Monitoring Wilson Disease Patients
with a Novel NCC Assay in Wilson Disease

Name

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Agenda

- Monitoring copper status in Wilson disease (WD)
- Challenges to measuring serum NCC
- Development of Orphalan copper speciation (NCC-Sp) assay
- Validation (technical & clinical) of Orphalan (NCC-Sp) assay

Monitoring Copper Status in Wilson Disease

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Normal Copper Homeostasis

- Copper (Cu) is an essential trace element required for normal growth, bone strength, immune function, metabolic function, energy development in the cells and formation of strong connective tissue [2]
- Dietary Cu is absorbed via Cu ion specific transporters in the gut epithelium and transported to the liver via portal vein [1]
- From the liver, Cu is distributed to the general circulation (bound to ceruloplasmin) to provide tissues with required amounts or secretion into bile (bound to ATP7B) [1]
- Bile is the major route for Cu elimination to the stool for defecation [4]
- Circulating Cu is either “free” or protein bound to amino acids, albumin, transcuprein or ceruloplasmin (CP). Cu that is not bound to CP is considered toxic.[3]

1. de Bie P, Muller P, Wijmenga C, Klomp LW.. J Med Genet. 2007;44(11):673-688.
2. Latorre M, et al. Part I. Chapter 4. Biological Aspects of Copper, on Kerkar N and Roberts E. Clinical and Translational Perspectives on WILSON DISEASE. 2019. Academic Press. ISBN 978-0-12-810532-0
3. Roberts EA and Schilsky ML. Hepatology. 2008 Jun;47(6):2089-2111.
4. Boyer JL..Compr Physiol. 2013 July;3(3):1035–1078

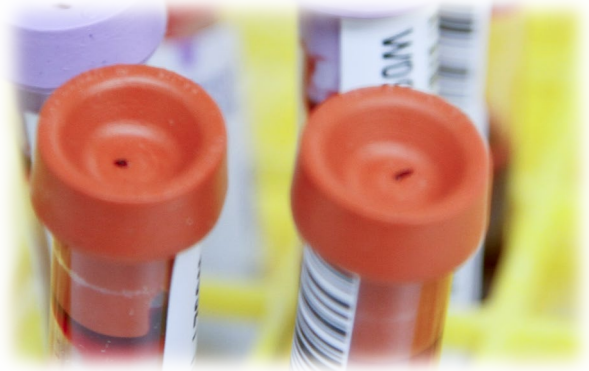
Monitoring Copper Status in WD Clinics

Urine

- 24 hr UCE

Blood

- Serum Cu
- Serum Cp
- Serum NCC / ExCu



24-Hour Urinary Copper Excretion (UCE)

- Widely used in clinical practice
- Does not reflect copper status of patient
- For patients receiving chelation therapy, provides assurances of compliance and drug effectiveness
- Limitations of UCE include:
  - High intra- and inter-patient variability
  - Contamination of container; incomplete collection
  - Intrusion for patients
  - Drug dosing and compliance and time since diagnosis will affect values
  - Pharmacokinetics / pharmacodynamics of chelation therapy relative to collection

Pfeiffenberger et al. J Inherit Metab Dis 2019

Treatment Targets: UCE

Target Range (Chelators): 150–500 µg/24-h (3–8 µmol/24-h)

- On treatment initiation is often >1000 µg/24-h but decreases over time to target range
- >500 µg/24h in patients previously at target suggest insufficient drug action (nonadherence to medication, poor drug absorption, inadvertently low dosing), acute liver injury (ALI), or excessive dietary copper
- <100 µg/24-h may signal overtreatment with excessive copper removal

Schilsky ML, et al. Hepatology 2023; 77(4):1428-1455.

Total Serum Copper (Cu)

- Not specific or sensitive as a biomarker for diagnosis, measure of compliance, disease progression
- Serum copper and ceruloplasmin (Cp) may be followed for trends
- Very high serum copper may indicate excessive copper intake
- Lower serum copper and ceruloplasmin could indicate overtreatment and total-body depletion

Schilsky ML, et al. Hepatology 2023; 77(4):1428-1455.

Treatment Targets: NCC

NCC normalizes with effective treatment (normal ≤15 mg/dL or ≤150 µg/L)

- Nonadherence to therapy, serum copper and exchangeable copper increase, and NCC may be elevated (>25 mg/dl)
- With overtreatment, serum copper and exchangeable copper are very low, as is the NCC (typically <5 mg/dl)

Serum Ceruloplasmin normal 20-40 mg/dl

- Suspect diagnosis with <20mg/dl

Schilsky ML, et al. Hepatology 2023; 77(4):1428-1455.

