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Analytical methodology

Copper dyshomeostasis in Wilson disease and Alzheimer's disease as shown by serum and urine copper indicators

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ABSTRACT

Abnormal handling of copper is the cause of Wilson disease (WD), a rare disorder typified by increased levels in plasma copper not-bound to ceruloplasmin (nCp-Cu, also known as 'free' copper). In Alzheimer's disease (AD), *meta*-analyses show that copper decreases in brain but increases in serum, due to the nCp Cu component increase. Despite the similarities, a direct comparison of copper biological status in the two diseases has never been carried out. To fill this gap, we evaluated serum copper, ceruloplasmin, nCp-Cu and Cu:Cp in 385 CE and 336 healthy controls previously investigated that were compared with 9 newly diagnosed WD patients. We then assessed 24 h copper urinary excretion in 24 WD patients under D-penicillamine (D-pen) treatment and in 35 healthy controls, and compared results with those of AD patients participating to a D-pen phase II clinical trial previously published.

After adjusting for sex and age, serum nCp-Cu and Cu:Cp resulted higher in AD and in WD than in healthy controls (both $p < 0.001$). While nCp-Cu was similar between AD and WD, Cu:Cp was higher in WD ($p < 0.016$). 24 h urinary copper excretion in AD patients (12.05 μg/day) was higher than in healthy controls (4.82 μg/day; $p < 0.001$). 77.8% of the AD patients under D-pen treatment had a 24 h urinary excretion higher than 200 μg/day, suggestive of a failure of copper control.

This study provides new insight into the pathophysiology of copper homeostasis in AD, showing a failure of copper control and the Cu:Cp ratio as an eligible marker.

1. Introduction

Copper is an essential micronutrient, serving as a component of many metalloproteins. In case of defective pathways, copper is dangerous promoting oxidative stress. Ceruloplasmin is a ferroxidase controlling the oxidation rate required for the release of iron from the storage sites [1]. Under normal conditions, copper in the blood is mostly tightly bound to ceruloplasmin (85–95%). While only small amounts of copper (5–30%) are loosely bound to and exchanged among albumin, small peptides and amino acids. This copper fraction is called non-ceruloplasmin-bound copper (nCp-Cu or so-called 'free' copper).

Copper: Ceruloplasmin is a copper index which serves as a useful internal quality control of ceruloplasmin.

Wilson disease (WD) is the paradigm of nCp-Cu overload disease. WD is an autosomal recessive disorder due to mutations in both copies of the *ATP7B* gene [2,3].

The defective function of the ATP7B copper pump determines failure of copper excretion into the bile, a drop of plasma ceruloplasmin along with a spillover of copper into the blood in the form of nCp-Cu [4].

Increased concentrations of nCp-Cu are evident also in Alzheimer's disease (AD), the most common form of dementia with a complex

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etiology [5].

The comparison between AD and WD for a panel of copper markers in serum and urine can improve our knowledge about copper disposition in AD, helping to address controversies still open. In fact, a direct comparison of the picture gleaned from laboratory tests between AD and WD has never been carried out so far. This investigation serves this purpose, analyzing in different sub-studies serum copper status (copper, ceruloplasmin, nCp-Cu and Cu:Cp) and 24 h urinary copper in healthy controls, AD and WD patients at baseline and under treatment with D-penicillamine (D-pen). 24 h urinary data are compared with those of a previous study of ours investigating the effects of D-pen in AD treatment [6].

2. Methods

2.1. Standard protocol approvals, registrations, and patient consents

Local institutional ethics committees approved the study. Subjects or their guardians gave written informed consent, in line with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and the standards established by the authors' Institutional Review Board.

2.2. Subjects

2.2.1. Subjects for copper status in serum between AD and WD patients

Data from 721 subjects investigated in previous studies [7,8] were re-evaluated for the comparison of markers of copper status among AD, WD patients and healthy controls. More specifically, data from 336 healthy controls [201 females (F), 59.8%)], median age 65 (interquartile range: 58–72), and 385 CE (280 F, 72.7%), median age 76 (70–80 years) were compared with those of a sample of 9 patients with WD (6 F; 66.7%), median age 13 years (10.5–14.5), recruited from the Liver Unit San Paolo School of Medicine, University of Milan, Italy.

As detailed elsewhere, healthy controls and AD [(NINCDS-ADRDA criteria) [9,10] and Mini-Mental State Examination (MMSE) score of 25 or less [11]], were enrolled by two specialized Neurological and Dementia care Centers in Italy: Fatebenefratelli Hospital, Rome, and at the Memory Clinic of the IRCCS Istituto Centro San Giovanni di Dio, Fatebenefratelli, Brescia, Italy, using common standardized clinical protocols and guidelines.

Serum samples from 9 cases with WD drawn at time of the diagnostic evaluation were provided by the Liver Unit San Paolo School of Medicine, University of Milan Italy. Diagnosis of WD was made using accepted criteria including the Leipzig score and confirmed genetically [12]. Fasting blood samples were collected in the morning and sera samples were separated by centrifugation (3000 rpm, 10 min, and 0 °C), divided into 0.5 mL aliquots and rapidly stored at −80° C. Serum samples were shipped to the Fatebenefratelli Hospital in Rome for assay.

