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ORIGINAL PAPER

ATP7B Variants as Modulators of Copper Dyshomeostasis in Alzheimer's Disease

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Abstract To understand the role of the key copper-regulating gene, *ATP7B*, in copper dyshomeostasis associated with Alzheimer's disease (AD), we analyzed the serum levels of copper, ceruloplasmin and 'free' (i.e., non-ceruloplasmin bound) copper in 399 patients with AD and 303 elderly healthy controls. We also performed analyses of informative variants of *ATP7B*. AD patients had higher levels of copper and free copper than controls. Individuals with free copper levels higher than 1.6 μmol/L (the upper value of the normal reference range) were more frequent among cases ($p < 0.001$). Among these individuals, those who were carriers of the *ATP7B* variants accounted for a large proportion of the free copper levels, specifically in the AD group ($p < 0.01$). Our results suggest the existence of a 'copper dysfunction' phenotype of sporadic AD which has a genetic basis. They also suggest that free copper is a risk factor for this disorder, modulating additional pathways leading to the disease cascade.

Keywords Alzheimer's disease · Copper · Ceruloplasmin · *ATP7B* · Wilson's disease

Introduction

Alzheimer's disease (AD) is a neurological disorder characterized by memory loss and progressive dementia. The late onset form of the disease is sporadic and has a complex disease etiology, with genetic background and age being the most widely accepted risk factors. The cause of the disease appears to be closely related to the aggregation within the brain of the beta-amyloid (Aβ) peptide and tau proteins in neurofibrillary tangles. Moreover, the epsilon4 allele of the apolipoprotein E (*APOE*) gene has been proven to increase the AD risk and decrease the age of onset, even though it accounts only for a percentage of AD heritability, leaving several genetic risk factors left to be identified. The 'amyloid cascade,' which has been claimed as the most popular Alzheimer hypothesis (Hardy and Higgins 1992), has now taken many forms as new details about the disease emerge. In fact, diverse pathogenetic pathways have been postulated to contribute to AD onset and progression. For example, in addition to Aβ oligomers and toxic tau aggregates, oxidative damage, and aberrant inflammation or impaired energy metabolism have been pointed out among the pathogenic pathways involved in the disease cascade that need to be targeted (Frautschy and Cole 2010). There is abundant evidence that oxidative

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stress, mainly via metal redox reactions, can cause damage to the AD brain (James et al. 2012; Faller 2011). Specifically, it has been proposed that the hydrometallation of the Aβ peptide can be at the basis of redox cycles of oxidative stress and H_2O_2 production, Aβ oligomer formation and precipitation (Bush and Tanzi 2008; Cherny et al. 1999; Bucossi et al. 2012). However, some in vitro studies pointed out that sub- and super-stoichiometric concentrations of copper prevent Aβ oligomer formation (Mold et al. 2013). These results confirm the evidence reported by other authors (Yang et al. 2009), who demonstrated that copper induces the precipitation of oligomeric Aβ, but not the increase in the beta sheet structure, and even that copper disrupts the already formed beta sheet aggregates into non-beta sheet aggregates. However, if it is protective or toxic, process is still debated. The study of Jin et al. (2011) can give some indications: Authors have described that copper dramatically affects Aβ aggregation (not in beta sheet formations) and enhances Aβ cytotoxicity. The topic is very complex and still not solved, even though many authors agree that the properties generated by copper and Aβ are regulated by their reciprocal concentrations and that their interaction to produce H_2O_2 appears the biochemical step to take into consideration when thinking about toxicity. A derangement of metal homeostasis leading to a labile pool of serum copper may feed the brain's copper reservoir which can enter Aβ-oxidative stress cycles, generating pleiotropic effects on the AD cascade (Frautschy and Cole 2010) (Squitti et al. 2006; Squitti and Salustri 2009). This notion is now sustained by diverse lines of evidence, and recent meta-analyses showed that AD patients have 0.41 units of standard deviation increase of copper (95 % CI 0.19–0.63) in serum in comparison with healthy controls (Bucossi et al. 2011b; Ventriglia et al. 2012). Moreover, a specific and relative high increase in copper non-bound to ceruloplasmin (also known as 'free' copper) in serum has been reported to be associated with some typical signs and cerebrospinal fluid (CSF) markers of AD (Squitti et al. 2006; Squitti and Salustri 2009). Furthermore, copper dyshomeostasis has been shown in CSF (Capo et al. 2008) and in some critical areas of the AD brain (James et al. 2012).