Challenges to Measuring Serum NCC

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Non-Ceruloplasmin Bound Copper (NCC)

- There are no FDA-approved assays for NCC
- Currently NCC is estimated using an immunological assay for ceruloplasmin
  - The immunologic assays do not differentiate between metal-bound and metal-free ceruloplasmin
- Assumes 6 Cu atoms bound to ceruloplasmin

  NCC = Total serum Cu (µg/dL) - [3.15 x Cp (mg/dL)]

  Cumbersome, inaccurate with negative value in ~ 20% of patients
- An unmet need in WD is the ability to measure NCC precisely and accurately
  - Studies to validate any novel diagnostic test should investigate the clinical utility, including:
    1. The potential to improve monitoring of disease (predict worsening of clinical manifestations or potential role as a biomarker)
    2. Adherence to treatment
    3. Facilitate dose titration
- 2022 AASLD Guidance encourages further studies to evaluate potential of these new assays as a monitoring tool for WD

Schilsky ML, et al. Hepatology 2023; 77(4):1428-1455.

NCC Tests

Technique	Acronym	Advantages	Disadvantage	Reference
Calculated NCC		Only requires Total Copper and Cp levels	Cumbersome, Inaccurate if immunodiffusion is used to determine Cp	1. Twomey 2008 2. Walshe 2010 3. Schilsky 2023
Exchangeable Copper	CuEXC	Doesn't depend on Cp	EDTA able to withdraws copper bound to Cp	1. Schilsky 2023 2. Medici 2022
Ultrafilterable Cu and CuEXC	CuUF and CuEXC	Simple 2-step method	CuUF extremely unstable; copper contamination from filters is possible; excludes other copper-binding proteins	1. McMillin 2009 2. El Balkhi 2009 3. Woimant 2019
Inductively Coupled Plasma Mass Spectrometry (appears to be what ARUP labs uses)	ICP-MS	Large range, low detection limit, high throughput, low sample volume, simple sample prep	Cost Expertise needed Specific laboratory needs, best for labs that run a lot of samples	1. Wilschefski 2019 2. aruplab.com
Strong Anion Exchange Chromatography coupled to triple quadrupole ICP-MS	SAX-ICP-MS-MS	Complementary technique to HPLC	More useful for separating proteins, peptides, and nucleic acids	1. Solovyev 2020
Novel ICP-MS (Alexion)	ICP-MS	Immunocapture using magnetic beads to specifically capture TPC	Specific to ALXN1840 2-step process (immunocapture + Chelation + Filtration and ICP-MS)	1. Liang 2022
Labile Bound Copper via ICP-MS Dual Filtration (Mayo Clinic Labs)	LBC-ICP-MS	Uses 2 filters - decreases cost and turnaround time	Unsure if EDTA incubation pulls copper from Cp; Research only done on healthy volunteers	1. Bitzer 2024
Liquid chromatography combined with ICP-MS	LC-ICP-MS	Allows for "speciation" of an element	Availability of Liquid chromatography device	1. Wilschefski 2019
High Liquid Chromatography coupled with ICP-MS (Orphalan)	HPLC-ICP-MS	Allows for "speciation" of an element; avoids EDTA	2-step process: ICP-MS for Total Cu and LC-ICP-MS for CpCu then calculate NCC by subraction; 2-week turnaround time Cost	1. Schilsky 2022 2. Del Castillo Busto 2021

Challenges with Current Available NCC Testing

- Biomedical analytical methods often rely on indirect measurements, such as immunoassays, which can lack effective traceability.
  - Limitations: No differentiation between metal-bound and metal-free ceruloplasmin, frequently leading to inaccurate NCC values.

- The challenge was addressed by the introduction of a parameter known as exchangeable copper “labile Cu” fractions, as measured by copper due to its determination *via* the incubation of blood serum or plasma with complexing reagents such as EDTA.
  - Limitations: Inaccuracies in the method due to the ability of the chelator to still remove a portion of ceruloplasmin- bound copper from the protein.

Medici et al. Lancet Gastro Hepatol 2022. 7(12): P1063-1065.
Solovyev et al Analytica Chimica Acta 2020 Feb 15;1098:27-36

Current AASLD Guidance
Maintenance Therapy

	Treatment Target		Over Treatment		Inadequate Treatment	
	NCC (µg/L)	UCE (µg/24h)	NCC (µg/L)	UCE (µg/24h)	NCC (µg/L)	UCE (µg/24h)
D-Penicillamine	50-150	200-500	<50	<100	>250	>500
Trientine	50-150	150-500	<50	<100	>250	>500
Zinc	50-150	<100	<50	<20	>250	>100

Schilsky ML, et al. Hepatology 2023; 77(4):1428-1455.

