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CLINICAL REPORT

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The co-occurrence of Wilson disease and X-linked agammaglobulinemia in one family highlights the promising diagnostic potential of proteolytic analysis

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Abstract

Background: We report the first case of a family with co-occurrence of Wilson disease (WD), an autosomal recessive disorder of copper metabolism, and X-linked agammaglobulinemia (XLA), a primary immunodeficiency disorder (PIDD) that features marked reduction in circulating B lymphocytes and serum immunoglobulins.

Methods and Results: Through utilization of a multiplexed biomarker peptide quantification method known as the immuno-SRM assay, we were able to simultaneously and independently identify which family members are affected with WD and which are affected with XLA using dried blood spots (DBS).

Conclusion: Being able to delineate multiple diagnoses using proteolytic analysis from a single DBS provides support for implementation of this methodology for clinical diagnostic use as well as large-scale population screening, such as newborn screening (NBS). This could allow for early identification and treatment of affected individuals with WD or XLA, which have been shown to reduce morbidity and decrease mortality in these two populations.

KEYWORDS

co-occurrence, immuno-SRM, newborn screening, Wilson disease, X-linked agammaglobulinemia

1 | BACKGROUND

Wilson disease (WD) is an autosomal recessive disorder of copper transport with a prevalence of at least 1:30,000 and a carrier frequency of 1:90 (Coffey et al., 2013; Schilsky, 2017). The condition occurs due to biallelic pathogenic variants in *ATP7B* (OMIM#606882), which lead to either decreased or absent ATP7B enzyme concentrations or the presence of dysfunctional ATP7B, a copper transporting ATPase that is primarily expressed in the liver and kidneys (Braiterman, Nyasae, Leves, & Hubbard, 2011). Impairment of the appropriate copper excretion from hepatocytes into the biliary system results in copper accumulation in various organs, causing variable degrees of liver disease, neurologic abnormalities, psychiatric manifestations, and the characteristic finding of

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Kayser–Fleischer rings in the cornea. Biochemical markers of WD can include low serum ceruloplasmin, subnormal serum copper, high urinary copper excretion, and increased hepatic copper concentration on liver biopsy (Mohr & Weiss, 2019). The diagnosis of WD can be confirmed with molecular testing in more than 95% of individuals, which can obviate the need for liver biopsy in many cases (Bennett & Hahn, 2011). Treatment is primarily targeted at reducing copper burden. This can be achieved via copper chelation, pharmaceutical interference with gastrointestinal absorption, and/or restriction of dietary copper intake. Orthotopic liver transplantation is often required in patients presenting with acute liver failure or advanced liver cirrhosis.

X-linked agammaglobulinemia (XLA) is one of the first recognized congenital primary immunodeficiency disorders (PIDD; El-Sayed et al., 2019). It occurs secondary to hemizygous pathogenic variants in *BTK* (OMIM#300300), which encodes a cytoplasmic protein that plays a critical role in B cell development (Conley et al., 2005). Due to its X-linked inheritance pattern, XLA primarily affects males and has an estimated incidence of 0.5–1:100,000 (El-Sayed et al., 2019; Winkelstein et al., 2006). Individuals with XLA demonstrate marked reduction in the number of circulating B lymphocytes and all classes of serum immunoglobulins. As a consequence, XLA patients are unable to form antibodies to infectious agents and to immunization exposure leading to recurrent bacterial infections, such as sinusitis, otitis media, pneumonia, meningitis, and sepsis. The diagnosis of XLA is confirmed by molecular testing. The mainstay of treatment is intravenous or subcutaneous immunoglobulin replacement with some centers also utilizing chronic prophylactic antibiotics (Berger, 2004). Decreased awareness of XLA resulting in delay of diagnosis and initiation of treatment has led to increased morbidity and mortality in this population. While newborn screening is not currently available for XLA, newborn screening (NBS) for severe combined immunodeficiency (SCID), using a T-cell receptor excision circle (TREC) assay has proven to be a both sensitive and effective population health screening tool, demonstrating the utility of NBS for PIDD (Taki, Miah, & Secord, 2019).

