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Review

Copper imbalance in Alzheimer's disease: Convergence of the chemistry and the clinic

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ABSTRACT

In this perspective we list the many clinical, histopathological, genetic and chemical observations relating copper to Alzheimer's disease (AD). We summarize how the coordination chemistry of the APP/Aβ system is centrally involved in neuronal copper transport at the synapses, and that genetic variations in the gene coding for the copper transporter ATP7B cause a subset of AD, which we call CuAD. Importantly, the distinction between loss of function and gain of toxic function breaks down in CuAD, because copper dyshomeostasis features both aspects directly. We argue that CuAD can be described by a single control variable, a critical, location-dependent copper dissociation constant, K_d^c. Loss of functional copper from protein-bound pools reduces energy production and oxidative stress control and is characterized by a reduced pool of divalent Cu(II) with $K_d < K_d^c$. Gain of redox-toxic function is described by more copper with $K_d > K_d^c$. In the blood, the critical threshold is estimated to be $K_d^c \sim 10^{-12}$ M whereas at synapses it is argued to be $K_d^c \sim 10^{-9}$ M. The synaptic threshold is close to the values of K_d for Cu(II)-binding to Aβ, prion protein, APP, and α-synuclein, implied in copper buffering at the synapses during glutamatergic transmission. The empirical support for and biochemical and pathological consequences of CuAD are discussed in detail.

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1. Introduction

Every day, thousands of people across the world are estimated to develop Alzheimer's Disease (AD) [1,2]. Despite its enormous prevalence and tragic consequences, currently available treatments only delay the disease by typically a few months [3,4], and the many recent failures of leading drug candidates have put renewed emphasis on alternative, more complete causal disease mechanisms [5–10]. The disease manifests first as mild cognitive impairment characterized by a gradual loss of episodic memory and leads to a slow, continuous impairment of cognitive abilities and life quality [3,11–13]. Neuron loss initiates in the cerebral cortex and commonly in the hippocampus [14].

Diagnosis of AD requires the observation of deposits of senile plaques outside the neurons and neurofibrillar tangles inside neurons [12,13]. The plaques contain oxidized and metal-bound β-amyloid (Aβ) peptides arranged in regular β-sheet fibril structures [15–17]. AD occurs most commonly with no apparent family history as "sporadic AD" (SAD); only a very small percentage relates to inherited mutations within a family, i.e. "familial AD" (FAD) [18]. FAD is caused by mutations in the genes coding for the β-amyloid precursor protein (APP) [19] and the presenilin isoforms PS1 and PS2; Aβ is produced from APP by the action of β- and γ-secretases [20,21], and PS1/2 is the catalytic subunit of γ-secretase which produces Aβ from APP together with β-secretase [22–26]. Thus, Aβ is centrally implied both by biochemical hallmarks and genetic risk factors, leading to the dominance of the amyloid hypothesis in current drug development efforts [15,27,28].

Other biochemical hallmarks include molecular oxidative stress [29], impaired glucose utilization [30,31], and disturbed concentrations of metal ions, notably calcium [32–34], zinc [14,35,36], copper [37–41], and iron [42,43]. Each of these hallmarks have associated theories of pathogenesis assuming them to be causative, e.g. the tau [44–47], metal ion [48,49] and oxidative stress [50,51] hypotheses. Age is however the main risk factor of the disease [52] and should accordingly be central to any disease mechanism.

This review focuses on copper's increasingly recognized role in AD. Copper's importance to the central nervous system (CNS) is well-known and involves processes such as catecholamine synthesis, neurotransmission, adenosine triphosphate (ATP) synthesis, antioxidant defense, and myelin formation, which are briefly reviewed below and discussed elsewhere in detail [14,53–56]. Copper is crucial for CNS development and takes part in complex regulatory networks of proteins and pathways that maintain CNS homeostasis via the two copper-transporting P-type ATPases (copper-ATPases) [57,58]. In recent years, it has become increasingly clear that impaired copper control plays a role in AD development and progression, mainly via an increase in serum Cu not bound to ceruloplasmin (noncp-Cu) [55]. Increases in noncp-Cu pertains to a percentage of AD patients, a copper-related AD subtype, which we call 'CuAD', characterized by high noncp-Cu levels and showing peculiar clinical characteristics linked to a perturbed copper homeostasis. These changes in noncp-Cu can be merged with the physical chemistry of copper-binding proteins which is also strongly related to AD [49,56,59,60]; the merging of these two fields to produce a united, consistent view of CuAD is the purpose of the present review paper.

2. The amyloid hypothesis and its main problems

The parallel between the plaque deposits in AD and the protein deposits in Creutzfeldt-Jakob disease inspired the hypothesis that the plaques in AD might be pathogenic [61]. When these plaques were found to consist primarily of aggregated Aβ peptides, the basis for the amyloid hypothesis was established [62] and further supported by the three major genetic risk genes of FAD coding for PS1/PS2 and APP [15,27,63].

The amyloid hypothesis is a "gain-of-function" hypothesis, i.e. it proposes that Aβ attains a toxic function in the brain that leads to disease [27,64]. Earlier it was commonly named the "amyloid cascade hypothesis", emphasizing a quantitative overload of toxic Aβ leading to AD [15,63]. The Aβ deposits in brains typically represent several years of total Aβ production [65]. Accordingly, drugs are pursued that prevent the formation of amyloids either by molecular interaction with Aβ [66–69] or by modulating its formation [70–76].