Development of Orphalan Copper Speciation (NCC-Sp) Assay

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Development of an Assay During the CHELATE Trial

- Serum NCC by Speciation (NCC-Sp) assay was developed to address FDA's concern:
  - No NCC assay available with desired precision and accuracy to assess copper load as a primary efficacy end point.
- Assay consists of two parts:
  - ICP-MS* assay for the quantitative measurement of copper in human serum (total: protein-bound and free)
  - LC-ICP-MS# assay for the separation of ceruloplasmin by LC and quantitative measurement of ceruloplasmin-bound copper by ICP-MS
- Non-ceruloplasmin-bound copper (NCC) is then calculated:

$$NCC = [Total\ Serum\ Cu]\left(\frac{ng}{mL}\right) - [Cp\ Cu]\left(\frac{ng.equiv}{mL}\right)$$

*Inductively coupled plasma mass spectrometry
Liquid chromatography inductively coupled plasma mass spectrometry

Schilsky et al Lancet Gastroentrol Hepatol. 2022; 12: 1092-1102

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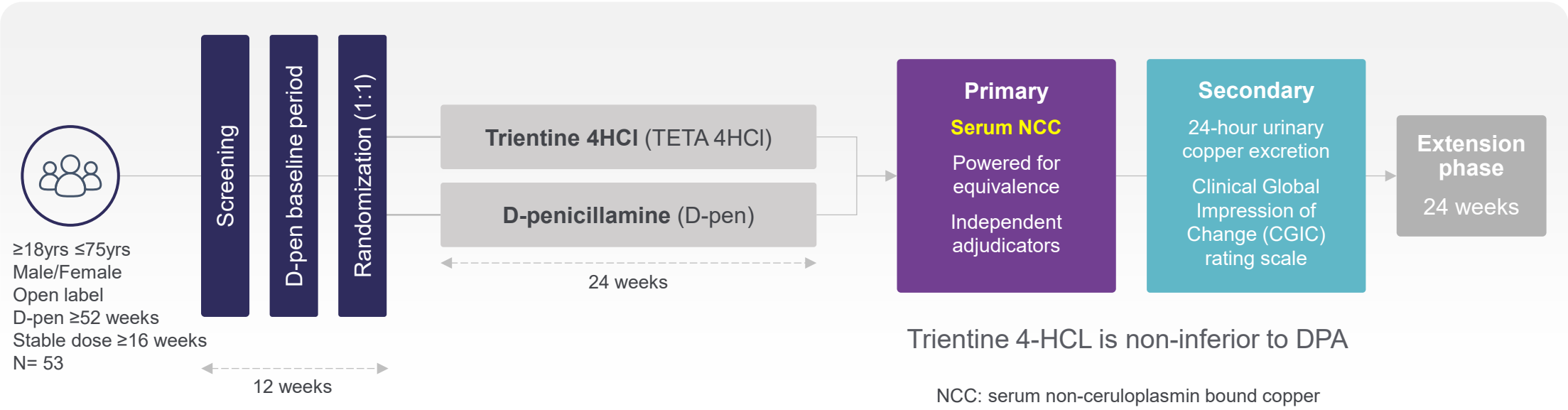
CHELATE Trial Design

- CHELATE is the first randomized study evaluating trientine vs. D-Penicillamine in stable patients
- Efficacy and safety end-points agreed with FDA; non-inferiority trial design
- Serum NCC (NCC-Sp) assay to measure copper levels in the serum