On these bases, we measured serum copper-related biochemical markers (copper, ceruloplasmin and free copper) and tested their association with some single nucleotide polymorphisms (SNPs) of a copper gene, expressly in AD patients and healthy elderly controls who had copper abnormalities—i.e., levels of free copper higher than the normal reference range. Specifically, we focused our attention on *ATP7B*. This gene encodes for the ATPase 7B protein that plays a key role in the regulation of copper homeostasis (Squitti and Polimanti 2012). In the hepatocyte, ATP7B receives copper from ATOX1 and delivers it to nascent ceruloplasmin. When intracellular copper exceeds cell needs, ATP7B moves toward the bile canalicular membrane to expedite its biliary excretion (Gaggelli et al. 2006). In order to investigate the role of *ATP7B* in copper dyshomeostasis, we selected informative SNPs on the basis of previous studies devoted to the analysis of the structure of the *ATP7B* gene (Gupta et al. 2007). In particular, we considered four SNPs: rs1801243 (missense substitution: Ser406Ala); rs2147363 (intronic variant: c.1544-53A>C); rs1061472 (missense substitution: Lys832Arg); and rs732774 (missense substitution: Arg952Lys), since they have been reported to be informative of *ATP7B* gene structure (Gupta et al. 2007; Squitti et al. 2013). In fact, in a recent study, we analyzed the genetic structure of *ATP7B* also in relation to the risk of having AD, and we found that, among the four SNPs studied, those which lay in the transmembrane domains had a stronger association with AD (Squitti et al. 2013). In this study, we correlated these informative genetic variants with free copper concentration in serum in order to identify the *ATP7B* gene regions associated with copper dyshomeostasis in AD and healthy individuals.

Methods

Subjects

The study was approved by the local IRB, and all participants or legal guardians signed an informed consent.

Alzheimer's disease patients and healthy controls were recruited by two specialized dementia care centers in Rome, Italy: the Department of Neuroscience of San Giovanni Calibita—Fatebenefratelli Hospital, and the Department of Neurology of Campus Bio-Medico University. Each used the same standardized clinical protocol (Giambattistelli et al. 2011). All AD patients had been diagnosed as 'probable AD' according to NINCDS–ADRDA criteria (Dubois et al. 2007; McKhann et al. 1984) and had a minimental state examination (MMSE) score $\leq$25 (Folstein et al. 1975). All AD patients underwent general medical, neurological, and psychiatric assessments. Neuroimaging diagnostic procedures and complete laboratory analyses have been performed to exclude other causes of progressive or reversible dementia, such as stroke or vascular effects.

The control sample consisted of healthy volunteers with no clinical evidence of neurological or psychiatric disease.

Exclusion criteria for both patients and controls were conditions known to affect copper metabolism and biological variables of oxidative stress (e.g., diabetes mellitus, inflammatory diseases, recent history of heart or respiratory failure, chronic liver or renal failure, malignant tumors, and

a recent history of alcohol abuse). About 55 % of the subject sample partially overlaps with the sample of previous studies (Squitti et al. 2007, 2011; Zappasodi et al. 2008).

Biochemical Investigations

Patients' fasting blood samples were collected in the morning and serum was rapidly stored at −80 °C. Serum copper concentration was estimated following the colorimetric method (Randox Laboratories, Crumlin, UK). An A Analyst 300 Perkin Elmer atomic absorption spectrophotometer, equipped with a graphite furnace with platform HGA 800, was also used (Foster City, CA, USA) (Squitti and Salustri 2009). Ceruloplasmin was analyzed by immunoturbidimetry assay (Horiba ABX, Montpellier, France) (Wolf 1982). For each serum copper and ceruloplasmin pair, we computed the amount of copper bound to ceruloplasmin (CB) and the amount of free copper following standard procedures (Appendix 1 of Ref. (Walshe 2003): "Calculation of 'free copper' concentration") (Walshe 2003); briefly: CB = ceruloplasmin (mg/dL) × 10 × n; $n = 0.0472$ (μmol/mg); free copper = absolute serum copper − CB (Walshe 2003). This calculation expresses free copper in μmol/L and is based on the evidence that ceruloplasmin contains 0.3 % copper (Walshe 2003). Values of free copper calculated for each subject entered the statistical analyses.

