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Total and Exchangeable Copper Assay Using Inductively Coupled Plasma Mass Spectrometry and Establishment of a Pediatric Reference Interval

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• ***Context.***—**Recently, an exchangeable copper (CuEXC) assay has been suggested as a robust and feasible diagnostic tool for Wilson disease (WD). Although WD is a disorder that requires lifelong treatment and monitoring, few data are currently available regarding the status of copper levels in children.**

Objective.—**To evaluate the performance of copper assays and establish a reference interval for total copper and CuEXC in the pediatric population.**

Design.—**Serum samples from children aged 1–5 (n = 122), 6–12 (n = 125), and 13–18 years (n = 120) were analyzed. Total copper and CuEXC concentrations were directly measured using inductively coupled plasma mass spectrometry, and relative CuEXC levels were calculated. Total copper reference intervals, CuEXC levels, and relative CuEXC levels were determined based on the 2.5th and 97.5th percentiles of the data with 90% confidence intervals.**

Results.—**There were significant differences in the median concentrations of total copper and relative CuEXC among the age groups. Reference intervals determined for total copper were 82 to 167, 75 to 139, and 64 to 133 μg/dL for children aged 1 to 5, 6 to 12, and 13 to 18 years, respectively. The reference intervals for CuEXC were 4.29 to 9.79, 4.02 to 9.09, and 3.55 to 8.25 μg/dL for children aged 1 to 5, 6 to 12, and 13 to 18 years, respectively. Among 11 patients with suspected WD, relative CuEXC values were elevated in all 3 diagnosed with WD.**

Conclusions.—**The pediatric reference intervals derived in this study are expected to be useful for the diagnosis, differential diagnosis, treatment, and monitoring of pediatric patients with WD.**

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Wilson disease (WD) is a genetic disorder associated with copper metabolism. It is an autosomal recessive condition caused by mutations in *ATP7B*, which encodes a transmembrane copper-transporting ATPase (ATP7B) that mediates copper excretion in the bile and ceruloplasmin binding.[1] Defective ATP7B causes free copper overload in the hepatocytes and results in the release of non–ceruloplasmin-bound copper (NCC, or free copper) into the circulation, which results in damage to various organs such as the liver, brain, and cornea. The prevalence of WD is approximately 1 in 30 000 worldwide[2]; however, recent molecular studies have estimated that the prevalence of WD associated with genetic causes is around 1 in 7500.[3,4] The prevalence of WD may be underestimated because of the various clinical manifestations of the disease, the unknown penetrance of *ATP7B* mutations, and the low sensitivity of certain biochemical diagnostic tests.[5] The spectrum of symptoms varies from asymptomatic liver disease to acute hepatic failure or cirrhosis, and it is difficult to distinguish the clinical symptoms of WD from those of other liver diseases. Wilson disease may also be accompanied by neurologic and psychiatric symptoms. In particular, WD is often more difficult to diagnose in pediatric patients than in adults because children are often asymptomatic, and conventional diagnostic criteria established for adults may not be appropriate for use in pediatric patients. Wilson disease is one of the few genetic diseases that can be successfully treated by early diagnosis and treatment; however, the failure to diagnose a WD patient can result in irreversible damage and is universally fatal. Unfortunately, no single test can currently definitively diagnose WD, and it is difficult to perform differential diagnosis because of the characteristics of the disease and the limitations of each test.[6]

Recently, the direct exchangeable copper (CuEXC) assay has been suggested as a robust and feasible diagnostic tool