Schilsky et al Lancet Gastroentrol Hepatol. 2022; 12: 1092-1102

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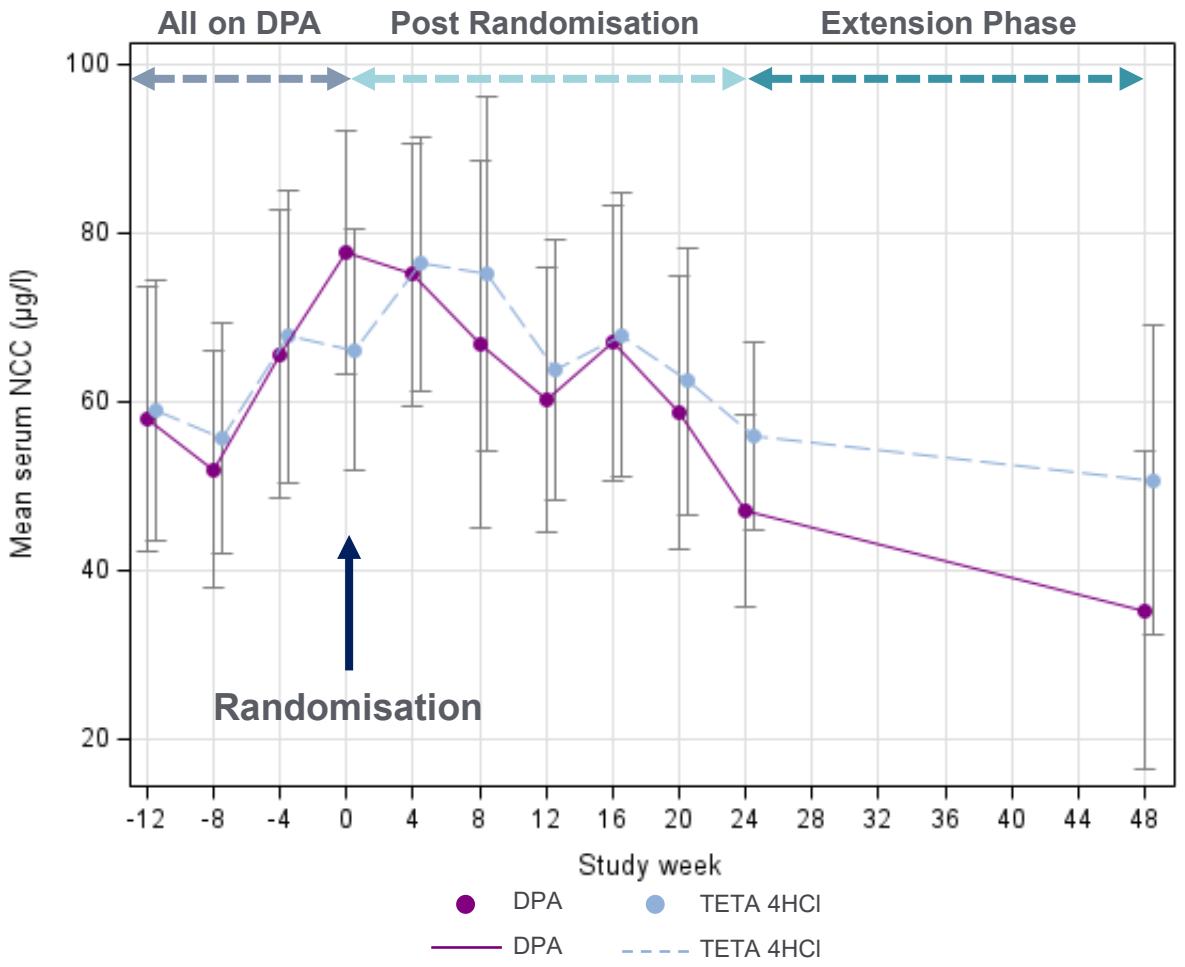
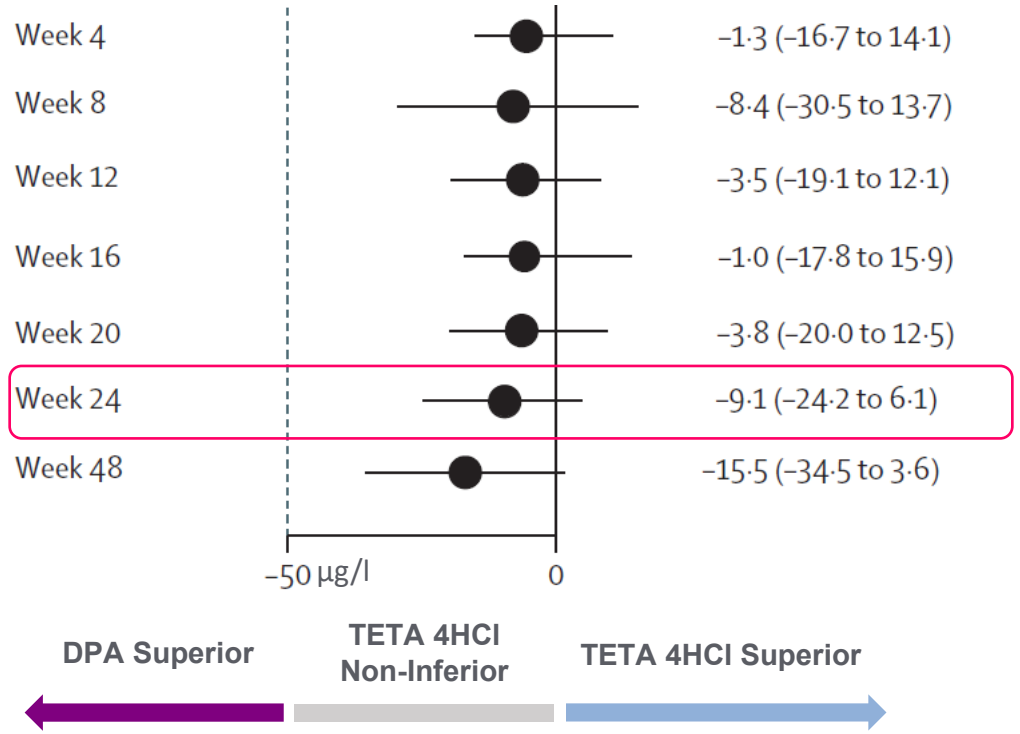
Primary Efficacy Measure:
Serum NCC by Speciation (NCC-Sp) at Week 24