2.2.2. Subjects for copper in 24 h urine in healthy controls and WD

For the comparisons of 24 h urinary excretion of copper, a new cohort of 35 healthy controls (24hU-CTRL) (female 57.1%) median age 59 (interquartile range: 47–73) and of 24 patients with WD [12] (female 50.0%) age 38 (interquartile range: 28–51) was recruited from the Clinical Laboratory Department, St. Ivan Rilski University Hospital, Medical University, Sofia, Bulgaria. Urine was collected in special containers over 24 h and kept cool. WD patients received a diagnosis following accepted procedures [12] adopted at the St. Ivan Rilski University Hospital, Sofia. Fifteen received genetic confirmation, 3 were not evaluated; 12 presented Kayser Fleischer rings; 14 had hepatic cirrhosis; 18 had neurological symptoms (tremor, vertigo, slurred speech, delaying speech, dysarthria, disorientation, emotional unstable, one presented multiple sclerosis). These WD patients were in stable chronic D-pen treatment (23 of 24 treated with D-pen, 1 of 24 was initially treated with trientine and then with D-pen).

Samples were then divided into aliquots and frozen at − 80 °C and shipped to the Fatebenefratelli Hospital in Rome for assay.

2.2.3. Biochemical investigations

All the biochemical analyses described were performed at the Fatebenefratelli Hospital in Rome. Total serum copper was measured by spectrometry, using an A Analyst 600 Perkin Elmer atomic absorption spectrophotometer [13].

Ceruloplasmin concentration was measured with an immunoturbidimetry assay (Futura system, Srl, Italy). Serum ceruloplasmin oxidase activity with *o*-dianisidine dihydrochloride as a substrate was measured spectrophotometrically as described in a previous study to further reference the concentration measurements [14]. Both ceruloplasmin concentration and activity were automated on a Pentra 400 (Horiba ABX, Montpellier, France) and performed in duplicate.

For the nCp-Cu index calculation we used values of ceruloplasmin obtained immunologically, as extensively discussed in a previous report (Methods section in [15]).

For each serum copper and ceruloplasmin pair, we computed the amount of copper bound to ceruloplasmin (CB) and the amount of non-Cp Cu, following standard procedures (Appendix A: "Calculation of 'free copper' concentration") [16] Briefly: CB = ceruloplasmin (mg/dL) * n; n = 0.472 (μmol/mg); nCp-Cu = total serum copper − CB [16]. This calculation expresses nCp-Cu in μmol/L and is based on the fact that ceruloplasmin binds 6 atoms of structural copper, which is equivalent to ceruloplasmin containing 0.3% copper (weight percentage of copper in Cp): [6 (atoms of Cu) *63 uma (Cu molecular weight)/ 132 KDa (Cp molecular weight)] * 100 = 0.3%; more details are available at http://www.j-alz.com/letterseditor/index.html#March2013. Equivalent data can be obtained calculating nCp-Cu from mg/L of ceruloplasmin and considering the conversion of 3.15 μg/Cu for mg of ceruloplasmin [17].

Copper: Ceruloplasmin ratio (Cu:Cp)[18,19] was calculated as reported in Twomey et al. [19]. These authors provided the equation as follows:

[copper μmol/L]* [132000 (g/mol)]/[Cp (mg/dL)*10^4].

A Cu:Cp value of 6.6 has been advocated as the theoretical optimal ratio for healthy subjects [6]. This value has been confirmed in a recent *meta*-analysis [4].

The Cu:Cp study in current healthy controls revealed that it has a value of 6.5 (1.0), indicating that copper and ceruloplasmin in the specimens analyzed are in the correct stoichiometry to applied the Walshe's formula [18,19].

2.2.4. 24 h urine methods

Urinary Cu concentration was measured in 24hU-CTRL and D-pen WD groups with Graphite Furnace Atomic Absorption GF-AAS equipped with a Longitudinal Zeeman Background correction (THGA AAnalyst 600 Perkin Elmer Instruments), following a specific method suggested from the company. In particular, the wavelength was 324.8 nm and the slit width was of 0.7 mm. A standard calibration were prepared with 0; 20; 50;100 μg/L of Cu diluting a 1000 mg/L Cu calibration standards (Perkin Elmer) in HNO_3 0.2% (v/v) (ultrapure grade from J.T.Baker®). 0.1%Triton (wetting agent, Perkin Elmer) and 0,1% Pd + 0,1%Mg $(NO_3)_2$ modifier (GF-AAS Modifier Perkin-Elmer) were added to the urine samples and references solutions, with a 1 + 1 (v/v) dilution. The thermic program employed was the one provided from Perkin Elmer with an Atomization temperature of 2100 °C. Data are expressed as μg/day, considering the volume of urine sample collected in 24 h. The same method was used in a previous study of ours, from which we have taken data regarding D-Pen AD study as described in details in the next paragraph [6].