SNPs Genotyping

Genomic DNA was purified from peripheral blood using the conventional method for DNA isolation (QLAamp DNA Blood Midi kit). Genotyping of SNPs rs1061472 was performed by the TaqMan allelic discrimination assay as described in Giambattistelli et al. (2011). The predesigned SNP genotyping assay IDs are ID_C_8713998_80 (rs1801243), ID_C_25473601_10 (rs2147363), ID_C_191 9004_30 (rs1061472) and ID_C_938208_30 (rs732774) (Applied Biosystems, Inc.). Direct DNA bidirectional sequencing was performed for 15 % of the PCR products, which were randomly selected and analyzed to confirm the genotypes. Apolipoprotein E (*APOE*) genotyping was performed according to the established methods (Hixson and Vernier 1990).

Statistical Analysis

A statistical analysis was carried out using SPSS 15.0. Demographic and clinical characteristics in our patient and control samples were described either in terms of mean ± standard deviation (SD) if quantitative or in terms of proportions. Student's t test, the Chi square (χ^2) test and ANOVA test were used to compare the characteristics of AD patients and controls. Correlations between age and copper variables were calculated. When significant, biochemical values were adjusted for the coefficient of their correlation with age before entering the ANOVA models. The Kolmogorov–Smirnov test was used to test the normal distribution of the biochemical variables. Accounting for the age effect, all biochemical variables results were normally distributed.

For the *ATP7B* genotypes, the Hardy–Weinberg equilibrium was evaluated using the exact test performed by SNPStats (Sole et al. 2006). Because the inheritance was unknown, different models were considered to evaluate the effect of the genetic changes on the markers of copper metabolism: codominant (each genotype has different effects), dominant (one copy of the allele is sufficient to increase the effect) and recessive (two copies of the allele are necessary to increase the effect). To estimate the haplotype phase of the individuals, the ELB algorithm was applied using Arlequin 3.5.1.3 (Excoffier et al. 2003). To complete the biochemical–genotype interaction analysis performed by the ANOVA test, we estimated that a sample size of 50 individuals would result in powers of 92 % (codominant model) and 96 % (dominant and recessive model) to detect a delta = 0.750 with an alpha of 5 %.

Results

Table 1 reports the demographic, clinical and molecular characteristics of the study population. Patients with AD and healthy elderly controls did not differ in sex and education, but they differed in age, *APOE* genotype and MMSE score ($p < 0.001$). Specifically, age correlated with copper ($r = 0.18$, $p = 0.012$), ceruloplasmin ($r = 0.132$, $p = 0.016$), but not for free copper ($r = 0.047$,

Table 1 Demographic, clinical and molecular characteristics of the study population

	AD patients ($n = 399$)	Controls ($n = 303$)	Significance
Age (years)	74.9 ± 8.1	66.5 ± 10.5	$p < 0.001$
Sex (female) (%)	67.7	68.3	$p = 0.889$
MMSE score	19.5 ± 4.5	28.6 ± 1.3	$p < 0.001$
APOE e4 carriers (%)	36.7	11.6	$p < 0.001$
Education (years)	8.9 ± 5.0	9.5 ± 4.5	$p = 0.634$
Copper (μmol/L)	14.98 ± 3.14	13.05 ± 2.98	$p < 0.001$
Ceruloplasmin (mg/dL)	26.88 ± 5.09	26.90 ± 5.20	$p = 0.959$
Free copper (μmol/L)	2.24 ± 2.25	0.28 ± 2.32	$p < 0.001$

The comparisons of biochemical variables are adjusted for age effect

$p = 0.395$). Accordingly, copper and ceruloplasmin values were adjusted for the age effect before entering ANOVA.

Copper and free copper levels were higher in cases than in controls ($p < 0.001$), whereas ceruloplasmin did not differ between them. Individuals with a level of free copper higher than the normal reference range [<1.6 μmol/L (Hoogenraad 2001)] were more frequent in the case than in the control group ($p < 0.001$).