PS1 mutations often produce lower amounts of all Aβ isoforms than the wild type protein [20,77,78]. The 99-residue C-terminal fragment of APP (C99, produced by cleavage of APP by β-secretase (BACE1), is subsequently trimmed by γ-secretase in consecutive steps [79], resulting in Aβ peptides that vary in length, most notably $A\beta_{40}$ and $A\beta_{42}$ [7,77], and the FAD mutations affect the precision of this cleavage [80,81] and tend to increase $A\beta_{42}/A\beta_{40}$ ratios [78,82–85], which, rather than any absolute amount of $A\beta_{42}$ or $A\beta_{40}$, is the main consistent feature of FAD mutations [28,78,86]. The long amyloids harbor additional hydrophobic amino acids and are thus more prone to aggregation [87,88] and more toxic to cells as these two features tend to correlate [15,89,90]. Thus, the current version of the amyloid hypothesis rests not on the total Aβ levels, but rather the local surplus of $A\beta_{42}$ leading to disease. The importance of the $A\beta_{42}/A\beta_{40}$ ratio is supported by its correlation with clinical age of onset of PSEN1 mutation carriers [91].

Many (estimated 20–40%) cognitively normal older people possess enough senile plaques [92] to satisfy currently applied AD diagnostic criteria [93,94]. The measured signatures of neurodegeneration and cognitive decline do not correlate significantly with plaque load [4,64]. Neuron loss generally begins in specific parts of the brain (e.g. hippocampus) but APP and Aβ is broadly distributed, implying other disease modifiers [95,96]. Rather than the plaques themselves, the intracellular soluble oligomers of Aβ are particularly toxic and are considered the pathogenic culprits in the current version of the amyloid hypothesis [97–102].

The toxicity of Aβ oligomers depends substantially on their structure and size [103–105], and the specific pathogenic forms that allegedly causes AD remain unknown [90,106,107]. Also the mechanisms of action remain unknown although many have been suggested [108], including general seeding of protein misfolding [87,98,109], toxic redox reactions in their metal-bound forms [110–113], and Aβ forming membrane channels that facilitate metal ion transport [114–118].

The physiological structures of Aβ peptides are central for understanding their aggregation and natural functions [7,15,88,119]. The structural disorder of the peptide [120] makes measured toxicity very dependent on concentration, pH, ionic strength, co-solvents, and the time scale of the experiment performed [89,121]. Whereas aggregated fibrillar Aβ attains a regular β-sheet structure as seen in senile plaques, the free Aβ monomer has a disordered coil-dominated structure with only a small amount of helix and β-strand [120,122–124] as reflected in structural models with PDB codes 1BA4 [125], 1IYT [122], 2LFM [126], 1Z0Q [123], and 1AML [127] (Fig. 1). However, in non polar co-solvents such as hexafluoroisopropanol and close to membranes, the helix content of Aβ monomers increases [127]. When Aβ is in a membrane-like chemical environment, the C-terminal of Aβ forms an extended, potentially membrane-spanning helix, an observation that fits well with the widely documented membrane channel properties of Aβ [115,117,128–130]. The conformation, aggregation tendency, and potential function of Aβ monomers

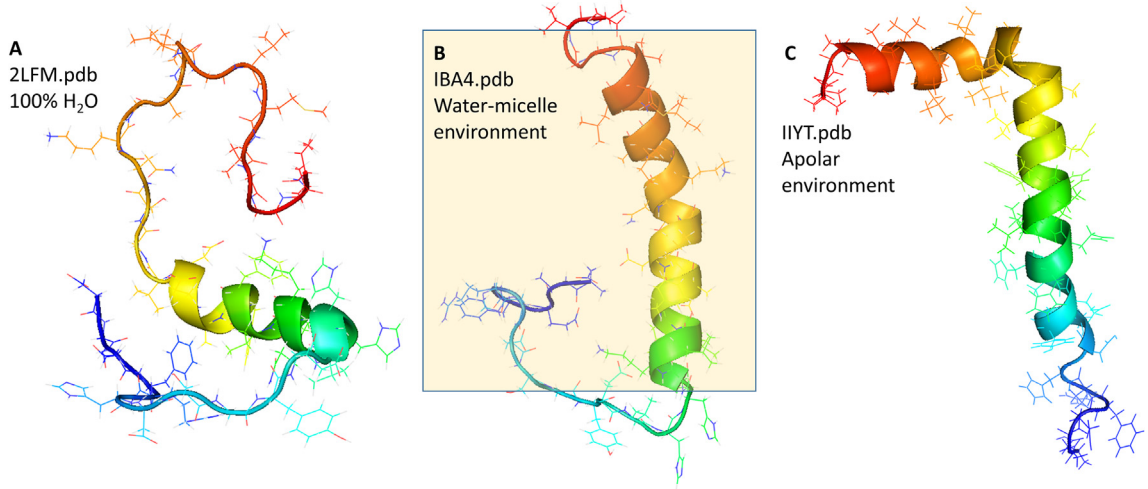

Fig. 1. Aβ structures derived from NMR experiments: A) $A\beta_{40}$ in water (PDB: 2LFM) [126]. B) $A\beta_{40}$ in a micelle membrane-mimicking environment (PDB: 1BA4) [125]. C) $A\beta_{42}$ in an apolar environment (20% water, 80% hexafluoroisopropanol) (PDB: 1IYT) [122]. N-terminals are shown in blue, and C-terminals in red.