Peptide immunoaffinity enrichment coupled with selected reaction monitoring mass spectrometry (immuno-SRM-MS) is a sensitive proteomic assay, involving antibody-mediated peptide capture that allows for concurrent quantification of multiple biomarker analytes. This method can be used to detect and quantify extremely low abundance proteins using their surrogate signature peptides. We have previously demonstrated that the immuno-SRM analysis can quantify picomolar concentrations of ATP7B protein in dried blood spots (DBS) and readily distinguish affected cases from normal controls by showing significantly reduced ATP7B in patient samples (Jung et al., 2017). In a multiplex analysis, immuno-SRM can accurately identify patients with XLA or Wiskott Aldrich Syndrome (WAS) from control groups (Collins et al., 2018). Signature peptides were significantly reduced or absent in affected patients compared to the controls and the assay performance showed high precision for the multiplex assay, raising the possibility of employing the approach for large-scale multiple newborn screening (NBS). Here, we report on the first case of a family with co-occurrence of WD and XLA detected by immuno-SRM.

2 | METHODS

2.1 | Ethical compliance

Clinical studies were approved by our Institutional Review Board (#00002183, #15194). Informed consent was obtained for the study #15194.

2.2 | Immuno-SRM evaluation

Proteolytic analysis with peptide quantification was simultaneously evaluated for ATP7B, BTK, and WASp using DBS. The proteolytic analysis was done according to the established protocol with minor changes (Collins et al., 2018; Jung et al., 2017). Detailed procedure is included in the supplemental method. *BTK* gene analysis was performed as previously described (Segundo et al., 2018).

2.3 | Subjects

The family cases include the mother, father, and six full siblings (four males, two females; Figure 1). Two male siblings have molecularly confirmed XLA and two siblings (one male and one female) have molecularly confirmed WD (Figure 1). All diagnoses had previously been made through established clinical or research-based biochemical and molecular testing methods prior to the performance of peptide quantification. For some family members, peptide quantification was performed years after their diagnosis had been made. Furthermore, it did not provide any family members with a new diagnosis that was not already known; however, molecular testing to confirm heterozygous status in some family members was performed after peptide quantification. Relevant laboratory results, including genetic testing results, are described below and included in accompanying tables and figures.

Neither the mother nor the father has any relevant medical history, including absence of known liver disease or other signs or symptoms of WD. The couple did have one either late miscarriage or stillbirth of a male fetus (P0) of uncertain etiology. The mother's ATP7B peptide quantification

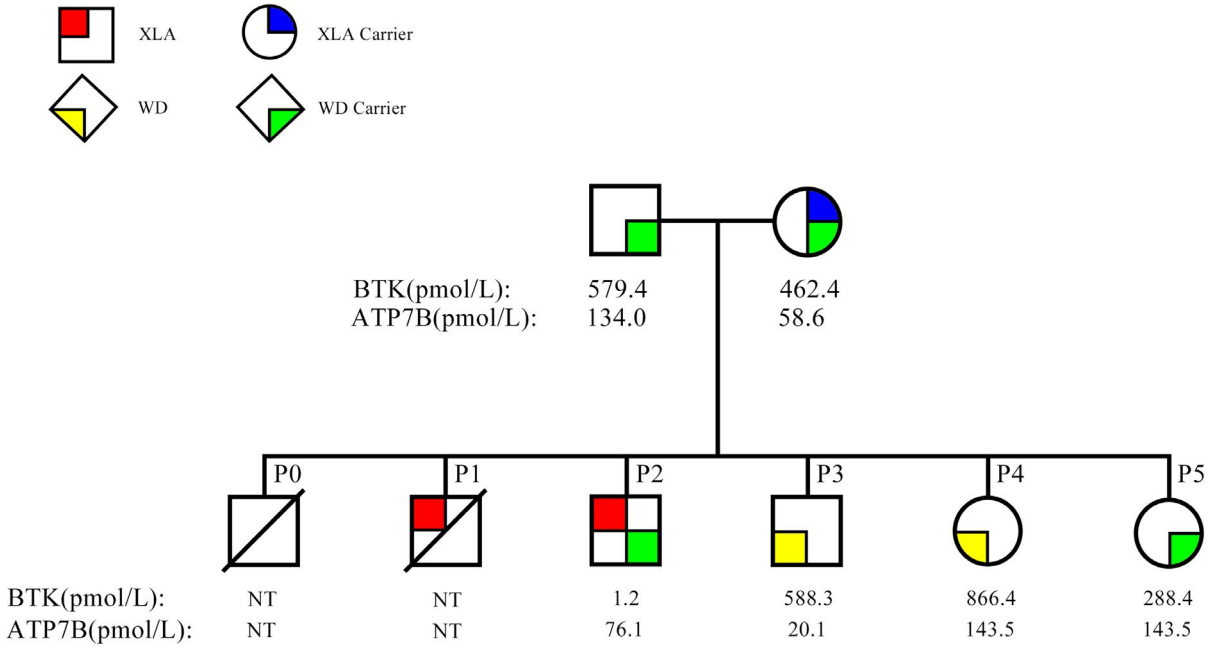

FIGURE 1 Family pedigree. NT, not tested; WD, Wilson disease; XLA, X-linked agammaglobulinemia

(58.6 pmol/ml) was less than half of the father's ATP7B peptide quantification (134 pmol/ml; Table 1), but both were above the current cut-off for ATP7B. Their BTK peptide quantifications were similar to one another. Flow cytometry in the mother showed very little BTK signal in platelets, suggesting she is heterozygous.