Mean difference (95% CI) in serum NCC by speciation (NCC-Sp) assay from baseline, µg/l, up to 48 weeks post randomisation



Non-inferiority margin was –50 µg/L NCC by speciation assay.

Schilsky et al Lancet Gastroentrol Hepatol. 2022; 12: 1092-1102

Analytical Validation of Orphalan NCC Assay

- Validated per FDA recommendations and in accordance with the Bioanalytical Method Validation Guidance for Industry, FDA (BMV Guideline May 2018)

*Assay is not commercially available

021-03517-y

RESEARCH PAPER

Validation Steps

Before a new medical test can be introduced into clinical practice, it should be evaluated for:

- **Accuracy and precision**
- **Analytical validity** - does the test work in the laboratory?
- **Clinical validity** - does the test work in the patient population of interest?
- **Clinical utility** - is the test useful? Can it lead to improvement in health outcomes?

Anion-Exchange HPLC-ICP-MS Separation of Serum Cu Proteins

Total serum Cu

ICP-MS

Non-exchangeable Cu(I)

HPLC-ICP-MS

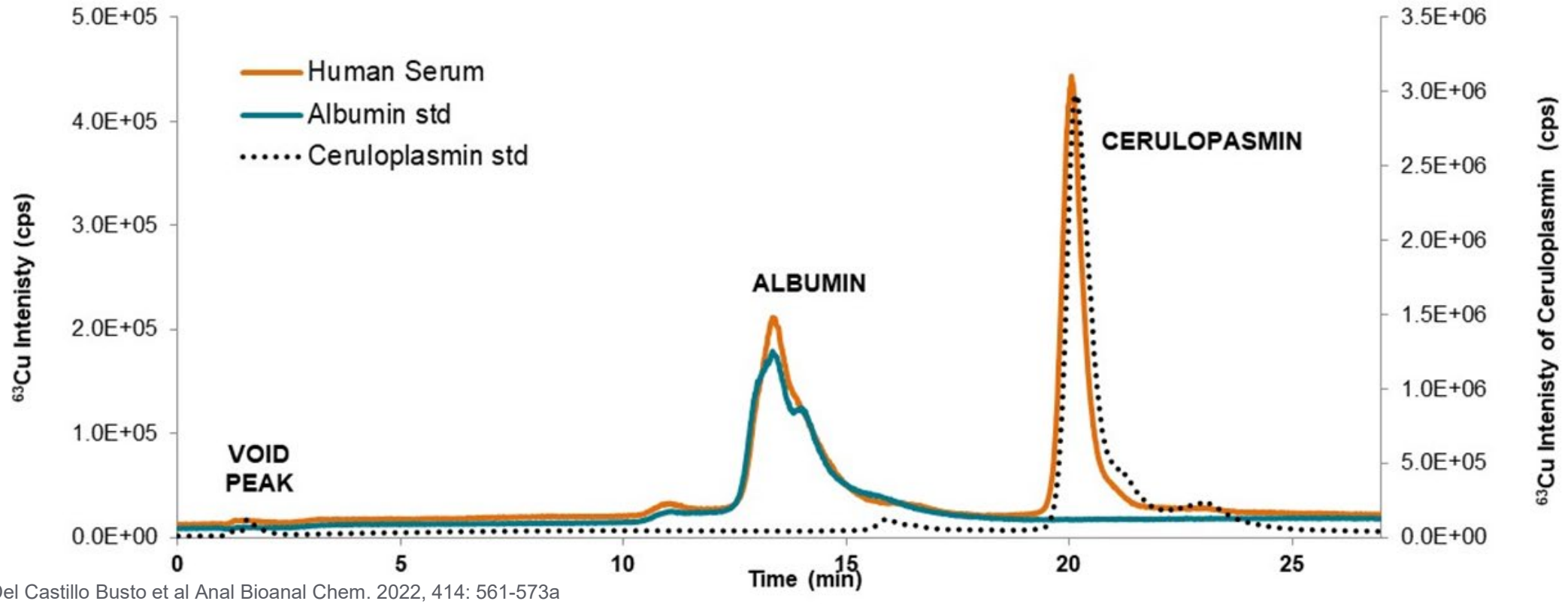
Exchangeable or labile Cu(II)