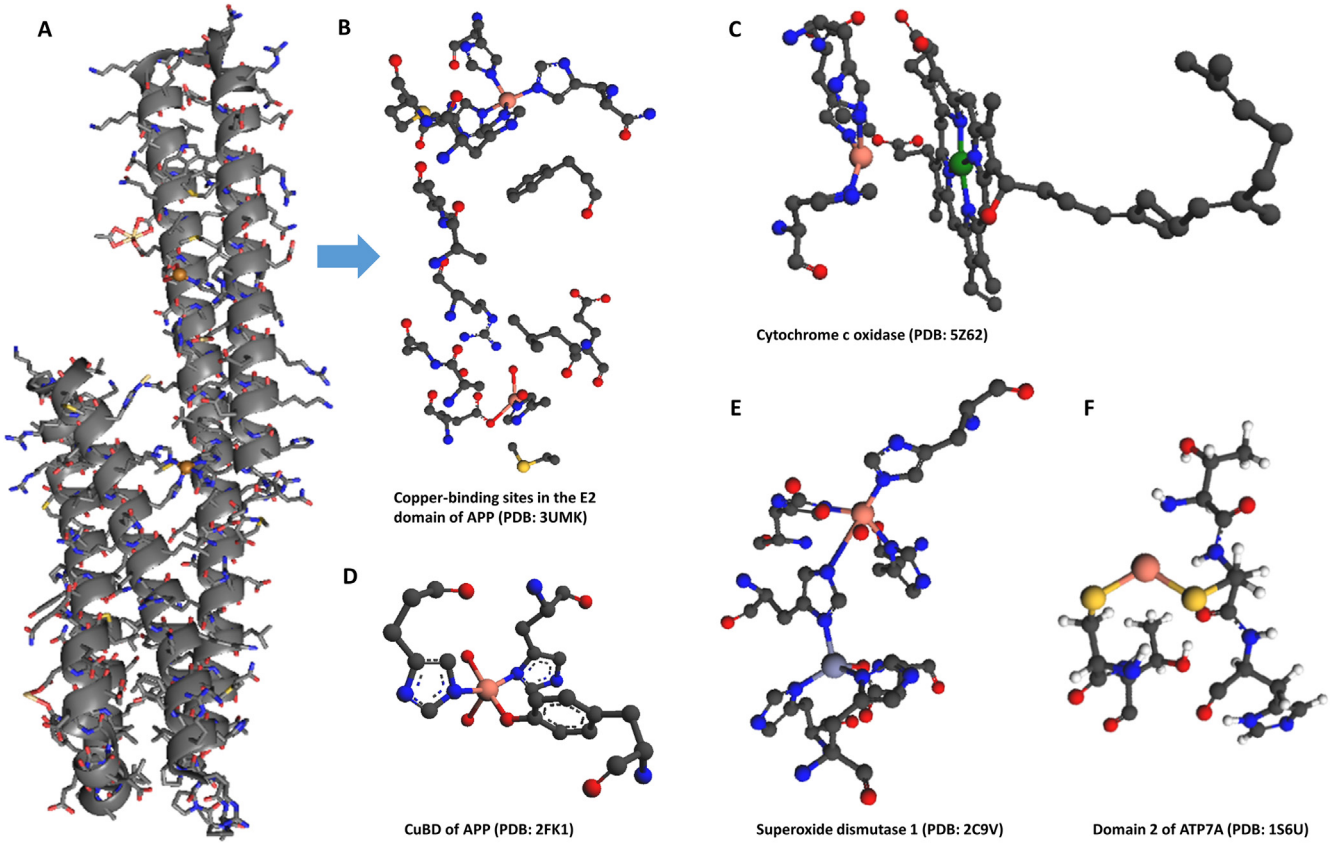

Fig. 2. Established copper sites in some key proteins of the brain: (A) and (B) Copper-binding sites in the E2 domain of APP (PDB: 3UMK) [139]. (C) Copper (center, orange) in CCO (PDB: 5Z62) [140]. (D) Copper in the CuBD of APP (PDB: 2FK1) [141]. (E) Superoxide dismutase 1 (PDB: 2C9V) [142]. (F) Domain 2 of ATP7A (PDB: 1S6U) [143].

and oligomers depends on the coordination environment imposed by metal ions [131–133], as discussed further below.

3. Brain copper homeostasis

Copper is an essential trace metal that is required for the activity of many enzymes and proteins; its homeostasis has been reviewed many times before but is briefly summarized below [14,134,135]. The redox cycling between its Cu(II) and Cu(I) oxidation states is utilized by numerous enzymes [14,136]. Copper is mostly transported and buffered in its Cu(I) state intracellularly, as for example bound to metallothioneins (MT), copper-transporter 1 (Ctr1), and ATPases ATP7A/B, but is functionally important as Cu(II) [137,138]. Vital functions involving copper directly in the catalytic active site include energy production via ATP synthesis in the mitochondria (cytochrome *c* oxidase, CCO), antioxidant defense (superoxide dismutase 1 and 3, SOD1 and SOD3), collagen maturation (lysyl oxidase), pigment synthesis