P1 was the oldest among his liveborn siblings and was diagnosed with XLA shortly prior to his death at 3 years of age. He had a history of recurrent inflammatory arthritis of the left knee, chronic sinusitis, and recurrent pneumonia. His cause of death was attributed to cardiac arrhythmia secondary to acute viral myocarditis on autopsy. Postmortem evaluation was also notable for very mild and focal leptomeningitis, chronic changes suggestive of bronchiectasis, and a lack of germinal centers in lymphoid tissue. Immunologic workup just prior to his sudden death revealed markedly reduced IgG, IgM, and IgA, as well as absence of measurable antibodies to pneumococcus, diphtheria, tetanus, and Hemophilus influenza despite previous immunization.I Intravenous immunoglobulin (IVIG) infusion had been recommended. A hemizygous variant, c.1567-12_1567-9delTTTG (NM_000061.2) was detected in P1 and P2 resulting in the loss of exons 14 and 15 and the in-frame deletion of amino acids 451–544 leading to a truncated BTK protein; this was confirmed by mRNA studies. This variant is absent in ClinVar and HGMD databases. Targeted testing confirmed that the mother is heterozygous for this variant.

P2 was started on monthly IVIG infusions just prior to 2 years of age for frequent infections, primarily otitis media, conjunctivitis, and pneumonia. He continues to receive them now as an adult and maintains symptoms of chronic sinusitis. His BTK peptide quantification was close to undetectable at 1.2 pmol/L, which was not noted in any of his siblings

TABLE 1 Genotype and peptide quantification analysis

	Genotype		Peptide analysis (pmol/L; cutoff)		
Subject	***ATP7B***	***BTK***	**ATP7B (<56.0)**	**BTK (<169.4)**	**WASp (<191.9)**
Father	p.Met645Arg/WT	NT	134	579.4	1,017.4
Mother	p.Alall35Glnfs*13/WT	c.1567-12_1567-9delTTTG/WT	58.6	462.4	1,548.4
P1	NT	c. 1567-12_1567-9delTTTG	NT	NT	NT
P2	p.Met645Arg/WT	c.1567-12_1567-9delTTTG	76.1	1.2	1,684.3
P3	p.Met645Arg/p.Alall35Glnfs*13	NT	20.1	588.3	1,486.6
P4	p.Met645Arg/p.Alall35Glnfs*13	NT	19.6	866.4	906.2
P5	p.Met645Arg/WT	NT	143.5	288.4	1,735.5

Abbreviations: NT, Not Tested; WT, wild type.

unaffected with XLA, and well below the diagnostic cutoff of 169.4 pmol/L (Table 1). His ATP7B peptide quantification was 76.1 pmol/L, which was above the ATP7B diagnostic cutoff and notably higher than that of his siblings who are affected with WD (P3 and P4). He does not have any known signs or symptoms of WD.

P3 presented with abdominal pain at 18 years of age and because of the known family history of WD in his younger sister (P4, described below), he was also evaluated for this diagnosis. His clinical picture is significant for elevated transaminases (AST 73 IU/L, ALT 198 IU/L), low serum copper (11 ug/dl, reference range 72–166 ug/dl), low serum ceruloplasmin (<3 mg/dl, reference range 16–31 mg/dl), and increased urinary copper excretion (289 mcg/24 hr, reference range ≤60 mcg/24 hr), absence of Kayser–Fleischer rings, absence of characteristic findings of WD on brain MRI, bilateral hand tremors of uncertain correlation with his WD, and diagnoses of attention-deficit hyperactivity disorder and depression. His current management includes dietary copper restriction and zinc gluconate supplementation. His ATP7B peptide quantification was low at 20.1 pmol/L, which is similar to his affected sister (P4), and below the diagnostic cutoff (Table 1). P3 and P4 are both compound heterozygous for two pathogenic variants; c.1934T>G (NM_000053.3 p.Met645Arg, rs121907998) in exon 6 and c.3402delC (p.Ala1135Glnfs*13, rs137853281) in exon 15. He has never had any symptoms suggestive of XLA. He has not undergone familial variant testing of *BTK*. His BTK peptide quantification is notably higher than his XLA-affected brother (P2) at 588.3 pmol/L.