Ceruloplasmin, CER (~70 – 95 %)

Albumin, ALB

α-2macrogobulin

Low-molecular weight compounds (e.g. free Cu)



Del Castillo Busto et al Anal Bioanal Chem. 2022, 414: 561-573a

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Real-World Evidence Study in Subjects With Wilson's Disease (REASON)

Ongoing Study Assessing NCC Assay

Currently available data:

- NCC-Sp range from 53 WD patients from CHELATE (~700 repeat samples)
- NCC-Sp range from 75 healthy subjects

Real World Evidence validation requires collection of NCC samples:

- Subjects with WD treated with Cuvrior® and other WD therapies over time outside of an RCT
- Newly diagnosed patients/ treatment naïve patients
- “Unstable” patients

Additional studies are being planned to validate NCC-Sp in different populations and under different conditions

Clinicaltrials.gov ID: NCT05783687

Assessment of Copper Status in WD
Summary

- UCE is cumbersome and dependent on voided volumes and patient compliance
- Reproducibility and reliability of estimated NCC measurement are known challenges among WD physicians
- There are concerns about direct measurements of free copper
- Immunological & enzymatic assays are not validated and imprecise
    - EDTA assay underestimates non-ceruloplasmin-bound copper
- Copper speciation assay* is more precise than currently available measurement tools and has been used in a registration trial as a primary endpoint in maintenance of Wilson Disease patients.
- Further real-world data are needed to validate the NCC-Sp assay in clinical practice in different populations

* Assay is not commercially available

Back up slides

Study Acronym – REASON

(**R**eal world **E**vidence to evalu**A**te the clinical utility of copper **S**peciati**ON** assay to measure free copper in patients with Wilson disease) Study

Multicenter, non-interventional, prospective, RWE study of adult Wilson Disease patients to assess clinical utility of measuring copper parameters, including a novel non-ceruloplasmin bound copper assay based on copper protein speciation (NCC-Sp) along with standard of care clinical and biochemical assessments

Clinicaltrials.gov ID: NCT05783687

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Study Objectives

- Primary:
  - Investigate the NCC-Sp range in WD patients over ~12 months
- Secondary:
  - Compare NCC-Sp assay results with standard clinical assessments including key WD parameters of copper metabolism from SoC laboratory testing
- Exploratory
  - Evaluate WD patient subgroups that could benefit from NCC-Sp

Target Population

- Adults ≥ 18 years with WD receiving treatment with DPA, any trientine or zinc
- Newly diagnosed or drug naïve (< 28 days)
- Physician considering a modification to therapy (dose and/or class of drug) because of either
  - elevated liver enzymes (ALT or AST > 1.5x ULN)
  - 24-hr urinary copper excretion outside of recommended range
    (chelators <200 mcg/L or >500 mcg / 24hr or zinc > 100mcg / 24hr

Clinicaltrials.gov ID: NCT05783687

Study Design – Overview
Target: 10 sites in U.S. / 50 patients

Population	Newly diagnosed / drug naïve OR Laboratory values (ALT, AST > 1.5x ULN; 24-hr UCE outside of recommended range)
Intervention	Non-interventional study (SoC)
Control	Non-interventional study (SoC)
Outcome	Descriptive analyses of laboratory assessments over time including: • Non-ceruloplasmin copper using speciation (NCC-Sp) • Non-ceruloplasmin copper (estimated using ceruloplasmin assay) • UCE • Liver enzymes Standardized assessments by physician and patient • UWDRS (Part II) • SF-12 QoL assessment • Morisky scale assessment for compliance (MMAS-8) • Patient Global Impression of Change (PGIC) • Clinical Global Impression of Change (CGIC)

Clinicaltrials.gov ID: NCT05783687

Inclusion Criteria

- Male or female patients, ≥18 years
- Able and willing to comply with study procedures and requirements, as judged by the treating physician
- Established diagnosis of Wilson's Disease (Leipzig score >4)
- Either:
  - Newly diagnosed;
  - Elevated liver enzymes (defined as ALT, AST ≥1.5xULN);
  - 24-hour urinary copper excretion outside of recommended ranges [chelation range 200-500mcg/24 hr; zinc >100mcg/24 hr]
- Adequate venous access to allow collection of blood samples

Clinicaltrials.gov ID: NCT05783687

Laboratory Investigations

Standard of Care (SoC)

- LFTs, biochemistry, hematology, coagulation and copper assessments,
- 24h urine for urinalysis

Extra Volume of blood (at the same time as blood draw for SoC investigations)