2.2.5. Statistical analysis

Current investigation reports data of three different studies,

regarding *i)* the comparison of copper status in serum among AD, WD and healthy controls; *ii)* the comparison of basal 24 h urinary copper excretion between AD and healthy controls; *iii*) a qualitative comparison of the 24 h urinary copper excretion between WD patients and AD patients both under D-pen treatment.

More specifically, as described in details in the '*Results*' section, data of the 24 h urinary copper excretion of AD and of D-pen AD patients analyzed in current study were taken from our previously published study [6]. In particular, data of 24 h urinary copper excretion of AD patients [6] have been compared with those of the 24hU-CTRL group, while data of the 24 h urine copper D-pen AD [6] have been compared with those of the D-pen WD group. The latter analysis has only a qualitative observational value: even though the WD patients were under a stable chronic D-pen treatment, the extent on length of their chronic treatment was not superimposable with that of the AD-pen group, who instead underwent to an homogeneous D-pen treatment protocol, derived from the phase II clinical trial study design [6].

All the statistical analyses were corrected for sex and age when appropriate.

Data were presented as mean (± standard deviation, SD) or, otherwise specified, as median (Interquartile range, IQR: 25th–75th percentile) or frequencies (%). A logarithmic transformation was applied to decrease the variability of data and to better approach a normal distribution.

In the study *i)*, we analyzed differences among AD, WD and healthy control groups in terms of sex and age distribution applying non parametric test (Chi-square Test, Kruskal Wallis test). Univariate ANOVA analyses were applied to explore the differences in the biochemical variables studied (Copper, Ceruloplasmin, non-Cp Cu, Cu:Cp). Cp activity data were excluded from the analysis since redundant with respect to the Cp concentrations values, as discussed in the '*Biochemical investigations*' Section.

ANCOVA analyses were employed to adjust the differences for sex and age. Multiple comparisons were performed applying Bonferroni adjustment. Mean differences and relative 95% Confidence Intervals (CIs) were reported. If the logarithmic transformation was applied, the results were converted back into the original scale using the antilog. Cohen's d was calculated to provide a standardized measure of size of the difference between groups. We tested the dimension of the effects by Cohen's d coefficient, calculated as the difference between the two pairwise means divided by the pooled standard deviations [20]:

$$\text{Cohen's d} = \frac{M_1 - M_2}{\sigma_{pooled}}$$

where

$$\sigma_{pooled} = \frac{\sqrt{(N_1 - 1)\sigma_1^2 + (N_2 - 1)\sigma_2^2}}{(N_1 + N_2) - 2}$$

N_1 and N_2 represents the numerosity of the two groups. A Cohen's d = 0.2 indicates a small effect size, 0.5 a medium effect size and higher than 0.8 large effect sizes.

A *p*-value less than 0.05 was significant. The statistical analysis was performed with SPSS version 16.0 (IBM corporation).

In the study *ii)* a T test, after logarithmic transformation, was applied for the comparison between 24 h urinary copper excretion values of the AD patients and 24hU-CTRL group. In the study *iii)* a T test was applied assuming unequal variances for the comparison between D-pen AD and D-pen WD.

3. Results

3.1. Comparison of markers of copper status in serum among AD, WD and healthy controls

Main demographic characteristics and the median and interquartile range of the serum markers of copper metabolism (copper, ceruloplasmin, nCp-Cu values and Cu:Cp) in the study groups are showed in Table 1, Table 2 and in Fig. 1. Mean value of ceruloplasmin activity in healthy controls was (mean ± SD) 103.96 ± 15.8, in AD patients was 98.62 ± 20.1, while in WD not treated 10.89 ± 3.92 and in WD D-pen 47.67 ± 32.9. The correlation between ceruloplasmin concentrations and ceruloplasmin activity in healthy controls was 0.980 ($p < 0.001$), in line with previous reports [14,21].

Table 1
Descriptive characteristics among the controls, AD and WD.

		Healthy Control	AD	WD	P
N		336	385	9	
Sex, F n (%)		201 (59.8%)	280 (72.7%)	6 (66.7%)	< 0.001
Age, years	mean; median (25th to 75th percentile)	65.3; 65.0 (58.0–72.0)	74.5; 76.0 (70.0–80.0)	13.1; 13.0 (10.5–14.5)	< 0.001

Data refer to the comparison *i*) as described in Methods (statistical analyses section) regarding the assessment of copper status in serum among AD, WD and healthy controls.