Our working hypothesis was to test whether those individuals with a clear copper dyshomeostasis had their copper abnormalities associated with *ATP7B* variants. We also wanted to determine whether this condition was more pronounced in the AD group. To address these questions, we firstly stratified our AD and healthy groups into three classes on the basis of their free copper levels in serum: 'low free copper' (<1 μmol/L), 'medium free copper' (≥1, <1.6 μmol/L) and 'high free copper' (≥1.6 μmol/L) (Fig. 1). Cases and controls had antithetic distribution: the 'low free copper' class accounted for 27 % of AD cases vs. 61 % of controls; the 'medium free copper' for 11 % of AD vs. 10 % of controls; and the 'high free copper' for 62 % of AD versus 29 % of controls.

Then, we analyzed the serum copper profile in relation to the *ATP7B* informative gene variants in the sole 'high free copper' class (patients with AD, $n = 109$; controls, $n = 53$; Table 2). The exact test revealed that all SNPs were in the Hardy–Weinberg equilibrium in both cases and controls. We compared markers of copper in serum and *ATP7B* SNPs through diverse genetic models, which were applied either on the whole study population or on AD cases and controls kept separate (Fig. 2). Details of the comparisons that reached a significance of 1 % are reported in Table 3. An analysis, conducted through a recessive model, revealed that those individuals who were carriers of at least one ancestral T allele in rs1801243 had decreased

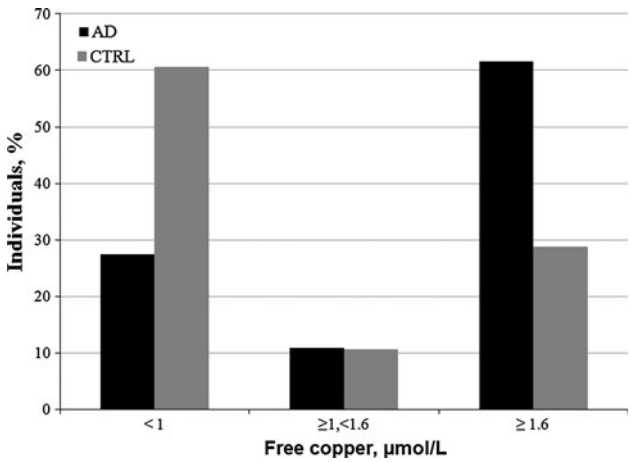

Fig. 1 Frequency distribution of free copper classes between AD cases and controls

levels of ceruloplasmin and copper. GG homozygous individuals for rs7323774 (codominant and dominant model) had increased free copper levels in serum, which was more prominent in AD. The analysis of the haplotypes for the four informative *ATP7B* SNPs revealed that individuals who were non-carriers of the haplotype with derivate alleles had increased free copper levels in the whole population analyzed. This increase was driven by the AD cases.

Discussion

The main result of our study is that individuals who were GG homozygous for *ATP7B* rs7323774 SNP had higher levels of serum-free copper, and this condition was more pronounced in the AD individuals.

Our data propose copper dyshomeostasis as a pathogenetic pathway toward AD. Our current findings show, in fact, that free copper distributions are antithetic between AD cases and controls and that this picture can be explained by gene variants within the *ATP7B* gene. Furthermore, our data indicate that informative *ATP7B* variants, located in genetic regions that encode the transmembrane domains of ATP7B protein, may be associated with copper dyshomeostasis in AD. However, since the investigated SNPs have been selected for their informativeness on the *ATP7B* gene structure and not for their impact on gene function, it is obvious that rs1801243, rs2147363, rs1061472 and rs732774, even though significantly associated with AD (Squitti et al. 2013), cannot be the loci responsible for the effects of the *ATP7B* gene on AD, either in terms of free copper derangement or in terms of an increased risk of the AD (Bucossi et al. 2012). Furthermore, our previous genetic studies revealed that *ATP7B* gene variants and *APOE* genotypes were independent risk factors for AD (Bucossi et al. 2011a, 2012; Squitti et al. 2013). In the current large study population, we found no significant correlations between copper-related biochemical variables and *APOE*

Table 2 Demographic, clinical and molecular characteristics of individuals within the 'high free copper' class

	AD patients ($n = 109$)	Controls ($n = 53$)	Significance
Age (years)	75.2 ± 7.3	65.7 ± 12.5	$p < 0.001$
Sex (female) (%)	69.6	66.0	$p = 0.648$
MMSE score	18.3 ± 5.6	28.3 ± 1.6	$p < 0.001$
APOE e4 carriers (%)	35.8	17.6	$p = 0.020$
Education (years)	8.3 ± 4.5	10.7 ± 5.1	$p = 0.259$