- Up to 10 mL for NCC-Sp, serum Cu, serum Zn

Subgroup Analysis by Treatment

Group	Treatment	Max Number of patients
A	Newly diagnosed (DPA / trientine / zinc for < 28days)	No upper cap*
B	DPA	30
C	Zinc	No upper cap*
D	Trientine (2HCl or 4HCl)	No upper cap*

*** Enrollment to stop when overall study sample size (n=50) achieved**

USA RWE STUDY SITES

Aim 50 patients

Fred Askari, University of Michigan, Ann Arbor, MI
Kalyan Bhamidimarri, University of Miami, FL
Amy Brown, Vanderbilt, TN
Amanda Cheung, Northwestern, Chicago
Valentina Medici, UC Davis, CA
Michael Schilsky, Yale, CT
James Hamilton, Johns Hopkins, MD
Regino Gonzalez-Peralta, Advent Health, FL
Steven Lobritto, Columbia University, NY

Diagnosis of Wilson Disease

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Wilson Disease Etiology

- First described in 1912 by Kinnier Wilson, WD is an autosomal-recessive disorder (both parents must pass on the impaired gene to the child) caused by a defective copper-transporting gene, ATP7B
- ATP7B dysfunction results in defective copper elimination from the liver leading to excess copper and deposition in liver, brain and other organs
- Affects adults and children with typical presentation range 5- 35 years old
- Misdiagnosis and delay in treatment are clinically relevant because untreated disease progresses to hepatic failure or severe neurologic disability and death

- Poujois A and Woimant F. Wilson's disease: A 2017 update. Clin Res Hepatol Gastroenterol. 2018 Dec;42(6):512-520.
- Nishito Y, Kambe T. Absorption Mechanisms of Iron, Copper, and Zinc: An Overview. J Nutr Sci Vitaminol (Tokyo). 2018;64(1):1-7
- Polishchuk RS. Part I. Cellular Physiology. Chapter 6. Cellular Function of ATP7B (Wilson ATPase) on Clinical and Translational Perspectives on WILSON DISEASE. 2019. Academic Press. ISBN 978-0-12-810532-0
- Lorincz MT. Chapter 18. Wilson disease and related copper disorders. Handbook of Clinical Neurology, Vol. 147 (3rd series) Neurogenetics, Part I D.H. Geschwind, H.L. Paulson, and C. Klein, Editors.
- Ferenci P. Wilson's Disease. Clin Gastroenterol Hepatol. 2005;3:726:733.

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AASLD 2022 Guidance for Diagnosis

- Search for a family history of WD, early death from liver disease or early onset neurological or psychiatric illness, a physical examination for signs of liver or neurological disease
- Biochemical testing for liver disease, followed by specific testing of copper metabolism
- Ophthalmologic examination for Kayser-Fleischer rings should be performed by slit lamp or anterior segment optical coherence tomography, a corneal imaging method that easily distinguishes and can quantify various anterior segment abnormalities.
- Laboratory testing should begin with clinical biochemical liver tests, blood counts, and coagulation parameters to assess for liver disease.
- Tests of copper metabolism specific for WD include serum ceruloplasmin, serum copper, basal 24-h urinary copper excretion (UCE) and liver biopsy for histology, histochemistry, and copper quantification.
- Genetic testing for *ATP7B* mutations may be used.

Schilsky ML, et al. Hepatology 2023; 77(4):1428-1455.

Diagnosis

- Circulating levels of ceruloplasmin are reduced in ~95% of patients presenting with WD (<20 mg/dl)
    - <14.0 mg/dl is more specific for diagnosis of WD than 20 mg/dl
- Urinary Copper Excretion (UCE) of symptomatic patients is typically:
    - >100 µg/24h, but a lower reference value of >40 µg/24-h (>0.6 µmol/24-h) may indicate WD in individuals who are asymptomatic or children
- Hepatic copper content determination has been extremely useful for disease diagnosis:
    - >250 µg/g dry weight liver for copper content

Schilsky ML, et al. Hepatology 2023; 77(4):1428-1455.