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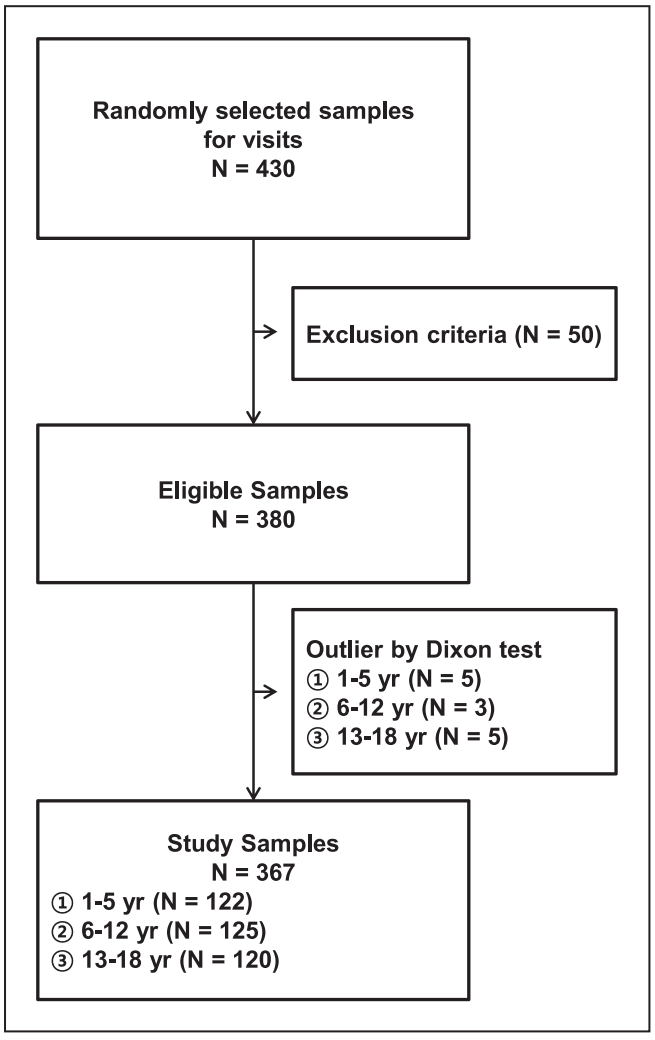

Figure 1. *Flow diagram depicting the inclusion and exclusion criteria for samples.*

for WD. Exchangeable copper corresponds to the labile fraction of copper complexed to albumin and other peptides but not complexed within ceruloplasmin; CuEXC is easily exchanged in the presence of high–copper-affinity chelating agents such as EDTA.[7,8] The technical approach to determine CuEXC was referred to in previously published studies.[7] Briefly, CuEXC is separated from the serum protein when samples are mixed with a chelating agent, and these samples are filtered by ultrafiltration before their analysis using inductively coupled plasma mass spectrometry (ICP-MS). El Balkhi et al[7] reported the possibility of diagnosing WD by determining the levels of relative CuEXC (REC; ratio between CuEXC and total copper). In a recent study, Guillaud et al[9] showed that CuEXC and REC can diagnose WD with high sensitivity and specificity. In this study, the performance of this test was evaluated by directly measuring CuEXC levels in the serum using ICP-MS and calculating the REC. The purpose of this study was to provide reference intervals of total copper, CuEXC, and REC for the pediatric population, who represent the main targets of early disease evaluation.

MATERIALS AND METHODS

Sample Collection

Blood samples were randomly collected from children visiting the tertiary referral, university-affiliated Severance Hospital, Seoul, Korea, from December 2018 to June 2019. Samples from subjects with known copper metabolism–related disorders, like protein-losing enteropathy, chronic diarrhea, nephrotic syndrome, chronic renal disorders, primary sclerosing cholangitis, primary biliary cirrhosis, chronic or acute hepatitis, malignant cancer, hematologic disorders, tuberculosis, and severe inflammation, and subjects receiving drugs that affect copper metabolism, like zinc salt, D-penicillamine, trientine, estrogen, and anticonvulsant medications, were excluded. Leftover serum after routine chemical measurements was used to analyze total copper and CuEXC levels. Medical notes and clinical details were assessed to exclude samples from patients with potential copper-associated abnormalities. More than 120 samples were collected for each age group (1–5, 6–12, and 13–18 years) based on previous studies regarding copper reference intervals in children.[10,11] A total of 430 specimens were collected and 380 were selected as the reference population after exclusion of samples from patients with potential copper-associated abnormalities (Figure 1). After exclusion of outliers, a total of 367 subject samples were used to generate the reference intervals. This study protocol was reviewed and approved by the institutional review board of the Severance Hospital, Seoul, Korea (IRB No. 4-2018-1020).