Logarithmic transformation of copper and Cu:Cp values was considered in all analyses. Adjusting for sex and age, the ANCOVA model revealed significant differences among the diagnosis groups in the serum levels of copper [$F(2725)=42.458$, $p < 0.001$)], nCp-Cu [$F(2725)=61.70$, $p < 0.001$)], ceruloplasmin [$F(2725)=30.495$, $p < 0.001$)] and Cu:Cp [$F(2, 725)=71.197$, $p < 0.001$; Fig. 1]. In particular, the adjusted levels of copper were higher in AD (adjusted geometric mean=14.44 μmol/L, 95% CI 14.13–14.76) than in controls (adjusted geometric mean=12.82 μmol/L, 95% CI 12.54–13.11; d = 0.67, $p < 0.001$) and in WD patients (adjusted geometric mean = 7.71 μmol/L, 95% CI 6.61–8.99; d = 3.27, $p < 0.001$). Levels of copper were lower in WD patients than in controls ($p < 0.001$; Table 2). On average, ceruloplasmin values in the AD group (adjusted mean = 26.69 mg/dL, Standard Error (SE) = 0.28) were greater than those of WD (adjusted mean = 12.10 mg/dL, SE = 1.98; d = 2.62, $p < 0.001$).The mean ceruloplasmin value of WD patients was lower than that of other groups ($p < 0.001$). Adjusted nCp-Cu values were significantly different between AD patients (adjusted mean = 2.20 - μmol/L, SE = 0.12) and controls (adjusted mean = 0.34 μmol/L, SE = 0.12; d = 0.83, $p < 0.001$), but not between AD and WD patients (adjusted mean = 2.17 μmol/L, SE = 0.79; d = 0.07, $p = 0.999$).

Sixty% of AD patients have nCp-Cu values higher than 2.3 μmol/L (corresponding to 150 μg/L) and 25% higher that 3.9 μmol/L (250 μg/L) considered respectively as uncertain or diagnostic of WD [17].

The adjusted values of Cu:Cp were higher in AD (adjusted geometric mean = 7.29, 95% CI 7.16–7.41), and in WD (adjusted geometric mean = 8.78, 95% CI 7.75–9.93) than in healthy controls (adjusted geometric mean = 6.39, 95% CI 6.27–6.50;$d_{AD\text{-}Ctrl} = 0.77$, $d_{WD\text{-}Ctrl} = 1.90$, all $p < 0.001$, Table 3, Fig. 2). Furthermore, WD patients exhibited higher values of Cu:Cp than AD subjects (d = 1.05, $p = 0.014$).

3.2. Comparison of copper in 24 h urine in healthy controls and AD

The 24 h urinary copper excretion of the 35 24hU-CTRL group from the St. Ivan Rilski University Hospital did not correlate with age (Pearson's r = −0.028, p=0.879). These data were compared with the baseline 24 h urine copper concentrations of 18 AD patients (F, 72%), median age 79 (interquartile range: 74–81) who completed the D-pen clinical trial and were described in details previously [6]. As shown in Fig. 3A, AD patients had 24 h urinary copper excretion higher than healthy controls ($p < 0.001$), being in 12.05-μg/day (median,

Table
Biochemical Descriptive characteristics among the controls and AD, WD patients.

		Healthy Control (336)	AD (385)	WD (9)	p value
Copper (μmol/L)	mean; median (25th to 75th percentile) Cohen's d§	13.2; 13.0 (11.3–15.0)	15.2; 15.0 (13.0–17.0) 0.67	7.7; 7.7 (7.45–8.10) 2.7	< 0.001*
Ceruloplasmin (mg/dL)	mean (sd) Cohen's d§	27.1 (5.02)	27.4 (5.17) 0.06	11.2 (1.58) 3.2	< 0.001
nCp-Cu (μmol/L)	mean (sd) Cohen's d§	0.4 (2.08)	2.2 (2.12) 0.86	2.4 (0.85) 0.97	< 0.001
Cu:Cp	mean; median (25th to 75th percentile) Cohen's d§	6.5; 6.6 (5.9–7.2)	7.4; 7.3 (6.6–8.0) 0.8	9.2; 9.2 (8.1–10) 2.2	< 0.001*

Data refer to the comparison *i*) as described in Methods (statistical analyses section) regarding the assessment of copper status in serum among AD, WD and healthy controls. Data are reported as mean (SD) or median (25th to 75th percentile).*Analysis was conducted on log transformed data.

§ Cohen's d is calculated as the difference between each of the pairwise mean differences divided by the pooled standard deviation, taken control group as reference group. A d = 0.2 indicates a small effect size, d = 0.5 a medium effect size and d = 0.8 large effect size [18].