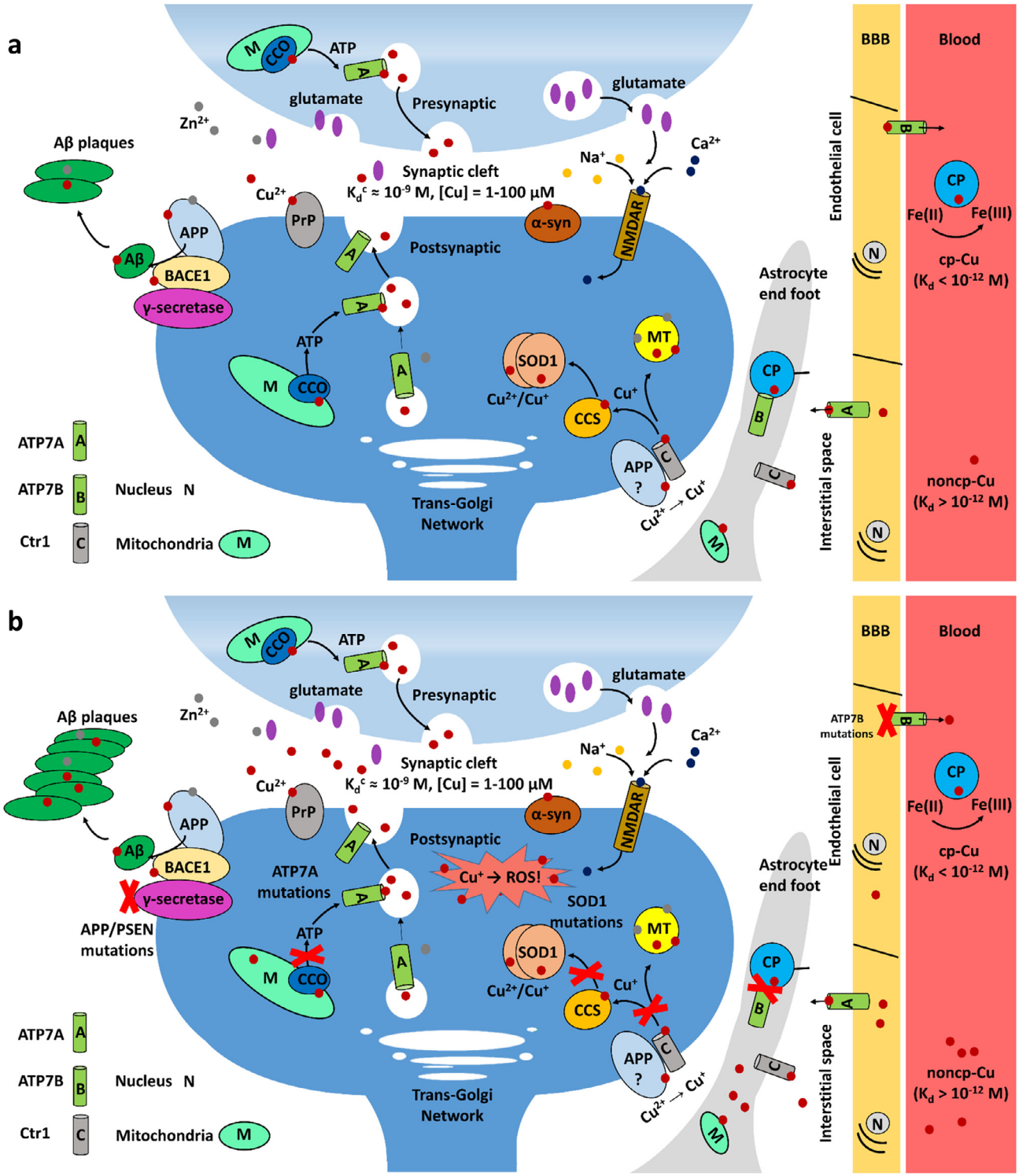

Fig. 3. Simple overview of synaptic copper homeostasis. (A) Normal healthy brain. (B) CuAD brain. The main differences are shown as red copper and red crosses of probable impaired pathways. CCO = cytochrome *c* oxidase; SOD1 = Superoxide dismutase 1; MT = metallothionein; PrP = prion protein; CP = ceruloplasmin. In CuAD (B), genetic (APP, PSEN1, or ATP7B) or life-style-induced changes in copper transport and balance leads to increased labile Cu, which disrupts ATP production (CCO), antioxidant defense (SOD1), and synaptic transmission.

(tyrosinase), and white and red blood cell production via hemoglobin synthesis and iron redox regulation (ceruloplasmin, hephaestin) [14,137].

Examples of established copper sites in relevant proteins are shown in Fig. 2A–2F. Most notably are the two copper sites in the extracellular E2 domain of APP (Fig. 2A and 2B), one having a 4-histidine coordination sphere similar to that in SOD1 (Fig. 2E), and the other E2 copper binding in a trigonal coordination geometry to one histidine, a carboxylate group of a glutamate residue, and a solvent water, with a potential coordinating methionine near-by [139]. Another copper site in APP is the so-called copper binding domain (CuBD) [144]; this copper binds two histidines

and a tyrosine and two solvent molecules in a distorted trigonal bipyramidal geometry (Fig. 2D) [141]. CCO, which is the terminal protein in the electron transport chain and thus required for energy production, contains both a Cu_A and Cu_B center. The Cu_B is coordinated by three histidines and is located uniquely close to a heme iron to assist in 4-electron-reduction of O_2 (Fig. 2C) [140]. Finally, the copper site in the ATPase is characterized by coordination to two cysteines in a distorted solvent-exposed coordination geometry; this structure is from solution NMR and thus less detailed (Fig. 2F) [143].

In the brain, copper is also centrally involved in catecholamine synthesis via dopamine β-hydroxylase (DBH), catecholamine degradation via monoamine oxidase (MAO), neuropeptide synthesis via peptidylglycine α-amidating monooxygenase, glutamatergic synapse modulation, excitotoxicity, and myelin formation [14,135]. Via its antagonistic relationship to zinc managed by the metallothioneins, copper also affects zinc levels and associated processes [38,145–148], which again affects calcium levels [149,150]. Via ceruloplasmin, copper modulates iron uptake and distribution by influencing the oxidative state of iron, since ceruloplasmin catalyzes the oxidation of Fe(II) to Fe(III), which is then loaded into transferrin [54,151].