Total Copper and CuEXC Level Determination

Collected samples were submitted to Green Cross Laboratories for total copper and CuEXC level measurements. All these specimens were deidentified according to the protocol of this study and kept frozen (–70°C) until use. Ultrafiltration was performed using an Amicon Ultra 0.5-mL centrifugal filter device with a molecular weight cutoff of 30 kDa (Millipore, Tullagreen, Ireland) in a centrifuge with a fixed-angle rotor (14 000*g*, 10 minutes, ambient temperature). Reagents, control materials, and standard materials used includes the following: diluent (NH_4OH, EDTA, Triton X-100); internal standard stock solutions: 1000 ppm of scandium (Sigma-Aldrich, Buchs, Switzerland), germanium (Wako Pure Chemical Ltd, Osaka, Japan), rhodium (Wako), indium (Sigma-Aldrich), iridium (Sigma-Aldrich), and bismuth (Wako) mixed with 100 mL of 1% HNO_3 and 0.5% HCl; ClinCheck serum base control (Recipe, Munich, Germany); Seronorm trace elements serum (Sero, Billingstad, Norway); environmental calibration standard part number 5183-4688 (Agilent Technologies, Santa Clara, California); copper standard solution (Wako); and National Institute of Standards and Technology (NIST) standard reference materials 1640a and 1643f. Protein concentrations were determined using the COBAS INTEGRA 400 Plus automated analyzer platform (Roche Diagnostics, Rotkreuz, Switzerland). Copper levels were determined using an Agilent 7900 ICP-MS (Agilent). Argon was used to form the plasma (purity 99.9%; CryoServices Ltd). Polyatomic interferences for copper were removed using collision reaction cell technology, which induced collision dissociation and kinetic energy discrimination using helium gas. Typical instrument parameters and operating conditions are shown in Table 1. Total copper determination was calibrated using a copper standard solution with a range of 0.5 to 310.6 μg/dL. A 7-point calibrator (blank, 0.50, 5.00, 10.00, 20.00, 100.00, and 200 μg/dL) was used by diluting Wako copper standard solution with a diluent (NH_4OH, EDTA, Triton X-100) because lower calibrator concentrations were required to determine the CuEXC levels. The analytical measurement range of CuEXC was 0.1 to 200 μg/dL. The procedure to determine CuEXC was as follows: 200 μL of serum was diluted with 200 μL of EDTA (1:1) in 0.9% NaCl solution and mixed by vortexing for 20 seconds. After 1 hour of incubation, the diluted serum was ultrafiltered using the centrifugal filter device. Then, serum CuEXC levels were determined using an Agilent 7900 ICP-MS instrument. All these steps were performed in accordance with a previously reported study.[8]

Table 1. Operation Parameters of the Agilent 7900 Inductively Coupled Plasma Mass Spectrometry Instrument

Parameters	Operating Conditions
RF power, W	1550
RF matching, V	1.70
Sample depth, mm	8.0
Plasma gas (Ar) flow, L/min	15.0
Nebulizer gas flow, L/min	1.00
Auxiliary gas (Ar) flow, L/min	0.90
Makeup gas flow, L/min	0.10
Integration time, s	0.1
Spray chamber temperature, °C	2.0
Reaction cell gas (He) flow, mL/min	6.0
Detector mode	Dual
Nebulizer pump, rps	0.1

Abbreviations: Ar, argon; He, helium; RF, radio frequency; rps, revolutions per second.

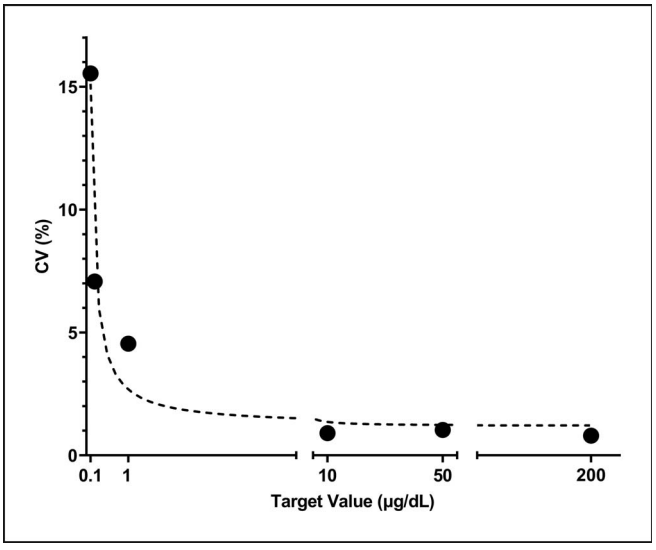

Figure 2. *Limit of quantitation (LoQ) determination of copper assay. LoQ was estimated by a nonlinear least-square fit of diluted sample replicate coefficients of variation (CVs) (N = 5).*

