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Relative Exchangeable Copper Confirms Wilson Disease and Supports Reclassification of the ATP7B p.Met665Ile Variant With Conflicting Pathogenicity Evidence

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ABSTRACT

Wilson disease (WD) is an autosomal recessive disorder of copper metabolism caused by ATP7B mutations. Diagnosis is usually straightforward in symptomatic patients, but can be challenging in children and adolescents with mild liver disease, borderline urinary copper excretion, or inconclusive genetic findings. Reduced penetrance of several ATP7B variants and the limited sensitivity of conventional biomarkers further complicate diagnostic assessment. Relative exchangeable copper (REC) has recently emerged as a highly accurate biomarker capable of distinguishing WD from other liver diseases and differentiating homozygotes from heterozygotes. We report a 14-year-old girl presenting with transient neurological symptoms and normal biochemical liver tests, except for markedly low serum ceruloplasmin. Standard urinary copper excretion was normal and only mildly increased after penicillamine challenge. Whole-genome sequencing revealed compound heterozygosity for the pathogenic ATP7B variant p.Gly626Ala and the variant of uncertain significance p.Met665Ile. Despite the inconclusive genotype, REC was markedly elevated (17.04%), indicating early impairment of copper homeostasis. Because REC is not yet validated for diagnosis in asymptomatic children, liver biopsy was performed, demonstrating steatosis and a hepatic copper content of 250 μg/g dry weight, confirming WD. This case illustrates the potential of REC as a sensitive metabolic biomarker able to detect early ATP7B dysfunction and to support diagnosis in patients with borderline biochemical findings or hypomorphic variants such as p.Met665Ile.

1 | Introduction

Wilson disease (WD) is an autosomal recessive defect in copper metabolism caused by mutations in *ATP7B* encoding an ATPase primarily expressed in the hepatocytes, and responsible for excretion of excess copper into bile (Członkowska et al. 2018). Common presentations are a variable degree of liver disease up to cirrhosis, and neurologic extrapyramidal symptoms and/or

Abbreviations: ALT, alanine aminotransferase; AMA, anti-mitochondrial antibodies; ANA, antinuclear antibodies; ANCA, anti-neutrophil cytoplasmic antibodies; ASMA, anti-smooth muscle antibodies; AST, aspartate aminotransferase; ATP7B, ATPase copper transporting beta; GGT, gamma-glutamyl transferase; IgG, immunoglobulin G; LKM, liver kidney microsomal antibodies; MRI, magnetic resonance imaging; NaEDTA, sodium ethylenediaminetetraacetic acid; REC, relative exchangeable copper; VUS, variant of uncertain significance; WD, Wilson disease.

psychiatric disease. WD is a composite diagnosis, and is rather straightforward in symptomatic patients, in whom usually a suspicion raised by first-line investigations (ceruloplasmin and urinary copper) is confirmed by genetic testing (Ferenci et al. 2003). However, urinary copper excretion—a paramount test in stepwise WD diagnosis—reflects time- and age-dependent systemic copper accumulation and can be falsely negative or borderline in children and adolescents with mild liver disease (Nicastro et al. 2009). In such cases, an inconclusive genetic test result poses considerable diagnostic challenges. Reduced penetrance has been reported for several WD-causing mutations, due to the modifier effect of the second *ATP7B* disease allele, that of other genes, in presence of hypomorphic variants, and for epigenetic and environmental factors (Kieffer and Medici 2017). This makes the attribution of pathogenicity for certain genotypes particularly challenging in the presence of asymptomatic individuals or those with no overt organ involvement (Stättermayer et al. 2019).

Relative exchangeable copper (REC) - the ratio between non-ceruloplasmin exchangeable copper (CuEXC) and the total copper in serum - has gained considerable interest since it has been reported to identify WD with the highest accuracy among liver disease from other causes, as well as to distinguish homozygotes from heterozygotes (El Balkhi et al. 2011; Djebrani-Oussedik et al. 2025). Recent guidelines by the European Society for the Study of the Liver have recognized the potential of the REC and stated that an abnormal REC result should prompt the initiation of the medical treatment, pending genetic testing confirmation (European Association for the Study of the Liver 2025). We report the case of an adolescent in whom—despite an inconclusive genetic test—the REC indicated an existing disturbance in copper metabolism that was not yet clinically or biochemically apparent, potentially allowing for a non-invasive diagnosis. Furthermore, we highlight that REC may offer unparalleled accuracy during the presymptomatic window in which other tests may fail to detect the disease.

2 | Methods

Ceruloplasmin in serum was measured by radial immunodiffusion

Urinary copper concentration was determined by flame atomic absorption spectrophotometry. For penicillamine challenge test, urinary copper was evaluated after administration of 500 mg of D-penicillamine 12 h apart during 24-h collection.