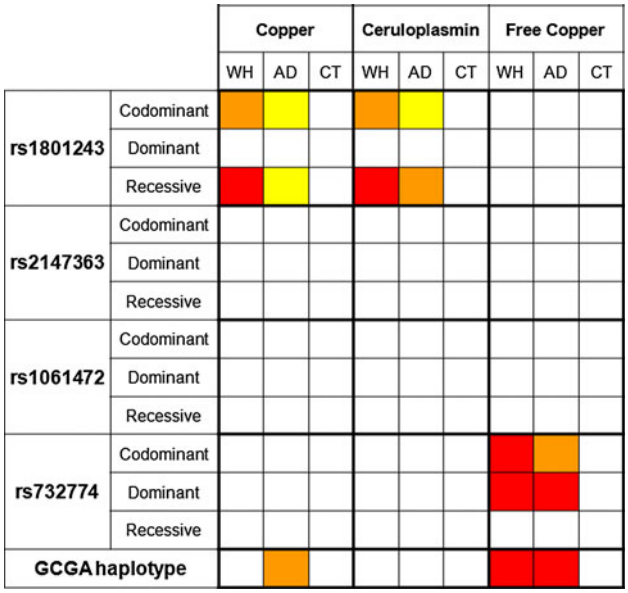

Fig. 2 Interaction analysis between biochemical (copper, ceruloplasmin and free copper) and genetic (ATP7B variants and haplotype) variables of copper dyshomeostasis. ANOVA test was applied considering different genetic models (codominant, dominant and recessive) and performed in whole study population (WH), AD cases (AD) and controls (CT). The *box color* represents the *P* value of the comparison: *white* ($p > 0.100$), *yellow* ($p < 0.100$), *orange* ($p < 0.050$) and *red* ($p < 0.010$) (Color figure online)

genotype, suggesting independent pathogenic pathways for *ATP7B* and *APOE*. Our results can be, instead, informative of the presence of hot spots for AD susceptibility in a specific *ATP7B* gene region, as we recently described (Squitti et al. 2013). Indeed, we hypothesized that some *ATP7B* functional variants can be hidden within this gene and that they can be genetic risk factors for AD (Squitti and Polimanti 2012). This scenario, proposed for AD, resembles the so-called ecological model of a disease caused by free copper toxicosis or accumulation, which is, exactly, Wilsons's disease (Brewer 2009; Hoogenraad 2001; Scheinberg and Sternlieb 1965). One of the main criticisms directed toward this statement is that neurodegeneration in Wilson's disease leads to a movement disorder with no particular effect on cognition, while AD is nothing but cognition loss. In other words, the two may both share copper toxicity, but the manifestations are so completely different that it seems unreasonable to claim the same gene is associated with both of them. Even though this concern is substantially correct, it does not actually refute our statement that the *ATP7B* gene can be a causative gene for Wilson's disease and a susceptibility gene for AD. In fact, diverse neurodegenerative disorders, such as AD itself, Parkinson's disease, amyotrophic lateral sclerosis and Huntington's disease, have a clinical phenotype which is dependent on the age of onset (Koedam et al. 2010; Halliday and McCann 2010; Scalfari et al. 2011). This is particularly true when a comparison is made between Wilson's disease, whose age of onset is typically in childhood, and sporadic AD, clinically defined as a late onset form of dementia with the age onset in adulthood, over 65 years of age by definition. Another example can be even more explanatory on this point, and it is given by a gene very close to *ATP7B*, which is *ATP7A*. *ATP7A* mutations have been typically associated with Menkes disease. However, it has been recently recognized that they can also been associated with a milder form of the disease called occipital horn syndrome (Moller et al. 2000). Moreover, striking recent evidence counterintuitively demonstrated that certain missense mutations of this gene can cause a syndrome restricted to progressive distal motor

Table 3 Comparisons with significance at 1 % between serum copper markers and *ATP7B* genotype/haplotype in the 'high free copper' class