Evaluation Methods

The performance of the test method was evaluated in accordance with the CLSI EP06-A guidelines[12] and the FDA *Guidance for Industry: Bioanalytical Method Validation*.[13] Accuracy, precision, linearity, and lower limit of quantification were evaluated. To evaluate the imprecision of CuEXC levels, the coefficient of variation was calculated from 5 replicates per day for 5 days using serum-based reference materials after ultrafiltration (Seronorm trace element level I and II: the mean values were 0.20 and 0.36 µg/dL, respectively) and NIST standard materials (NIST 1643f and 1640a; the certified values were 2.166 ± 0.071 and 8.575 ± 0.051 µg/dL, respectively) (Supplemental Table 1; see supplemental digital content containing 4 tables and 1 figure). To evaluate the imprecision of total copper levels, the diluted and nondiluted serum-based reference materials (Seronorm trace element level I and II; the target values were 108.80 and 185.0 µg/dL, respectively), at concentrations of 27.20, 54.40, 108.80, and 185.00 µg/dL, were analyzed 5 times for 3 days. Accuracy was evaluated by calculating the copper recovery using samples with concentrations of 0.1, 0.5, 1.1, 4.3, 54.4, 92.5, 200.0, and 300.0 µg/dL, which were produced by diluting the reference materials (NIST, Seronorm, 1000 µg/dL environmental calibration standard, and 100 000 µg/dL Wako copper standard solution) (Supplemental Table 2). These samples were analyzed 5 times for 5 days (total, 25 replicates). Recovery was reported as the percentage of measured concentrations relative to the expected concentrations (mean of measured concentration/expected concentration × 100). The acceptable criteria for precision and accuracy were whether coefficient of variation and percentage recovery were within 15% or not. The limit of quantitation was assessed by determining the lowest concentration with a precision of less than 20%. The linearity was evaluated by analyzing 6 concentrations (0.1, 0.2, 1.0, 10.0, 50.0, and 200.0 µg/dL) of copper solutions, which were prepared by diluting the standard (Wako copper standard solution). In addition, the filter contamination potential was evaluated by measuring the copper concentration in the saline blank and NIST standard materials before and after filtration. Finally, the filtration performance was evaluated by determining the serum protein levels before and after ultrafiltration.

Establishment of Reference Intervals and Statistical Analysis

Reference intervals were calculated using a nonparametric method based on the 2.5th and 97.5th percentiles with a 90% confidence interval. To help decide whether to partition the reference interval by subclass—as suggested by Harris and Boyd[14]—the standard normal deviation test was used to verify the statistical significance of the difference in the subclass mean. Parametric and nonparametric statistical analyses and regression were performed using Analyse-it (Analyse-it Software Ltd, Leeds, United Kingdom), GraphPad Prism (GraphPad Software, Inc, San Diego, California), and Microsoft Excel (Microsoft, Redmond, Washington).

RESULTS

Method Validation

The analytical measurement range (0.1–200.0 µg/dL; to convert to micromoles per liter, multiply by 0.157) was validated during 5 days using Wako copper standard solution of a known concentration ($y = 1.011x - 0.004$; $R^2 = 1.000$; N = 5). The limit of quantitation was 0.10 µg/dL, which was determined using diluted NIST standard reference materials at the lowest concentration showing a coefficient of variation of less than 20% (Figure 2). Total imprecision (coefficient of variation) was less than 2% at 185.42 µg/dL (29.11 µmol/L) and less than 20% at 0.20 µg/dL (0.03 µmol/L) (Supplemental Table 1). Accuracy was expressed as recovery (mean measured value/expected value × 100), which was 101.4%, 108.0%, 100.5%, 99.1%, 103.2%, 104.7%, 102.7%, and 101.0% at 0.1, 0.5, 1.1, 4.3, 54.4, 92.5, 200.0, and 300.0 µg/dL, respectively (Supplemental Table 2). A major concern with any trace element analysis is the potential for contamination with external elements. We evaluated the potential for copper contamination originating from the Amicon filter devices by filtering normal saline and NIST standard reference materials and comparing copper levels in the ultrafiltered specimens with those determined before ultrafiltration. No statistically significant differences were observed between the filtered and unfiltered samples (Supplemental Table 3). Filtration efficiency was evaluated by comparing the protein concentrations in 5 serum samples with those in the corresponding ultrafiltered specimens. An average of 99.9% (SD = 0.01%) of protein was removed by filtration for more than 20 minutes, and this was consistent with the manufacturer's claims (Supplemental Table 4).