As discussed in detail below, the chemical and pathological features of disease tend to converge in the synapse. A simplified view of the copper homeostasis at the synapse is displayed in Fig. 3. The healthy normal situation is shown in Fig. 3A. The integral membrane protein Ctr1 imports Cu(I), which is then loaded onto copper-dependent enzymes via chaperone proteins such as copper-chaperone for SOD1 (CCS) and MTs. The two copper-transporting ATPases are encoded by the genes *ATP7A* and *ATP7B*. Both ATPase7A and ATPase7B remove excess Cu from cells, control influx of copper into vesicles, whereas primarily ATPase7B delivers Cu for incorporation into Cu-dependent enzymes. In the gastrointestinal tract, copper is pumped out by ATPase7A and then transported to the liver bound to albumin or α2 macroglobulin or amino acids via the portal vein. In the liver, ATPase B incorporates copper into ceruloplasmin. The majority of the plasma copper is strongly bound to ceruloplasmin (called here cp-Cu), whereas the remainder is loosely bound and is exchanged among albumin, α-2-macroglobulin, amino oxidases, ferroxidase(II), SOD3, metallothioneins, and amino acids (called here noncp-Cu) [135,152,153].

In the central nervous system (CNS), astrocytes appear to play an important role in copper transport from the blood and cerebrospinal fluid (CSF) to the neurons [154,155], at the astrocyte foot (Fig. 3A). At this site ATPase7A and ATPase7B control brain copper balance, starting from the early phases of development [156]. ATPase7A is expressed before birth, primarily in the hippocampus and cerebellum [157], then its levels decline. The protein is involved in synaptogenesis and axonal outgrowth and contributes to seizure resistance in hippocampal CA2 pyramidal cells [157]. ATPase7A is expressed in the basolateral membrane. While in the enterocyte it controls copper absorption into the body, in the endothelial cell of the blood brain barrier (BBB) it facilitates copper transport from the blood to the brain [158] and modulates synapses of glutamatergic neurons [159]. Inactivation of ATPase7A in Menkes disease disturbs the transport of Cu into the brain, which leads to neurodegeneration [155,160]. ATPase7B is expressed at the apical side of endothelial cells of the blood cerebrospinal fluid barrier and facilitates copper transport into the CSF, as well as copper sequestration into the choroid plexus [156]. ATPase7B is expressed mainly in the hippocampus, in the cerebellum and brain cortex, and acts as a mediator of the synthesis of the Cu-dependent enzymes mentioned above [160].

Several other proteins locate at neuronal membranes and bind copper, most notably APP, α-synuclein (α-syn), and the prion protein (PrP), a glycoprotein located at synapse [161–163]. PrP is involved in copper uptake and efflux, and the metal facilitates PrPc internalization [164,165]. The interplay between copper and PrP appears to modulate glutamatergic transmission [166–169], and PrP also binds to Aβ [170]. Moreover, the binding of copper to the PrP increases its conversion to the infectious protease-resistant, β-sheet-rich form of the protein [171–173].

Much of the bioinorganic chemistry of neuronal copper occurs at the synaptic cleft, where Cu(II) is released from the postsynaptic terminal upon stimulation of the N-methyl-D-aspartate receptor (NMDAR) during glutamatergic neurotransmission (Fig. 3A). Cu (II) is presumably in a 'free' form when it is released at the synaptic cleft and modulates glutamate neurotransmission by down-regulating the NMDAR activity [156]. Copper is also released from presynaptic vesicles during transmission [174] in a recycling process regulated by PrP [159,167,168,175–178], which is a natural synaptic Cu(II) binding protein [164,166,171,172]. Due to the small volume of the synaptic cleft, Cu(II) can transiently reach micromolar concentrations and peak levels can be up to 100-fold higher [166].

While actual "free" exchangeable copper in the cytoplasm is virtually negligible at concentrations as low as 10^{-18} M [14,179,180], the distinction between loosely bound and strongly bound copper is important. The strongly bound pool of copper is characterized by a tight coordination geometry of non-solvent ligands as seen in active sites of copper proteins [137]. In the plasma this fraction is mainly bound to ceruloplasmin (cp-Cu). The remaining plasma Cu (noncp-Cu) is loosely bound to albumin, α-2-macroglobulin (transcuprein), and random peptides and amino acids and exchanged among them [136] and comes out from the liver; this type of copper crosses the BBB, is chelatable and engages in toxic Fenton-like reactions that produce very reactive radicals such as hydroxyl, which can contribute to oxidative stress in the aging neuron [14,179,181,182].

4. The critical K_d of CuAD

To differentiate the pathological and physiological Cu(II) pools, we use a threshold (or critical) dissociation constant K_d^c: Since K_d = [Cu][P]/[CuP] where P is the copper protein, [Cu] is the "free" copper concentration and [CuP] is the complex, a small K_d corresponds to strong binding, whereas a large K_d represents copper that is relatively exchangeable. The Cu(I) oxidation state binds generally much more strongly to proteins, reaching attomolar K_d ($\sim 10^{-18}$ M or lower) in high-affinity copper storage and transport proteins such as MTs and Ctr1[183]. Since the copper concentrations and the affinities of the copper proteins vary with redox state and location, the relevant K_d^c is context-dependent, and we thus focus on the K_d^c of Cu(II) at the synapse which reflects the discussed pathology. Evidence suggests that dyshomeostasis will spread from location to location, as described below for the impact of *ATP7B* variants on brain copper homeostasis [184–186]. The threshold K_d^c represents the affinity that separates, for the metal ion in the given location, the exchangeable solvent-accessible copper (all copper bound with $K_d > K_d^c$) from the strongly bound, inaccessible copper (all copper bound with $K_d < K_d^c$). K_d^c is a constant of the redox state and location, whereas concentrations of the two pools of copper can vary. We define the copper dyshomeostasis as the situation where the ratio $[Cu](K_d > K_d^c)/[Cu](K_d < K_d^c)$ increases.