Copper concentration in dried liver tissue was determined by flame atomic absorption spectroscopy according to Kingston and Jassie (Kingston and Jassie 1986) (normal range = 6–50 μg/g of dry weight).

For exchangeable (free) copper, plasma measurements (Li-heparin) were performed after treatment with NaEDTA, ultrafiltration using Amicon Ultra-4 Millipore devices, and ultracentrifugation. The REC was the ratio between exchangeable copper and total serum copper, both quantified by atomic absorption spectroscopy (Pinnacle 800, Perkin-Elmer).

Whole genome sequencing analysis was performed according to the previously published protocol (Lucca et al. 2025). Consent for publication of anonymized data was obtained by the patient's parents.

3 | Case Report

A 14-year-old girl was admitted for the onset of neurological symptoms characterized by brief, sudden involuntary movements affecting all four limbs, myoclonus and vertigo. Emergency brain MRI was unremarkable. Biochemistry showed normal blood cell count, normal renal function, AST 21 IU/L (normal 0–40), ALT 19 IU/L (normal 0–40), GGT 24 IU/L (normal 0–36). Ammonia was 32 mmol/L (normal 0–50), blood gas analysis and lactate were normal. Total IgG, anti-cardiolipin antibodies, lupus anticoagulant, ANA, ASMA, LKM, ANCA, and AMA (including liver disease Western blot panel), as well as a throat swab for *Streptococcus pyogenes*, were all negative/within normal limits. Urinary δ-aminolevulinic acid and porphyrins were in the normal range. Ophthalmologic examination did not reveal any noteworthy abnormalities, but serum ceruloplasmin was 5 mg/dL (normal > 20).

24-h urinary copper excretion was 33 μg (normal 0–60), and increased to 508 μg/24 h after D-penicillamine challenge test. Given the very low accuracy of this test in children with mild liver disease (specificity 24% if > 200 μg/24 h; specificity 51% if > 500 μg/24 h) (Nicastro et al. 2010), a whole genome sequencing on the patient/parents trio was requested.

The patient's neurological symptoms resolved spontaneously within a few days without medical treatment. She also reported two episodes of panic attacks. A psychological assessment revealed significant social and school-related anxiety.

Repeated brain MRI, performed with and without contrast, showed no cortical abnormalities and no significant signal alterations within the cerebral parenchyma—particularly in the basal ganglia—normal diffusion-weighted sequences, while perfusion imaging demonstrated symmetric, harmonious patterns without focal abnormalities (Figure 1A,B). An organic cause of the transient movement disorder was thus highly unlikely.

The patient resulted compound heterozygous for two *ATP7B* variants: (i) c.1877G>C (p.Gly626Ala) of paternal origin—located in exon 6—has been extensively described in literature and classified as pathogenic; (ii) c.1995G>A (p.Met665Ile), of maternal origin, present in the databases with conflicting classifications of pathogenicity, including 16 reports of uncertain significance and 2 of likely benign (Figure 1C). Expanding the metabolic work-up, the patient had a total serum copper of 20 μg/dL, with an exchangeable fraction (CuEXC) of 3.41 μg/dL, and a REC of 17.04% (suggestive of WD if ≥ 15%), thus indicating a definite diagnosis of WD. However, since REC is still not validated in asymptomatic children as a diagnostic tool in WD—and in presence of a Leipzig score of 3—a liver biopsy was performed as further step.

Liver histology showed polymorphic hepatocytes with clear, swollen cytoplasm, and marked macro- and micro-vesicular steatosis involving 30% and 40% of the hepatocytes, respectively, with no fibrosis (Figure 1D). Rhodanine stain was negative.