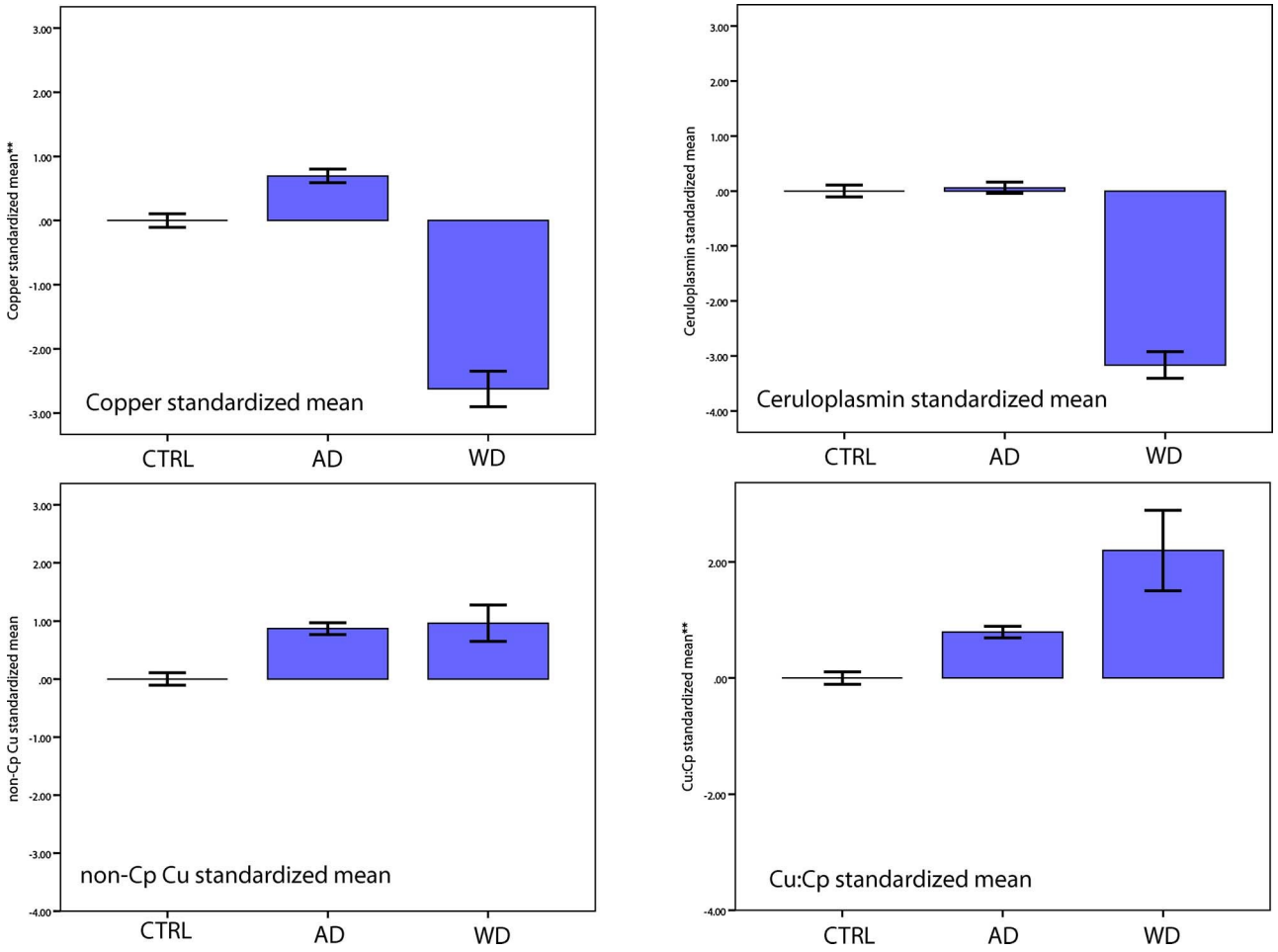

Fig. 1. Data refer to the comparison i) as described in Methods (statistical analyses section) regarding the assessment of copper status in serum among AD, WD and healthy controls. Mean and 95% confidence interval of the serum markers of copper metabolism* for each diagnosis group (Healthy control, AD and WD). *Each graph is based on the values of the variables standardized according to the mean and standard deviation of controls. **The initial values are on the logarithmic scale.

interquartile range: 7.85–22.50) and in the 24hU-CTRL group 4.82 μg/day (median, interquartile range: 3.31–7.43), with 7 of 18 AD patients having 24 h urinary copper excretion higher than 20 μg/day. In a subgroup of 16 elderly 24hU-CTRL [median age 70.5 (interquartile range: 64.5–75.0)] 24 h urinary copper excretion had a mean value of 4.48 μg/day (interquartile range: 3.1–7.9), and the comparison with those of the AD group remain significantly different ($p < 0.001$).

3.3. Comparison of copper in 24 h urine in D-pen AD and D-pen WD

Measurements of 24 h urinary copper excretion of the 9 D-pen AD from the clinical trial previously described [6] were compared with the 24 D-pen WD from St. Ivan Rilski University Hospital. As shown in Fig. 3B, 24 h urinary copper excretion was lower ($p = 0.001$) in the D-pen AD group, being 237.3 μg/day ± 49.97, than in the D-pen WD group 519.3 μg/day ± 264.6). Considering a cutoff of 200 μg/day as suggested in [22] 87.5% of D-pen WD and 77.8% of D-pen AD had 24 h urine copper values higher than 200 μg/day.

4. Discussion

This study was aimed at exploring the disposition of copper in AD living patients, as revealed by a panel of copper markers in serum and urine analyzed in WD and AD, showing what it is shared and what it is different between the two.

Meta-analyses published in the latest 5 years clearly show copper involvement in AD. Nevertheless, literature reports controversies surrounding the conclusions of these studies [23]. Current study, showing

Table 3
Multiple comparisons.