As mentioned, the concentrations of copper vary by orders of magnitude dependent on redox state and location, and the amount of exchangeable copper varies according to the K_d of the local copper-proteins. In blood serum, the moderate-affinity copper-proteins albumin (10–15% of copper [187], mostly exchangeable, $K_d < 10^{-12}$ M [188]) and α-2-macroglobulin (5–15% of copper,

which exchanges with albumin, $K_d \sim 10^{-12}$ M [189]) and the high-affinity ceruloplasmin (40–70% of copper, not exchangeable, $K_d = 10^{-15}$ M) ensure very little exchangeable Cu(II). The threshold of exchangeable Cu(II) in this location is then arguably close to K_d of albumin ($K_d^c \sim 10^{-12}$ M).

However, at the pathologically relevant synaptic cleft we argue that $K_d^c \sim 10^{-9}$ M: First, average [Cu^{2+}] at the synapses (100–250 μM)[190] is ~100–1000-fold that of the CSF (0.3–0.5 μM) [191] and the extracellular space (~1 μM) [192] and the exchangeable [Cu^{2+}] vary by 100-fold during synaptic transmission, whereas it is kept small and constant in blood and CSF. Second, K_d is surprisingly similar, typically 0.1–10 nM, for the disordered proteins suspected to be involved in synaptic Cu(II) transport and regulation, all being main culprits of neurodegenerative diseases, i.e. Aβ, PrP, and α-synuclein [193,194]. We argue that this is not a coincidence but reflects the K_d^c at the synapses. Third, the amount of copper in Aβ plaques (~440 μM) [17] is similar to the average [Cu^{2+}] at the synapses (100–250 μM) [190] indicating that the K_d of Aβ is close to the threshold value for synaptic [Cu^{2+}]. Fourth, micromolar K_d values (10^{-6} M) in typical assays represent unspecific binding of labile metal ions such as Zn(II) with small binding free energy [14,193]. For Cu(II) at the same concentrations as Zn(II), a threshold K_d^c value 1000-fold smaller (~10^{-9} M) can be argued from K_d of complex formation to typical N-donor and O-donor ligands, i.e. the Irving-Williams series [195]. Fifth, we argue that the if ratio $[Cu](K_d > K_d^c)/[Cu](K_d < K_d^c)$ is increased in the blood as measured directly by noncp-Cu, this will also affect the ratio in other places. One example that we discuss further below is the analogy with failed copper incorporation into nascent ceruloplasmin upon defects of the *ATP7B* gene that cause Wilson's disease (WD), an autosomal recessive disease distinct from AD but with some biochemical features being similar to CuAD [54,160,196,197].

The K_d of the involved copper proteins may change by mutation or post-translational modification such as oxidations of sulfur ligands, and this will accordingly change the proposed pathogenic ratio $[Cu](K_d > K_d^c)/[Cu](K_d < K_d^c)$. Most notably, Aβ exists *in vivo* in various modified forms, with emphasis on N-terminal modified peptides of the type $A\beta_{4-x}$. Since these modifications affect the primary copper binding site, they also affect the K_d of copper and can reach much higher affinity than the full peptide and thus be functionally important [198–203].

The definitions above have centered on the thermodynamic binding equilibria of Cu(II). Cu(II) binding may be under kinetic control if release of copper becomes rate-limiting for equilibrating the copper pools described above. The lability is the appropriate term if the ligand-metal interaction is kinetically controlled, measured as the life time of the ligand's association with the metal ion or the rate constant of ligand substitution [204]. The thermodynamic (K_d) and kinetic (lifetime) definitions of pathological metal ions thus potentially differ, as indicated by some recent studies [205]. For high-affinity copper sites in proteins, kinetic control of copper pools may arise from barriers to release copper depending on the steric and electronic nature of the ligands. However, for the low-affinity synaptic proteins with $K_d \sim 10^{-10}$ M–10^{-9} M, exchange is very fast. NMR relaxation recently showed that the pathogenic A2V mutation of Aβ exchanges Cu(II) much faster than the protective A2T and wild type Aβ, despite having similar K_d values [206], suggesting a role of kinetics over thermodynamics in Cu(II) buffering. Rapid changes in [Cu^{2+}] at the synapses can produce other barriers than induce kinetic control despite fast metal-ligand dissociation, for example due to slow vesicle copper release or membrane transport at the synapses. Under such conditions, low affinity proteins such as the synaptic Aβ, α-syn and PrP, will be much more adequate in restoring the copper pools, as copper is rapidly released from these sites on the timescale of synaptic transmission, supporting of a relatively high K_d^c at the synapses.

