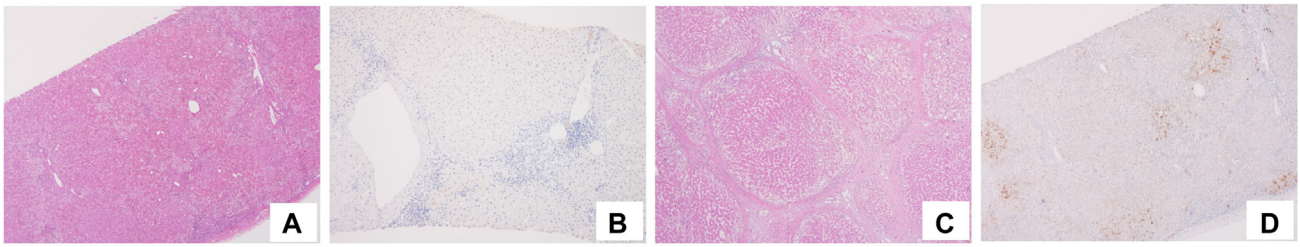

Figure 2.
H/E (A, C) and corresponding MTH IHC (B, D) in control cases. (C) No staining and (D) weak staining. IHC, immunohistochemistry; H/E, hematoxylin and eosin; MTH, metallothionein.

Nonetheless, an overall sensitivity of 88% underscores the utility of this immunostain to aid in the diagnostic workup for potential cases of WD.

MTH IHC is also a substantial improvement over histochemical copper staining in the histopathologic workup of WD. Histochemical stains have historically been used to demonstrate copper in cases of WD, but regardless of the type of stain used, the reported sensitivity is <60%. In our prior study of WD cases, we demonstrated that rhodanine had a sensitivity of 55%.[18] In a comparison study published by Lecca et al,[27] the limited sensitivity of each of the histochemical stains (Timm, orcein, and rhodanine) was shown and the authors, at that time, advocated for performing a panel with all 3 stains. They also concluded that despite the abundant copper in the liver in patients with WD, the copper was "not yet demonstrable with any of the 3 histochemical techniques utilized." A third comparative study by Pilloni et al[28] similarly found histochemical stains all had a sensitivity <60% and the sensitivity of rhodanine, in particular, was poor. In WD, copper accumulates within the cytoplasm as free copper primarily before being bound by copper-associated proteins. This contributes to the poor sensitivity of orcein and rhodanine, which highlight copper-binding proteins and lysosomal copper bound to proteins. Timm silver stain stains free copper in the cytoplasm and so has a higher sensitivity than other histochemical stains. However, the Timm silver stain method, which is not widely used in contemporary practice, requires sufficient cytoplasmic copper to react with sulfides to precipitate silver. As such, its low sensitivity is not surprising. MTH production by hepatocytes is stimulated by heavy metal ions including copper. The copper ions bind to the promoter region of the MTH genes leading to increased production of MTHs.[29,30] Increased MTH is therefore an early metabolic change in WD and this explains the sensitivity of IHC for MTH in this context. Taken together our published data and those in the literature suggest that rhodamine histochemistry should not be considered a reliable diagnostic test for WD.[28,31,32]

Although quantitative tissue copper testing has historically been a mainstay of WD diagnosis, liver biopsy specimens often do not contain enough tissue for extensive histologic workup as well as quantitative tissue copper testing. In our laboratory at Mayo Clinic, Rochester, ~2 mg of tissue (1 cm of an 18-G needle biopsy) is required for quantitative testing. Conversely, MTH IHC can provide useful diagnostic information using only a single unstained section making it accessible and feasible for consulting expert pathologists.

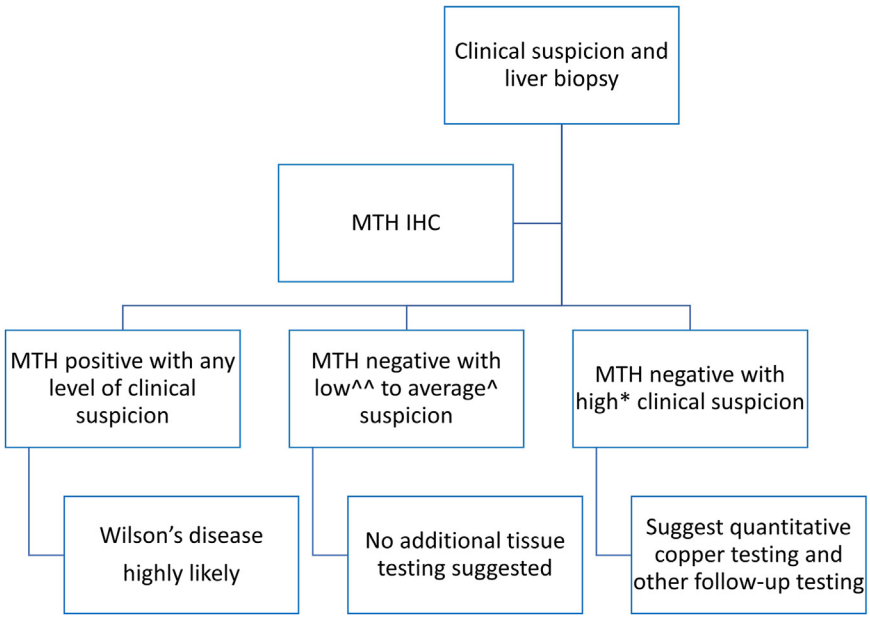

Figure 3.
New proposed algorithm for the screening and diagnosis of Wilson's disease. *Clinical/biochemical evidence of Wilson disease known to pathologist. ^ No clinical information is available to the pathologist. ^^ There is evidence to support alternative cause.

Given that patients with WD who receive early treatment have better outcomes compared with those with delayed or no treatment,[2,9,10] an important advantage of the MTH IHC is that it may reduce diagnostic delays. We propose a practical algorithmic approach to the evaluation of potential patients with WD that includes MTH IHC on liver biopsies (Fig. 3). The proposed algorithm is specifically intended to aid practicing pathologists who may have limited or incomplete access to clinical information and laboratory findings. This algorithm includes the level of clinical suspicion as well as immunostain reactivity. If the clinical suspicion is high and the stain is positive, then a diagnosis of WD is highly likely. If the clinical suspicion is low or average and the stain is negative, then no further workup is required. If the clinical suspicion is high and the stain is negative, then quantitative copper testing on liver tissue and close follow-up with further testing is recommended. Indeed, Wiethoff et al,[21] whose study followed our initial report and used the same IHC we reported, showed that MTH could be included in modified Leipzig criteria[33] in place of serum ceruloplasmin or 24-hour urinary copper without reducing sensitivity for the diagnosis of WD.

In summary, this multiinstitutional multinational study of 91 patients with WD effectively shows that MTH IHC is an excellent tool for histopathologic WD workup. Furthermore, we propose an algorithm for using the immunostain intended to aid practicing pathologists.

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Author Contributions

N.S., A.P. and R.G. participated in performing the study and writing the manuscript. All other authors participated by contributing cases to the study and reviewing and editing the manuscript. All authors read and approved the final paper.

Data Availability

The data sets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Declaration of Competing Interest

The contributing authors and their corresponding institutions do not have any personal or financial conflicts of interest. All coauthors have had the opportunity to review the contents of the manuscript. We declare that the content of this research is an original work and not under review by any other publisher.

Ethics Approval and Consent to Participate

This study was approved by the Mayo Clinic Institutional Review Board.

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