P4 first presented with epigastric pain, emesis, and diarrhea at 9 years of age, ultimately leading to the family's first diagnosis of WD. Her clinical evaluations have included elevated transaminases (AST 109 IU/L, ALT 71 IU/L), low ceruloplasmin (<4 mg/dl, reference range 16–31 mg/dl), and elevated urinary copper excretion (105 mcg/24 hr, reference range ≤60 mcg/24 hr). Liver biopsy demonstrated copper accumulation with an elevated copper content of 1,412.9 ug/g (reference range 15–55 ug/g) and mild inflammation. Her most recent brain MRI revealed possible evidence of copper accumulation in the bilateral globus pallidus and substantia nigra. She also has a history of headaches, a normal evaluation by Neurology, absence of Kayser–Fleischer rings, and recent decline in school performance. Her current management includes dietary copper restriction and zinc acetate supplementation. Her ATP7B peptide quantification was 19.6 pmol/L, which is notably lower than her WD-unaffected older brother (P2) and younger sister (P5), but similar to her affected brother (P3) and below the diagnostic cutoff of 56.0 pmol/L (Table 1). Her BTK peptide quantification was 866.4 pmol/L, which is notably higher than her XLA-affected brother (P2) and her XLA-heterozygous mother.

P5 is the youngest sister in this family. She does not have any known medical concerns and is not affected by WD. Her ATP7B peptide quantification was 143.5 pmol/L and her BTK peptide quantification was 288.4 pmol/L. Both values are significantly above diagnostic cutoffs (Table 1). Her BTK peptide quantification is suggestive of likely heterozygous state, but confirmatory molecular testing has not been performed.

Of note, three of the siblings (P2, P3, and P4) also have a platelet function disorder of uncertain etiology that is not explained by either WD or XLA. P3 has been prescribed clotting medications as needed, but none of the three siblings receive regular medications nor transfusions related to this platelet problem.

3 | DISCUSSION

In this family with co-occurrence of both WD and XLA, we were able to identify independently and accurately individuals affected with WD (P3, P4) and XLA (P2) by multiplexing peptide quantification of ATP7B and BTK using DBS. This demonstrates the feasibility of utilizing this method as a straightforward diagnostic assay for these disorders from small volumes of noninvasively collected DBS samples. Additionally, it demonstrates the possibility of using immuno-SRM as a screening method as there are currently no reliable biomarkers for either disorder available for NBS. Interestingly, the specific diagnosis of XLA for P2 was not originally reported by the family when P3 and P4 were enrolled for DBS peptide analysis for ATP7B and this was not revealed until P2 was seen in clinic himself to clarify his specific diagnosis. This further emphasizes the usefulness of multiplexing with a peptide screening approach as there are likely additional families with co-occurrence of multiple genetic conditions.

Evaluation of ATP7B peptide quantification successfully identified individuals in this family who are affected with WD in the setting of two *ATP7B* variants, p.Met645Arg and p.Ala1135Glnfs*13, that had not previously been evaluated by this assay (Jung et al., 2017). This provides support for the sensitivity of the assay across an expanded set of pathogenic variants in *ATP7B*. However, a limitation of sensitivity could still occur from variants that affect protein structure or function without decreasing quantity. Since the maternally inherited variant in this family is a frameshift variant, which is expected to result in decreased peptide quantity and, therefore, detection by the assay, this report does not necessarily broaden that aspect of the assay's sensitivity. The p.Ala1135Glnfs*13 variant is known to be the most common variant in Venezuelan patients accounting for 26.9% of the 26 studied families (Paradisi, Freitas, & Arias, 2015). Genotype–phenotype correlation in patients carrying this variant was not absolute. In the Brazilian patients carrying the mutation, 6 out of 8 homozygotes had neurologic involvement (Deguti et al., 2004).

However, these findings are difficult to extrapolate to our patients who are compound heterozygous for this variant. Neither of our affected patients with this variant had clinical neurologic symptoms that could be related to WD, although P4 did have questionable copper accumulation on neuroimaging.