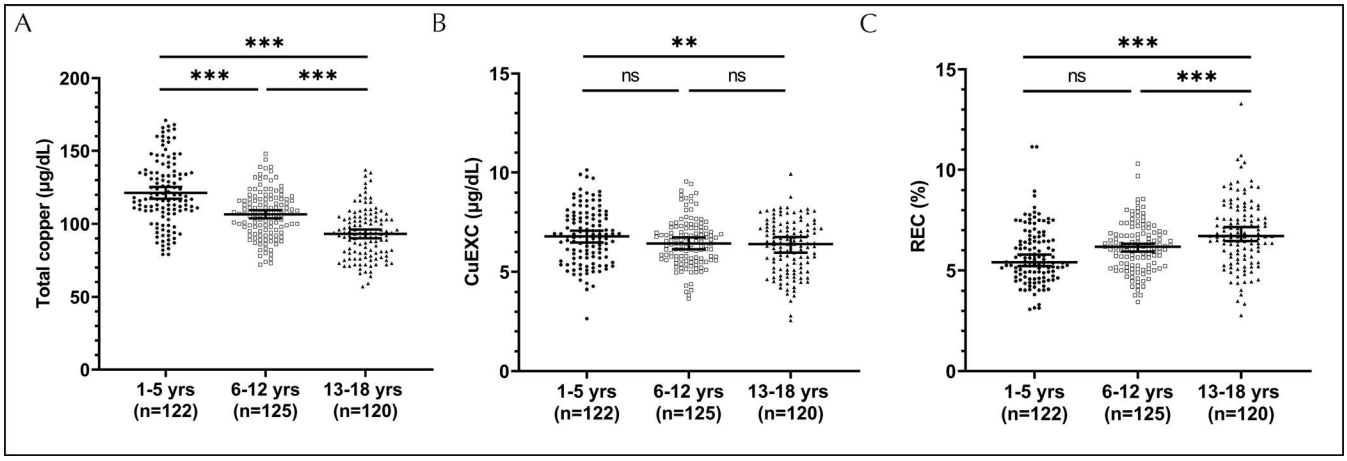

Figure 3. *Comparison of total copper (A), exchangeable copper (CuEXC) (B), and relative exchangeable copper (REC) (C) levels according to the age groups. Horizontal line: mean with 90% confidence interval. Abbreviations: ns,* $P > .05$; **, $.001 < P \leq .01$; ***, $P \leq .001$.

Reference Interval Establishment

In total, 367 samples were analyzed in this study, with participants ranging in age from 1 to 18 years. The frequency distributions of serum total copper and CuEXC levels are shown in Supplemental Figure 1. The total copper and CuEXC levels showed non-Gaussian distributions; thus, we used nonparametric analysis and established reference intervals as the 2.5th and 97.5th percentiles. The median concentrations for total copper and CuEXC were 107 µg/dL (range, 57–171 µg/dL) and 6.55 µg/dL (range, 2.56–10.13 µg/dL), respectively. Subclasses were formed according to the 3 age groups (1–5, 6–12, and 13–18 years), and the criteria suggested by Harris and Boyd[14] were used to confirm the statistical significance of differences in the subclass means.[15] The distribution of total copper in the 3 subclasses followed a Gaussian distribution. Thus, differences between groups were compared using analysis of variance tests and Tukey post hoc multiple comparisons tests. As shown in Figure 3, A, mean levels of total copper exhibited significant differences with respect to age groups. The age-dependent reference intervals for total copper were 82 to 167, 75 to 139, and 64 to 133 µg/dL for those in the 1 to 5, 6 to 12, and 13 to 18 years groups, respectively. The CuEXC and REC data followed a non-Gaussian distribution. Nonparametric analysis of these data suggested no significant differences between the CuEXC concentrations among the age groups (Figure 3, B). However, REC levels showed significant differences among the age groups (Figure 3, C). The reference intervals for CuEXC levels were 4.29 to 9.79, 4.02 to 9.09, and 3.55 to 8.25 µg/dL for individuals in the 1 to 5, 6 to 12, and 13 to 18 years age groups, respectively. The reference intervals for REC levels were 3.18% to 8.93%, 3.81% to 8.56%, and 3.50% to 10.51% for individuals in the 1 to 5, 6 to 12, and 13 to 18 years age groups, respectively (Table 2).