Routine Tests to Diagnose WD[1]

Test	Typical Finding	False Negative	False Positive
Serum Ceruloplasmin	Decreased by 50% of lower normal value	Normal levels in patients with marked hepatic inflammation Overestimation by immunologic assay Pregnancy, estrogen therapy	Low levels in: - Malabsorption - Aceruloplasminemia - Heterozygotes
24-hour Urinary Copper	>100 µg/24h[2] >40 µg/24h[2] in children	Normal: - Incorrect collection - Children without liver disease	Increased: - Hepatocellular necrosis - Cholestasis - Contamination
Serum Free Copper	>100 µg/L[2]	Normal if CP overestimated by immunologic assay	
Hepatic Copper	<50 µg/g[2] dry weight	Due to Regional Variation - in patients with active liver disease - In pateints with regenerative nodules	Cholestatic Syndromes
Kayser-Fleischer Rings	Present	Absent - in up to 50% of hWD - in most asymptomatic siblings	Primary Biliary Cirrhosis

CP: ceruloplasmin
hWD: hepatic Wilson disease presentation

1. Adapted from: Ferenci et al. *EASL Clinical Practice Guidelines: Wilson disease*. J Hepatol 2012; 56 (3) 671-685
2. Schilsky ML, et al. Hepatology 2023; 77(4):1428-1455

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Measurement of NCC

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Measurement of NCC

NCC-Ex

”Exchangeable Copper”

1) NCC chelated with EDTA

2) Ultrafiltration removes ceruloplasmin

3) Cu measured in filtrate

EDTA Cu trapped in filter

NCC-Sp

”NCC by protein speciation”

1) Protein speciation by AE HPLC
(high performance liquid chromatography)

2) Measure Cu in each protein peak by MS (mass spectrometry)

3) NCC-Sp = Total Cu ·(1-CpCu fraction)

El Balkhi-A. Anal Bioanal Chem 2009; 394:1477

Solovyev N. Anal Chem Acta 2020; 1098:27-36
Del Castilo Busto ME. Anal Bioanal Chem 2021; 414:561

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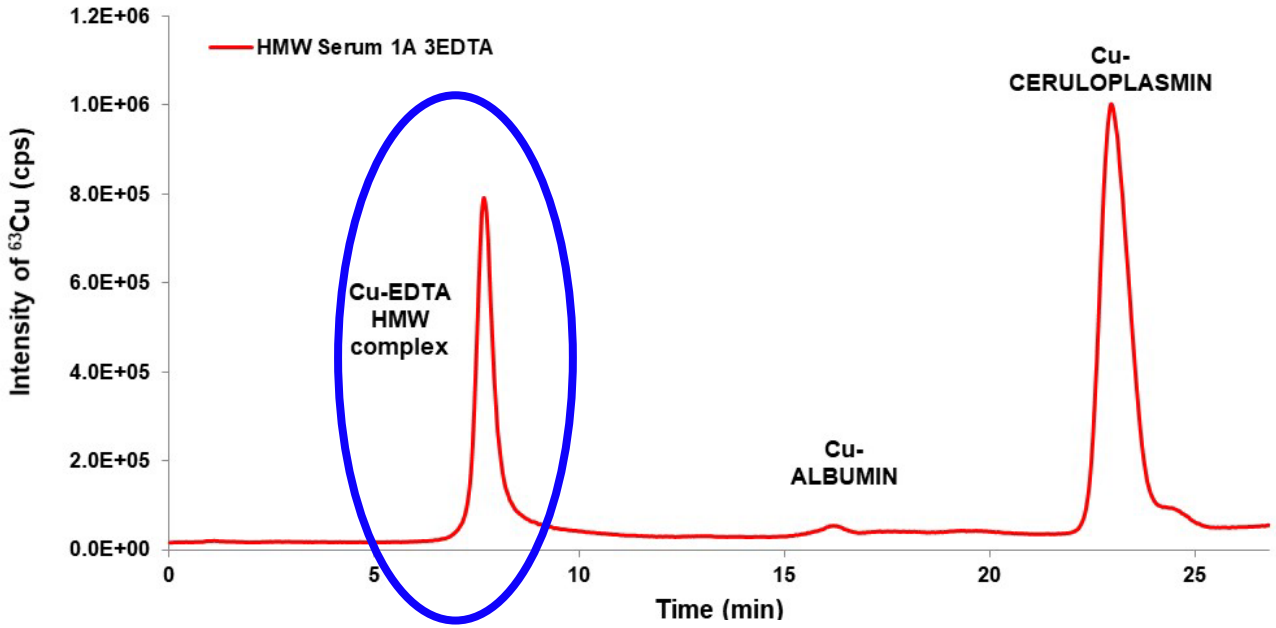
Exchangeable Copper Assay – Influence of EDTA on Accuracy



- Examined the effect of incubating the serum with 3g/L EDTA.
- Distribution of Cu-containing proteins was altered
- Identified Cu-EDTA HMW Protein complex
- Cu-EDTA HMW complex remained above the filter and so underestimated NCC in filtrate
- In the CHELATE Trial this HMW complex effect led to the assay for exchangeable copper underestimating NCC by 35% compared to LC-ICP-MS

Del Castillo et al *Analytical Bioanalytical Chem* 2021
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