The p.Met645Arg variant is the most common pathogenic variant in Spanish patients (Margarit et al., 2005). Despite the high prevalence of p.Met645Arg in this population, there remains a scarcity of reported homozygotes in the literature suggesting mild, later-onset effects of p.Met645Arg in homozygous individuals. The one reported homozygous patient in the literature was from a pediatric cohort that did not provide detailed clinical information regarding this patient (Nicastro et al., 2009). The p.Met645Arg variant displayed Cu-uptake indistinguishable from wt-ATP7B in the expression study using the Baculovirus expression system in Sf9 cells (Huster et al., 2012), thus questioning the pathogenicity.

It is notable that the ATP7B peptide levels in the father and P5 who carry the p.Met645Arg variant were significantly higher than that in the mother carrying the frameshift variant. However, this was not demonstrated in P2, who is also heterozygous for the p.Met645Arg variant. In the two patients with WD, P3, and P4, the peptide level was very low, despite the fact that they carry the p.Met645Arg variant in one allele, and may have been expected to have a less severe *ATP7B* reduction. One hypothesis for this discrepancy is the targeting and rapid degradation of misfolded ATP7B proteins by cellular chaperones in a similar mechanism to misfolded Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) in patients with cystic fibrosis (Kim & Skach, 2012). Unlike for the CFTR protein responsible for cystic fibrosis, no tertiary structures or co-translational assembly have been known to exist for co-translated ATP7B protein from each allele. However, endogenous ATP7B exists in the complex with Clusterin and COMMD1, and they facilitate the degradation of abnormal ATP7B resulting from disease-causing variants (Materia, Cater, Klomp, Mercer, & Fontaine, 2012). A recent study has shown that ATP7B proteins form stable dimers and retain their dimeric form in HEK293 cells regardless of the absence of the N-terminal metal-binding domains (Jayankathan et al., 2017). Based on this proposed structural model, a frameshift mutation within the core of ATP7B, such as p.Ala1135Glnfs*13 reported here, may potentially interfere with the quaternary structure assembly of ATP7B, theoretically giving signals for degradation by Clusterin and COMMD1. If true, this may explain the detrimental reduction of ATP7B in the presence of a mild variant, p.Met645Arg, that is, the misfolded protein from one allele may affect the other translated protein causing posttranslational misfolding of the assembly. This hypothesis may explain why the patients carrying two variants had significantly reduced level of ATP7B peptide in DBS when carrying the mild variant in one allele.

The definitive diagnosis of XLA requires gene sequencing to confirm a mutation in *BTK* and/or the absence of BTK protein expression in monocytes or platelets by flow cytometry (Futatani, Watanabe, Baba, Tsukada, & Ochs, 2001). As these tools are not commonly available in small clinics or developing countries, a definitive diagnosis of XLA may not be able to be established in a timely manner. It is therefore expected to see families like the one we describe here in which the index case presents with a catastrophic event that leads to an undesirable outcome, especially in cases where the clinical phenotype of XLA is mild and with nonspecific symptoms.

The use of DBS for screening and diagnostic testing has multiple advantages, including less invasive sampling, increased stability for storage, and easy sample transportation. Additionally, as demonstrated by this study and in current NBS programs, the use of a DBS card also allows for multiple independent biochemical evaluations from a single sample. Here, we successfully identified both WD and XLA independently via multiplexed peptide analysis in a single DBS. This provides support for the feasibility of using immuno-SRM analysis as a clinical screening method and the incorporation into population screening programs, such as NBS. Both WD and XLA can result in significant morbidity and mortality in affected individuals if left untreated. However, as both diagnoses have effective treatments available, a swift diagnosis is key to providing best outcomes for these patients. Therefore, the ability to detect these conditions through NBS could allow for earlier identification and treatment of individuals that may otherwise suffer unnecessary medical consequences from a delay in the diagnosis and initiation of appropriate medical management.

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AUTHOR CONTRIBUTIONS

S.A.P. and J.T. were involved in acquisition of clinical patient data, drafting the manuscript, revising the manuscript, creating and editing the table and figure, and preparing the manuscript for publication. C.C. was involved in study design, peptide analysis performance, and drafting multiple sections of the manuscript. C.T.M. was involved in performing molecular testing and interpretation. R.D. and F.Y. were involved in sample preparation, peptide analysis, data analysis, and reviewing the draft. P.D. was involved in IRB preparation, consent, and revising the manuscript. I.C. contributed to the acquisition of clinical patient data. H.D.O. and T.R.T. were involved in study design, acquisition of clinical patient data, and revising the manuscript, table, and figure. S.H. conceptualized and designed the study and was involved in data analysis, interpretation, drafting, and revising the manuscript. All authors revised the manuscript critically for important intellectual content and gave final approval of the version to be published. All authors agreed to be accountable

for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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