Levels of CuEXC in Patients With WD

To evaluate the potential clinical usefulness of the results regarding free copper levels, specimens were obtained from 11 patients with suspected WD (Table 3). Among these patients, 2 were confirmed to have WD arising as a result of biallelic *ATP7B* pathogenic variants, as detected by molecular genetic testing. One patient was diagnosed with WD after clinical manifestations, and biochemical testing showed low serum copper and ceruloplasmin concentrations and increased urinary copper levels. Molecular genetic testing of this last patient revealed only heterozygous *ATP7B* pathogenic variants. All 3 patients diagnosed with WD showed REC values beyond the reference interval upper limit. Only 1 of 8 patients who were not diagnosed with WD showed an REC value (14.40%) higher than the upper reference limit (10.51%), however, which was lower than the cutoff value (18.5%) reported previously.[7]

DISCUSSION

Wilson disease is rarely diagnosed very early in life, because it takes time for copper to become deposited and for the symptoms to manifest. It is common for children to be accidently identified with abnormal liver functioning after 1 year of age, but WD is rarely symptomatic before 5 years of age.[16] If WD is not diagnosed and treated at the appropriate time, it will progress and result in liver failure and organ damage, including brain damage, which is irreversible and can even be fatal. Although WD is one of the few hereditary

Table 2. Age-Specific Reference Intervals for Total Copper and Exchangeable Copper (CuEXC) Levels and Ranges of Relative Exchangeable Copper (REC) Levels

	Total Copper, µg/dL		CuEXC, µg/dL		REC, %	
Age Group, y	Lower Limit (90% CI)	Upper Limit (90% CI)	Lower Limit (90% CI)	Upper Limit (90% CI)	Lower Limit (90% CI)	Upper Limit (90% CI)
1–5	82 (79–87)	167 (163–171)	4.29 (2.65–4.87)	9.79 (8.98–10.13)	3.18 (3.08–4.03)	8.93 (7.82–11.15)
6–12	75 (72–80)	139 (134–148)	4.02 (3.66–4.97)	9.09 (8.72–9.56)	3.81 (3.45–4.24)	8.56 (8.12–10.31)
13–18	64 (57–69)	133 (122–137)	3.55 (2.56–4.10)	8.25 (8.09–9.94)	3.50 (2.78–4.40)	10.51 (9.43–13.29)

Table 3. Relative Exchangeable Copper (REC) Levels in Patients With Suspected Wilson Disease (WD)					
Subject No.	Age, y	Total Copper, μg/dL	CuEXC, μg/dL	REC, %[a]	WD Diagnosis
B-85	11	98	5.57	5.70	No
B-122	11	90	6.77	7.55	No
B-136	12	100	5.67	5.67	No
C-4	14	106	5.75	5.43	No
C-47	13	9	5.59	**59.88**	Yes
C-49	14	190	11.08	5.84	No
C-54	16	95	6.61	6.95	No
C-79	18	72	6.44	8.90	No
C-81	18	8	4.56	**54.75**	Yes
C-83	13	10	4.67	**47.37**	Yes
C-143	16	31	4.5	14.40	No

Abbreviations: CuEXC, exchangeable copper.

[a] Bold text indicates REC values in patients diagnosed with Wilson disease.

metabolic disorders in which early medical intervention is possible and important, early diagnosis is often challenging, especially in case of pediatric patients. Children are often asymptomatic, and conventional diagnostic criteria established for adults may not be suitable for use in pediatric cases.[6,16] Pharmacologic therapies should be administered lifelong, and treatment monitoring is essential for successful WD management. However, because of this disorder's characteristics, which include clinical variability, overlapping symptoms of various severities, and the limitations of biochemical tests, there have been difficulties in clearly identifying and diagnosing WD patients and monitoring their subsequent treatment.