5. Clinical evidence: copper dyshomeostasis in AD

Metal ion imbalances are a consistent feature of AD [15,33,34,37,50,104,182]. Several meta-analyses focusing on copper have been performed during the last 10 years merging data from more than 35 studies [207–211]; the most updated studies are shown in Table 1. The meta-analysis of copper in the brain shows that AD patients have lower levels of copper in their brains than healthy controls [185]. Meta-analyses monitoring the serum/-plasma show increased copper in AD patients vs. controls [207,210–213]. This latter increase can be explained by the fact that the chelatable portion of copper (noncp-Cu) is increased in AD patients [209], in analogy with WD. A recent study comparing directly WD and AD patients showed that while the two diseases differ in terms of serum copper and ceruloplasmin levels, they share increased levels of noncp-Cu, indicative of a failure a copper control [197]. Expansion of the weakly bound Cu(II) in AD coincides with decreased levels of functional protein-bound Cu(II) in the brain [208], as has been found in autopsy studies [214]. Analysis of copper in cerebral cortex Brodmann areas 22 and 46 showed reduced copper in AD patients, associated with 20% increase of labile copper in the same Brodmann areas [214], as also confirmed in other studies [214,215]. The results for copper differ from those for many other metal ions, including Zn, implying their possible antagonistic relationship [216].

While there is some disagreement on the ceruloplasmin concentration levels in AD [217], data on ceruloplasmin activity, which importantly reflect fully constituted copper-bound protein, are univocal, and largely similar for all the reported studies [151,191,217–220]. Ceruloplasmin is a 132 kDa multicopper oxidase that acts as an acute phase reactant scavenging oxygen-derived free radicals during inflammation states [54,187,221,222]. Ceruloplasmin is mainly synthesized in hepatocyte, where the ATPase 7B protein loads six copper atoms into apo-ceruloplasmin, generating an active holo-ceruloplasmin that is then transferred into blood circulation. It is also expressed in the kidney, mammary gland, placenta, and choroid plexus in the brain [187]. A ceruloplasmin isoform linked to the cell membrane by glycophosphatidyl inositol (GPI) is expressed endogenously by the CNS and is abundant in astrocytes in the vicinity of neurons [223]. A key process catalyzed by ceruloplasmin is the oxidation of Fe^{2+} to Fe^{3+}, which reduces the availability of Fe^{2+}, promoting instead iron transport to various organs by transferrin, which accepts only Fe^{3+} [224]. Ceruloplasmin also scavenges H_2O_2 thus preventing the production of dangerous oxygen species [225]. Holo-ceruloplasmin synthesis strictly depends on the amount of available copper in the liver [224] and on ATPase7B function [135,187]. Consequently, reduced copper availability or ATPase7B protein dysfunction increases the fraction of apo-ceruloplasmin, which has no ferroxidase capability and is rapidly degraded in plasma [135,187].

The specific activity of ceruloplasmin decreases in AD and higher values in serum were shown to reduce risk of the disease [217]. The holo-form of ceruloplasmin in CSF [191], along with ceruloplasmin activity in serum [217,220] are decreased in AD patients. Low-molecular-weight fragments of ceruloplasmin derived from apo-ceruloplasmin degradation have been detected in AD samples and are suggestive of a defective activity of the ATPase7B copper pump [226]. Same mechanisms can be applied to the brain, positing a defective loading of the metal into other copper proteins and enzyme. Supporting this notion, AD risk variants of *ATP7B* (rs732774 and rs1061472) have been found to cause abnormalities in the expression [227] and function of the ATPase7B [57]. As a whole this evidence suggests a disturbed activity of ATPase7B in loading the metal in copper proteins and enzymes in AD at liver but also at brain level.

Table 1
List of the most updated meta-analyses of copper in Alzheimer's disease, in developmental disorders, and epidemiological studies of copper in cognition.[a]

Most updated meta-analyses copper in Alzheimer's disease

Reference (Author, year)	Cu biomarker	Tissues sample sources	Studies analyzed	Number of AD Cases	Number of controls	Trend	p value
Li et al. 2017 [211]	Cu	Serum	35	2128	2889	Increase	<0.0001
Squitti et al. 2014 [209]	nCp-Cu	Serum/plasma	10	599	867	Increase	<0.001
Bucossi et al. 2011 [207]	Cu	Serum/CSF	5	116	129	No diff in CSF, increase in serum	
Schrag et al. 2011 [208]	Cu	Brain	7	115	123	Decrease	=0.0003

Meta-analyses of copper in neurodevelopmental disorders

Reference (Author, year)	Condition	Tissues sample sources	Studies analyzed	Number of Cases analyzed	Number of controls	Results
Saghazadeh et al. 2018 [409]	Febrile seizures	Serum/plasma	4	99	92	Cu increases in patients
Saghazadeh et al. 2015 [409]	Epilepsy	Serum/plasma	10	934	899	No changes in Cu