Biological Variable	Neurological disorders			Mean Diff	95% CI	P	Cohen's d
Copper*	AD	vs	CTRL	1.13	1.08;1.17	< 0.001	0.67
			WD	1.88	1.54;2.28	< 0.001	3.27
	CTRL	vs	WD	1.66	1.38;2.00	< 0.001	1.90
Ceruloplasmin	AD	vs	CTRL	−0.28	−1.27;0.71	0.999	0.05
			WD	14.58	9.67;19.50	< 0.001	2.62
	CTRL	vs	WD	14.86	10.18;19.54	< 0.001	2.83
nCp-Cu	AD	vs	CTRL	1.86	1.43;2.28	< 0.001	0.83
			WD	0.17	−1.92;2.27	0.999	0.07
	CTRL	vs	WD	−1.68	−3.67;-0.31	0.205	0.75
Cu:Cp*	AD	vs	CTRL	1.14	1.11;1.18	< 0.001	0.77
			WD	0.83	0.71;0.97	0.016	1.05
	CTRL	vs	WD	0.73	0.63;0.84	< 0.001	1.90

Data refer to the comparison *i*) as described in Methods (statistical analyses section) regarding the assessment of copper status in serum among AD, WD and healthy controls.

* analyses was performed on log transformed data and the results were converted back into the original scale via antilog. §Cohen's d is calculated as the difference between each of the pairwise mean differences divided by the pooled standard deviation, taken control group as reference group. A d = 0.2 indicates a small effect size, d = 0.5 a medium effect size and d = 0.8 large effect size [18].

the performance of the four markers analyzed in serum (Fig. 1) can help explaining counterintuitive findings at the basis of these controversies. Through a direct comparison between AD and WD patients, for the first time we show that nCp-Cu in AD reaches concentrations commonly found in WD. The Cohen's d demonstrates a large biological effect of nCp-Cu variation in AD vs. healthy controls, similarly to WD. Conversely, copper is increased in AD vs. healthy controls, with a Cohen's d indicating a medium biological effect, while ceruloplasmin does not change.

Fig. 1 clearly shows that the expansion of the nCp-Cu serum component explains copper increase in AD, and indicate a copper dyshomeostasis. The main result of current study concerns the Cu:Cp that appears the eligible marker to detect a gradient of copper dysfunction. Cu:Cp merges the information derived from copper, ceruloplasmin and nCp-Cu measurements in a unique index, and shows both AD and WD patients differing from healthy controls, but also markedly differing between each other. In fact, the Cohen's d value of 0.8 indicates a large biological effect of the Cu:Cp in AD vs. healthy controls. Cu:Cp Cohen's d further increases in WD to 2.2.

In WD, either copper (driven by the drop of ceruloplasmin biosynthesis) or ceruloplasmin itself are markedly decreased vs. healthy controls; the ceruloplasmin Cohen's d confirms the biological relevance of this difference, being 0.06 in AD and 3.2 in WD.

Results from the 24 h urinary copper section show that copper excretion is increased in AD patients compared to healthy controls. This is in agreement with the expansion of the nCp-Cu component in plasma, suggesting a renal elimination of excess copper as small ultrafilterable molecules. The qualitative comparison between WD (patients in chronic stable D-pen treatment) and AD patients (under D-pen treatment for 6 months) is suggestive of abnormalities in copper excretion in AD patients.

It is known that normally human dietary consumption and absorption of copper exceed the metabolic need: homeostasis of this element is maintained almost exclusively by the ATP7B-mediated level of biliary excretion. Thus, abnormalities in nCp-Cu levels can be reasonably ascribed to this pathway.

WD is definitely a disease of copper overload; however, the total serum copper is usually decreased. This is due to the drop of ceruloplasmin. The ATP7B copper pump controls copper loading into ceruloplasmin, copper entry into lysosomes and copper excretion into

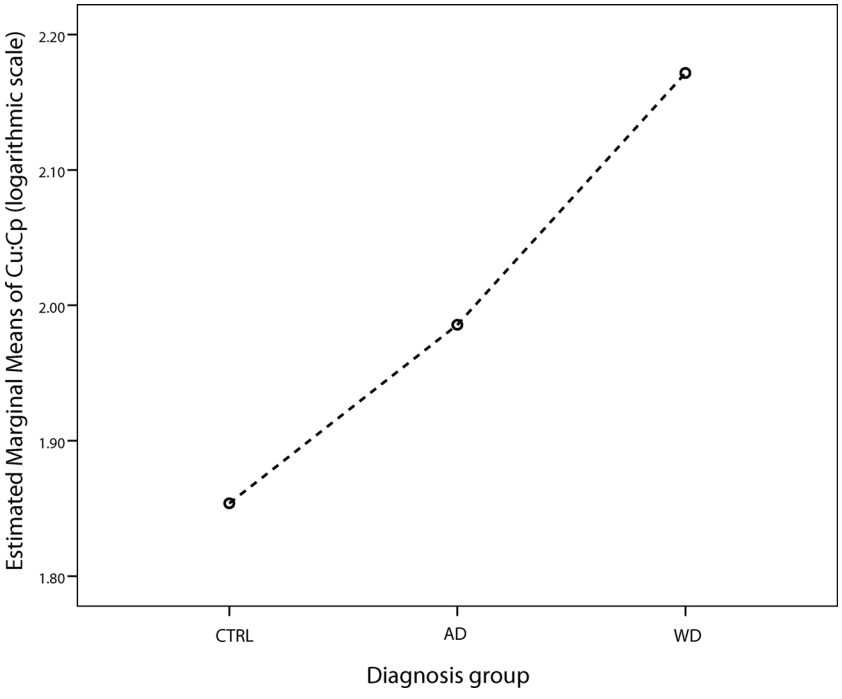

Fig. 2. Data refer to the comparison *i*) as described in Methods (statistical analyses section) regarding the assessment of copper status in serum among AD, WD and healthy controls.
Estimated marginal means of Cu:Cp in each diagnosis group (Healthy control, AD and WD).