The basic diagnostic approach for WD includes a variety of biochemical tests, such as tests for serum ceruloplasmin, serum total copper, NCC, and 24-hour urinary copper excretion. Wilson disease is difficult to distinguish from other liver diseases based on clinical symptoms alone; thus, biochemical tests play an important role in its diagnosis, especially in case of children with WD who are asymptomatic. Non–ceruloplasmin-bound copper can be calculated using serum total copper and ceruloplasmin levels (total serum copper concentration in μg/L − ceruloplasmin-bound copper in μg/L [3.15 × ceruloplasmin in mg/L]).[6] The conventional calculation method for NCC is limited by its dependence on the accuracy of the methods used for measuring serum copper and serum ceruloplasmin concentrations.[6,17] Some direct methods for determining free copper levels have been developed, including ultrafiltration, but these are unreliable because of the continuous exchange between free copper and plasma proteins; therefore, they are not commonly used.[8] In addition, the amount of copper excreted in urine during 24 hours is routinely used to diagnose and monitor patient treatment. However, it is difficult to accurately collect urine in children, which results in diminished assay sensitivity and specificity.[16,18]

Recently, several studies have reported that reliable CuEXC measurement enables sensitive, fast, and accurate WD diagnosis.[7,9,19–21] It is known that copper in the body loosely binds to the ceruloplasmin complexes, albumin, or amino acids like histidine, and only 2% to 5% of the copper in the body is present as free copper. Weakly bound copper becomes easily exchangeable in the presence of high-affinity copper chelators like EDTA, and the CuEXC concentration represents the NCC concentration.[8]

We validated a method to determine CuEXC levels in serum samples using ultrafiltration and ICP-MS. This method—CuEXC abundance determination using ICP-MS—can be performed after a simple sample preparation process, making it appropriate for routine analysis. The validation results of our method showed that the approach was robust and precise within an appropriate linear measurement range. We used this method to generate reference intervals for CuEXC levels directly measured in the Korean pediatric population. Pediatric reference intervals are often difficult to establish because of the challenges associated with obtaining sufficient numbers of blood samples from appropriately qualified children. In this study, we applied exclusion criteria involving all related factors, such as infection, medication, other liver disorders, and copper-related disorders. Reference interval partitioning was based on total copper levels, which exhibited the most significant differences with age. In contrast, CuEXC showed a slight decline with age, with no statistically significant differences between age groups.

We have not found any studies that describe pediatric CuEXC reference intervals. El Balkhi and colleagues[8] set reference intervals to determine the total copper and CuEXC levels. Their calculated reference intervals were 74.5 to 183.4 μg/dL and 0.64 to 1.12 μmol/L (4.08–7.12 μg/dL) for total copper and CuEXC, respectively, based on 5th to 95th percentiles in 44 presumably healthy adult subjects. Lin at al[11] described pediatric reference intervals for total serum copper as 75 to 153, 64 to 132, and 57 to 129 μg/dL for individuals aged younger than 10.3, 10.3 to 12.5, and older than 12.5 years. They noted that there were significant differences in the copper reference intervals categorized according to each age group.

Of the 11 suspected WD patients included here, 3 who were actually beyond the total copper reference range were later identified as those having WD. Although this represents a small number of patients, this is consistent with the finding that it is easy to confuse WD with other liver diseases, as reported in other papers.[7,9,19,20]

This study has some limitations. Because samples are rarely collected in a trace element tube from pediatric patients showing conditions not related to WD, this study, performed with residual samples, could not perform the evaluation using metal-free dedicated tubes. However, a study[22] has been published showing no statistical difference in the measured copper concentrations between sample

collection tubes, that is, the serum separator tube and trace element tubes. In addition, we could not determine an REC cutoff value because we did not enroll a sufficient number of WD patients; this cutoff value differs with respect to the reference interval, which can be statistically calculated using only healthy individuals. Further studies with adequate numbers of WD patients are needed to identify the optimal REC cutoff value in pediatric patients.

Despite these limitations, this study is meaningful in that the reference intervals for CuEXC and REC were developed to interpret these results for the early diagnosis or treatment monitoring of pediatric WD patients. Currently, even the total copper status in growing children is rarely provided. Wilson disease is a disorder that requires lifelong treatment and monitoring, and pediatric WD patients who have already been diagnosed will continue to grow during the monitoring period. Adjustment for age-specific reference intervals is required during such maturing periods for sensitive monitoring.

Determination of CuEXC levels—which represent the NCC—and REC calculation is a new tool that can be used to diagnose WD. It was found to be an analytically reliable and robust method for diagnosing and monitoring WD treatment. The reference intervals for pediatric patients derived in this study are expected to be useful for the diagnosis, differential diagnosis, and treatment monitoring of pediatric patients, who are the primary targets for early WD diagnosis.

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