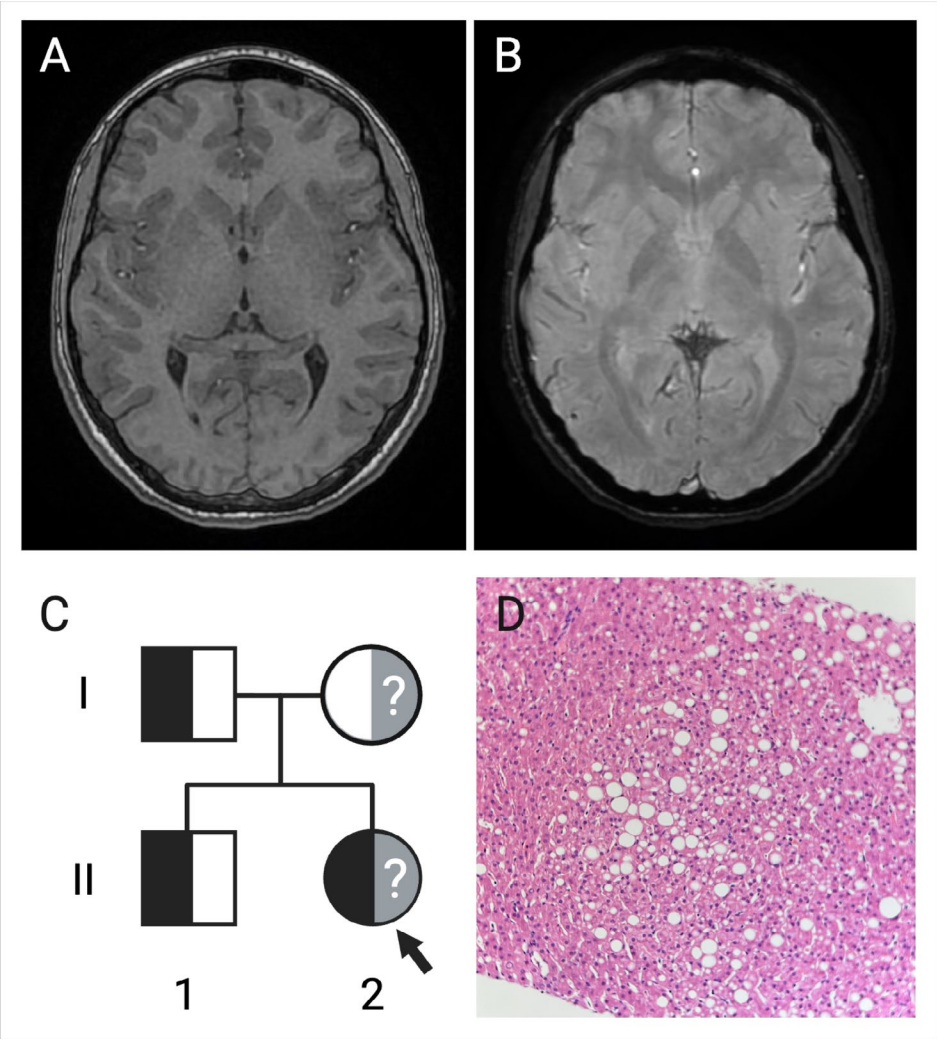

FIGURE 1 | Neuroimaging, pedigree, and liver histology of the patient. (A, B) Brain MRI performed 2 months after neurologic symptoms onset. Axial T1-weighted (A) and T2-weighted Susceptibility-Weighted Imaging (B) sequences show no cortical abnormalities, no significant signal changes in the cerebral parenchyma, and normal appearance of the basal ganglia, with no evidence of diffusion restriction or focal perfusion defects. (C) Pedigree of the family. The proband (arrow) is compound heterozygous for ATP7B variants p.Gly626Ala and p.Met665Ile, whereas the brother carries only the pathogenic p.Gly626Ala allele. Shading indicates the genotype status for each allele. (D) Liver biopsy (H&E stain, 20×) showing polymorphic hepatocytes with clear, swollen cytoplasm and marked macrovesicular (30%) and microvesicular (40%) steatosis without fibrosis. These findings, together with a hepatic copper content of 250 μg/g dry weight, confirmed the diagnosis of Wilson disease.

Liver copper content was equal to 250 μg/g dry weight (normal < 50), definitely confirming the diagnosis of WD.

The patient was started on zinc acetate 50 mg three times per day and is well after 6 months of treatment.

The p.Gly626Ala heterozygous 17-year-old brother exhibited a serum ceruloplasmin of 20 mg/dL, urinary copper of 48.5 μg/24 h, a total serum copper of 46 μg/dL, an exchangeable copper fraction of 2.89 μg/dL, and a REC of 6.28%. A summary of the patient and the sibling diagnostic tests is displayed in Table 1.

4 | Discussion

The p.Met665Ile (c.1995G>A) variant in ATP7B is currently classified as of uncertain significance (VUS) in public databases. First, computational predictions are uncertain whether this variant is neutral or deleterious (REVEL: 0.655), and functional studies are lacking. One compelling argument for pathogenicity relates to population-level data. Although few homozygotes with ascertained WD have been described (Loudianos et al. 1998; Khabou et al. 2026; Tampaki et al. 2020), the p.Met665Ile variant has been identified at relatively high frequency in control populations (0.15%)—including homozygous individuals in large datasets such as gnomAD—without documented WD phenotype, suggesting that individuals carrying two copies may remain asymptomatic or develop only subclinical disease (Mikó et al. 2021; Nilles et al. 2023).