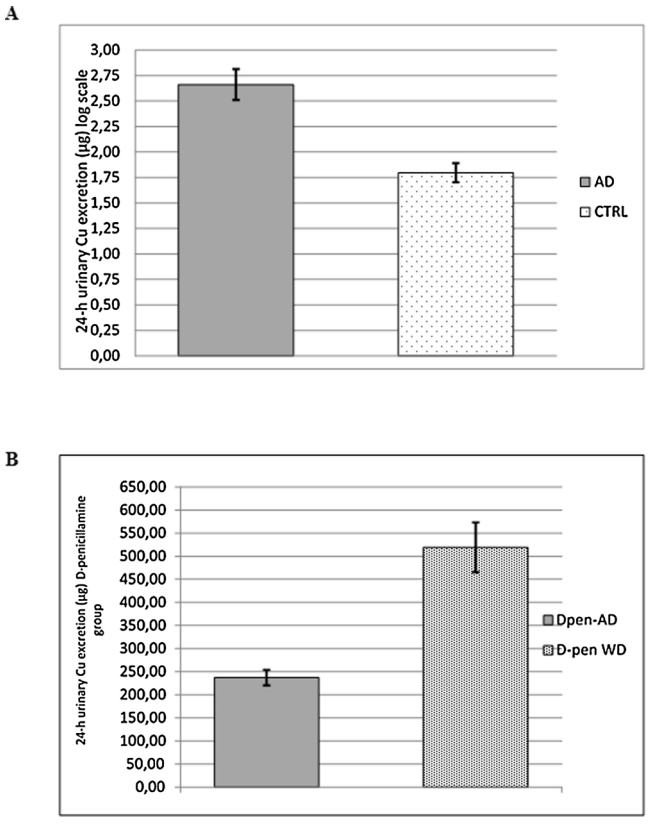

Fig. 3. A Data refer to the comparison *ii*) as described in Methods (statistical analyses section) regarding the assessment of copper status in urine among AD, and healthy controls at baseline; data represent mean values of 24 h urine copper in 24hU-CTRL and in AD patients. Copper is higher in AD than in healthy controls.
B. Data refer to the comparison *iii*) as described in Methods (statistical analyses section) regarding the assessment of copper status in urine between AD and WD patients both in treatment with D-pen; data represent mean values of 24 h urinary excretion. Copper in urine is higher in D-pen WD than in D-pen AD patients.

the bile, accounting for the pleiotropic effects of *ATP7B* gene mutations on copper homeostasis [4,24]. nCp-Cu pool expansion in AD can likely reflect slight abnormalities in copper entry into lysosomes and copper excretion into the bile. In fact, ceruloplasmin in AD shows only slight, though significant, decreases in specific activity [7,25–28], as also reflected by increased fragments of the apo-form of protein detected in AD serum [29]. The drop of ceruloplasmin in WD affects iron metabolism (ceruloplasmin controls the oXidative state of the metal), likely producing effects in diverse vital pathway involving the metal. Moreover, ceruloplasmin directly is involved in lipoprotein metabolism, in the reduction of cholesterol, and in the regulation of the HDL cholesterol/total cholesterol ratio.

Along with the study of copper status in serum we carried out a series of qualitative observations on the 24 h urine copper in WD and healthy controls comparing these data with those collected in a previous study of ours in which 600 mg/day of D-pen was administrated to AD patients for 6 months [6].

Mainly from the experience in WD, it is known that in untreated patients the 24-h urinary excretion of copper reflects the amount of nCp-Cu in the circulation.

Literature about 24 h urinary copper excretion in AD patients is null, apart our study of 2002 [6]. The comparison of copper in the 24 h urine shows that copper excretion was three times higher in AD than in healthy controls. However, it is lower than 40 µg/day, upper limit of normal (ULN; reported as representative of a 'normal' value of human copper excretion) and threshold for WD diagnosis (as reported in Leipzig score) [17]. Literature on D-pen effects on copper excretion in healthy controls is very limited [30,31], while that in WD is ample, but it is generally referred to pediatric patients [22]. Nevertheless, some conclusions can be draw. A study in 1992 [32] proposed the "D-pen challenge test" as a useful screening test for the diagnosis of WD, specifically for asymptomatic patients (generally in pediatric age). "D-pen challenge test" studies were performed with the purpose of obtaining further evidence in support to the WD diagnosis. This test consists in the assessment of the 24 h urinary copper after the administration of D-pen 500 mg at the start of the 24-h urine collection and then again at the 12-h (half-way) point. Values higher that the cut-off of 1500 µg/day (namely > 25 µmoles/day) were considered supportive of WD diagnosis [17]. Copper excretion under D-pen is highest at the beginning. With chronic D-pen administration, urinary copper excretion generally decreases to 200–500 µg/day on treatment. [33] In fact, after a first burst of copper mobilization from parenchymal deposits, the amount of copper in urine stabilizes to levels about three times lower, achieved in few months [33].