	WH						AD
	rs1801243 (recessive)						
	T			GG			
Copper (μmol/L)	15 (2.7)			17 (5.5)			-
Significance	$p = 0.008$						-
Ceruloplasmin (μmol/L)	25 (4.2)			28 (6.2)			-
Significance	$p = 0.003$						-
	rs732774 (codominant)			rs732774 (dominant)		rs732774 (dominant)	
	GG	GA	AA	GG	GA+AA	GG	GA+AA
Free copper (μmol/L)	6 (6.4)	3 (1.5)	4 (2.4)	6 (6.4)	4 (2.0)	7 (7.2)	4 (2.1)
Significance	$p = 0.009$			$p = 0.004$		$p = 0.005$	
	GCGA haplotype			GCGA haplotype			
	Non-carriers		Carriers	Non-carriers		Carriers	
Free copper (μmol/L)	5 (4.4)		3 (1.9)	6 (5.2)		3 (1.8)	
Significance	$p = 0.002$			$p < 0.001$			

Data are mean (SD). The comparisons of biochemical variables are adjusted for age effect

WH, whole study population; AD, patients with Alzheimer's disease

neuropathy without overt signs of systemic copper deficiency (Kennerson et al. 2010). Besides Wilson's and Menkes diseases, other examples of diseases with neurological signs related to genes involved in the regulation of metal homeostasis are present in the literature. Patients with *HFE*-related hemochromatosis showed iron accumulation in different brain areas, suggesting that *HFE* mutations modify the risk of developing neurodegenerative disorders (Nandar and Connor 2011). Genetic variation in the ceruloplasmin (*CP*) gene showed an association with the risk of having Parkinson's disease (Hochstrasser et al. 2005).

Finally, data presented support a simple paradigm linking genetic, biochemical and clinical evidence in a causative triangle, explaining copper dyshomeostasis in a percentage of AD cases: Unknown *ATP7B* functional variants can cause free copper derangement. This, in turn, increases the susceptibility for AD by, for example, activating or accelerating additional pathogenic pathways of the disease cascade, as the amyloid precursor protein (APP) or Aβ hypermetallation (Bush and Tanzi 2008; Squitti and Polimanti 2012). Specifically, our data suggest that rs732774, a non-synonymous variant associated with an amino acidic substitution from arginine to lysine in position 952 in the transmembrane domains of the ATP7B protein, can be likely associated with this pathogenic pathway. This evidence, together with the results of a previous genetic association study of ours (Squitti et al. 2013), suggests that *ATP7B* SNPs located in the transmembrane domains are linked to copper dyshomeostasis in AD. However, additional regions of the *ATP7B* gene or even additional genes in the copper pathway can be potential susceptibility loci for AD.

Even though it is not the determinant of AD, copper dysfunction can be assumed as a causative, rather than associated, risk factor for AD, as sustained by solid clinical (Squitti et al. 2005, 2006; Squitti and Salustri 2009), epidemiological (Lam et al. 2008; Morris et al. 2006), experimental (Cherny et al. 1999; Multhaup et al. 1996; White et al. 1999), meta-analysis (Loef and Walach 2012; Schrag et al. 2011; Bucossi et al. 2011b; Ventriglia et al. 2012) and genetic (Bucossi et al. 2011a, 2012, Squitti et al. 2013) evidence.

Adjusting data for the possible confounder is a common and reliable statistical procedure used to control, in our case, the effect of age, which differed between AD and healthy populations. Specifically, AD patients were approximately 9 years older than controls on average. In a perfectly age-matched design, the age adjustment is still useful if not necessary, but it was mandatory in our case in an attempt to achieve a reduction in this potential confounding factor. Accordingly, all the significant outcomes obtained in the present study are confirmed in the crude and adjusted analyses. Furthermore, the case–control design assumes that controls should have the possibility to 'become cases,' as theorized by many epidemiologists (Rothman 1986). From the above, in our study population, the possibility that some controls are in the condition to convert to AD while they age another 9 years makes our controls and cases closer and our estimate of the association between *ATP7B* and AD more conservative. This can also account for the relatively high percentage of controls having high free copper levels.

Even though additional studies with bigger sample sizes are needed, we suggest the existence of a 'copper dysfunction' phenotype of AD, which could help to explain contrasting results in the literature and hopefully to provide some AD patients with tailored therapeutic approaches that might prevent, or even counteract, disease progression.

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Conflict of interest All authors and their family members report no financial relationship related to the manuscript or the topic and no conflicts of interest.

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