From a mechanistic perspective, the substitution of methionine by isoleucine at codon 665 might result in a "milder" impact on ATP7B function compared to truncating or canonical loss-of-function mutations. Nevertheless, codon 665 lies within the ATP-binding domain (N-domain). Even conservative substitutions may subtly affect folding, phosphorylation cycle kinetics,

TABLE 1 | Diagnostic items and Leipzig score for the index patient and her heterozygous sibling.

Item	Normal range	Index patient (II, 2)	Sibling (II, 1)
ATP7B genotype		p.Gly626Ala/p.Met665Ile compound heterozygous	p.Gly626Ala heterozygous
Ceruloplasmin (mg/dL)	> 20	5	20
24 h urinary copper (μg/dL)	0–60; 0–40*; 0–100**	33	48.5
24 h urinary copper PCT (μg/dL)	0–500 or ≥ 5 × basal UCu	508	—
Liver copper (μg/g)	0–50	250	—
Leipzig score w/o liver biopsy		3	1
Leipzig score with liver biopsy		5	—
Serum CuEXC (μg/dL)	2.9–7.4 μg/dL***	3.41	2.89
REC (%)	2.3–8.5*** (WD ≥ 15)	17.04	6.28

Abbrevations: PCT, penicillamine challenge test; UCu, urinary copper; CuEXC, non ceruloplasmin exchangeable copper; REC, relative exchangeable copper; WD, Wilson disease.
*Optimal threshold for children with mild liver disease.
**Threshold for adult patients.
***Hovden Christensen S, et al. *Clin Chim Acta* 2025.15;565:119978.

or vesicular trafficking (Squitti et al. 2014). This hypomorphic effect might preserve partial copper-transport activity, sufficient under physiological conditions to avoid overt copper overload, or delay its accumulation—which could explain why clinical manifestations appear late, remain mild, or never manifest.

So, many reasons for a reduced penetrance exist, and *ATP7B* alleles' interaction, as well as other genetic, epigenetic, environmental (dietary copper intake, hepatic stressors), or stochastic modifier factors contribute to the resulting phenotype (Wallace and Dooley 2020).

In this context, our case—with hypoceruloplasminemia, elevated REC and histological evidence of steatotic liver disease—supports the notion that p.Met665Ile determines a "latent" pre-symptomatic form of WD.

Had REC been incorporated into the composite diagnostic elements of the Leipzig score, a definite diagnosis of WD could have been established without the need for an invasive liver biopsy in the described patient. From the biochemical and metabolic perspective, unlike urinary copper—which witnesses copper accumulation only after it has already occurred—REC appears to be more sensitive to subtle deviations in copper handling in the initial disease course. In fact, REC reflects the fraction of copper loosely bound to albumin and other low-affinity ligands, and therefore increases early in the course of impaired biliary excretion, even when total serum copper remains within normal limits. In a mechanistic model of human copper metabolism looking for sensitive ATP7B gene therapy-induced activity change biomarkers, REC proved highly sensitive to small changes in ATP7B activity, and clearly outperformed urinary copper excretion (Lindauer et al. 2025).

The REC was also specific enough to discriminate between affected and heterozygous siblings in the same family as previously suggested (Djebrani-Oussedik et al. 2025). The discrepancy between REC and conventional biomarkers in this patient highlights a broader conceptual point: metabolic copper biomarkers exist along a continuum of sensitivity, with REC potentially representing the earliest detectable signal of impaired ATP7B-dependent copper flux.

This has relevance for screening individuals with VUS in *ATP7B*, especially children and adolescents lacking overt symptoms. For variants such as p.Met665Ile, REC may represent a surrogate functional readout, serving as an in vivo functional test for residual ATP7B activity, thus bridging genotype–phenotype uncertainty and improving diagnostic confidence, ultimately allowing earlier identification of individuals at risk before irreversible hepatic injury develops.

The clinical implications are significant: reliance solely on traditional diagnostic criteria could miss such individuals, delaying diagnosis and potential chelation/zinc therapy. Therefore, combination of sensitive biomarkers (e.g., REC), histology, and close follow-up may be particularly important when dealing with "non-classical" *ATP7B* variants such as p.Met665Ile. Nonetheless, functional studies and longitudinal data are needed to definitively confirm its pathogenicity and define its penetrance.

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The authors have nothing to report.

Ethics Statement

In accordance with local regulations, Ethics Committee Lombardia 6 approval is not required for case reports, provided that GDPR compliance, as attested by the institutional Data Protection Officer, full data anonymization, and written informed consent for publication from the patient or legal guardians are ensured.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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