A study published by Nicastro and colleagues [22] proposed a less restrictive D-Pen challenge test cut-off of 200 µg/24 h (5x ULN), to identify asymptomatic WD patients [22], also reported within the Leipzig score [34]. The D-pen test, however, is not disease-specific. Individuals suffering from hepatic cirrhosis, for example, also show excretion values higher than the mentioned cut-off. In AD patients under D-pen treatment [6], values higher than the 200 µg/24 h is suggestive of failure a copper control. In fact, applying this cut-off, we found that 87% of D-pen WD and 78% of D-pen AD had values of 24 h urine copper higher than 200 µg/day. Furthermore, it has to be considered that the 24 h urinary excretion study in the D-pen AD cohort was performed after a 6 month treatment. In fact, the values achieved were close to those expected in WD on stable D-pen treatment (200–500 µg/day on treatment). For example, previous studies [33] report 354.87 ± 238.11 µg/24 h urinary copper excretion in 115 WD under chronic D-pen treatment. Levels of copper excretion in D-pen AD were higher than those reported in other studies and diseases [30,31]. For example, Muijsers and colleagues reported that, after a single dose administration of 1000 mg of D-pen, healthy volunteers had copper excretion in the 48 h urine of 292 µg/48 h [31], while Milanino [30], reported a value of 109.3 µg/24 h [30] in rheumatoid arthritis.

ATP7B WD mutations in a heterozygous manner, along with specific *ATP7B* variants, have been reported to increase the risk for AD (from 1.63 to 5.16 Odds Ratio; reviewed in [35]). Recently, the AD-related *ATP7B* gene's variant K832R has been described as a loss-of-function mutation in drosophila [36]. This variant has a frequency of 66% in AD patients typified by high levels of nCp-Cu [37]. As a matter of a fact, in complex diseases harboring a risk variant does not mean to develop the disease. The actual development of the disease phenotype depends in large part on person's genetic make-up, environment and lifestyle, as for example diet [38]. Furthermore, a nucleotide deletion at the 5'UTR region of a single allele of the *ATP7B* gene was indicated as a risk factor for late onset parkinsonism [39]. The authors [39] posited the hypothesis that Parkinson's disease may represent a heterozygote form of WD. In this line of reasoning, we suggest *ATP7B* gene's variants K832R (rs1061472) [37] and rs2147363 [40] as risk factors for AD. Nevertheless, despite the similarities including those shown in this study, WD and AD have essentially a different etiology. Wilson's disease is an autosomal rare disorder caused by an inborn error of copper metabolism [2,3], while AD is a multifactorial disorder with a complex etiology, in which a number of genes interacting with each other and with environmental factors have effects in on beta-amyloid and tau pathways. In this view, copper dysfunction could account as one of the potentially modifiable risk factors contributing to global susceptibility for cognitive decline along with diabetes mellitus, midlife hypertension, midlife obesity, physical inactivity, depression, a major depressive disorder, smoking, and limited education [9]. In fact, beside large population studies [10–13] and *meta*-analyses [4,14,15], clinical investigations in diverse longitudinal cohorts showed that patients with AD increase about threefold their risk of the disease when abnormal

values of serum exchangeable copper, nCp-Cu (higher than 1.6 μM [16,17], are present [18,19].

The current study has many limitations, specifically related to the small size of the drug free WD patient sample analyzed. Also, WD cases with atypical normal levels of copper or ceruloplasmin are underestimated. WD is a rare disorder and it is treated, generally starting at childhood. This limited the size of our drug-free WD sample, which can have influenced the precision of our statistical analyses of the copper markers in serum and limits our conclusions. Data about copper status in serum of patients under D-pen treatment are irrelevant, since the copper-bound to the drug cannot be distinguished by the copper not bound. Also, the D-pen qualitative study is only suggestive of abnormal urinary excretion, but it suffers for study design problems derived from the different extent on length of WD patients under chronic treatment, and cannot be taken as indicative of WD diagnosis for the AD patients.

Beyond its limitations, this study provides new insight into the disposal of copper dysfunction in AD, suggesting the potential of Zinc therapy for those AD patients typified by altered copper metabolism, as our laboratory has recently pioneered with the notion of different "types" of AD [21,35,37,41–43]. Nevertheless, if Zinc therapy can improve cognitive deficits in this percentage of AD patients, as it might be expected from the WD experience, has to be proven through a dedicated phase II clinical trial.

Declaration of interest

R Squitti was consultant CanoX4drug SPA (Italy) in the past 3 years. Other authors declare:

Conflicts of interest

Other authors declare; none'.

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