Epidemiological studies of copper and cognition

Reference (Author, year)	Type of analysis	Number of Cases analyzed	Results	p value
Morris et al. 2006 [234]	Longitudinal, 9 years follow-up	3718 (CHAP study)	Among persons whose diets were high in saturated and trans fats, higher copper intake was associated with a faster rate of cognitive decline (participants in the highest fifth of cu intake combined with a diet high in saturated fats lost cognition three times faster than expected)	<0.01
Lam et al. 2008 [235]	Cross-sectional, and cores on 11 of 12 cognitive function tests Plasma analysis	1551 (Rancho Bernardo Study)	In women copper concentrations had inverse linear associations with Buschke total, long and short-term recall and Blessed scores (cognitive test).	<0.001
Shen et al. 2014 [233]	Copper concentration in soil in relation to annual mortality of AD	Chinese general population, from 1991 to 2000, in 26 provinces, 3 districts	relative risk of mortality in the regions with the highest copper concentrations (60–80 mg/kg) was high [OR: 2.63 (95% CI: 2.626–2.642)]	<0.001
Zhou et al. 2016 [410]		826 children	Significant association between high copper levels and poorer working memory in boys (unstandardized regression coefficient (b) = −0.099, robust standard error (s.e.) = 0.009, t = −10.99, p = 0.008, 95% CI (−0.138, −0.060)	
Kicinski et al. 2015 [237]			A two-fold increase in blood Cu was associated with a 0.37 standard deviation decrease in sustained attention (95% CI: −0.67 to −0.07, p = 0.02) and 0.39 standard deviation decrease in short-term memory (95% CI: −0.70 to −0.07, p = 0.02)	
Pujol et al. 2016 [387]		2836 children aged from 8 to 12 years.	The results establish an association between environmental copper exposure in children and alterations of basal ganglia structure and function	P < 0.001
Takeuchi et al. 2019 [238]	gray matter volume (rGMV), mean diffusivity (MD), and cognitive performance	924 healthy young adults	Cu in hair associates with aberrant cognitive functions, greater rGMV in extensive areas, greater MD (which are indicative of low density in brain tissues) in subcortical structures in the healthy young adults	P < 0.0001 for rGMVs
Alemany et al. 2017 [411]		1645 scholar children from the BREATHE project	*ATP7B* rs1061472 (P for interaction 0.016) and *ATP7B*-rs1801243 (P for interaction 0.003) polymorphisms modified the association between indoor copper exposure and inattentiveness	

[a] Risk is reported as OR = Odds ratio; CI = Confidence Intervals.

Elevated levels of noncp-Cu in serum increase the risk of AD by approximately three-fold [228,229] and contribute to a worse prognosis of the patients [230]. Some AD patients display increased levels of noncp-Cu correlating with altered patterns of electrical brain activity and brain atrophy, along with pathological levels of Aβ and tau-protein in the CSF, and more severe clinical outcome [55]. In one study, subjects with mild cognitive impairment (MCI) with elevated noncp-Cu levels have a 3-fold increased probability of AD and faster conversion to AD [212]. Total copper and the ratio of copper to non-heme iron distinguished progressive MCI subjects from cognitively stable MCI subjects in a five-year follow-up study [37]. Other cross-sectional studies also demonstrated that noncp-Cu is higher in MCI than in healthy controls [219,231,232]. Large population studies suggest that copper imbalances occur at early stages of cognitive loss and AD development, and copper in the soil has been associated with increased mortality relating to AD (Table 1) [233].

Aside from AD, other studies have related copper levels to cognitive performance: A high copper diet combined with high levels of trans- and saturated fatty acids associated with a 3-fold increase in loss of cognition (Table 1, [234]). High copper plasma concentrations have been associated with loss of cognitive performance in normal women (Table 1) [235], and a poorer working memory and attention in children [236] and adolescents [237] (Table 1). A recent study found that copper in hair correlates with low cognitive abilities, high regional gray matter volume over widespread areas of the brain, and larger indices of low density in brain tissues (Table 1) [238]. As a whole, these studies indicate that elevated noncp-Cu is associated with loss of cognitive performance. These indications are further supported by a study investigating a neuropsychological battery of 19 tests in a cohort of healthy elderly women [239].

Several studies indicate that noncp-Cu may serve as a biomarker for specific sub-group of AD [240]. AD patients stratified on the basis of a noncp-Cu cut-off [219] have different clinical [241,242] and genetic features that relate to the frequency of specific variants of the *ATP7B* gene [243]. This evidence supports the existence of a detectable metabolic subtype of AD associated to copper abnormalities AD [240], called here CuAD. The AD risk variants of *ATP7B* (rs732774 and rs1061472) have been found to cause abnormalities in the expression [227] and function of ATPase 7B [57].

Many *in vitro* or *in vivo* studies have increased our knowledge about the likely causative mechanisms of copper dysregulation in AD [39,40,49]. *In vivo* studies in animal models demonstrated that small amounts (0.13 ppm) of Cu ingested through drinking water double plasma concentrations of noncp-Cu, reduce CSF Aβ clearance via the BBB in wild type mice, and increase Aβ production in a mouse model of AD [244].

It is known from many studies that cholesterol can enhance Aβ pathology in models of disease [245–248]. However, it was found that this tendency varied depending on the quality of water given to the animals, such that distilled water gave less pathology compared to tap water [249]. Strong evidence for a direct link between copper homeostasis and Aβ-related AD pathology came from the report that the reason is probably that trace amounts of Cu(II) (0.12 ppm) in water induce Aβ accumulation and senile plaque-like structures in the hippocampus and temporal lobe of cholesterol-fed rabbits, with associated retardation of learning ability [250]. These findings were later confirmed in another study with controls against other metal ions, Zn and Al, with no such effect [251]. These findings fit well with *in vitro* Aβ aggregation seen in many studies being caused by trace metals in contaminated laboratory glassware [252].

A further indication of Cu dyshomeostasis in AD comes from the fact that MT expression changes in AD [253,254]. These small cysteine-rich proteins naturally bind and buffer the concentrations of copper and zinc [145,179,255]. The MTI and MTII isoforms are found in almost all organs, whereas MTIII is predominantly expressed in the CNS. MTIII possesses a unique neuronal growth inhibitory activity distinct from the MTI and MTII isoforms [145,256]. MTI/MTII biosynthesis is induced by a range of factors including glucocorticoids, cytokines, reactive oxygen species, and metal ions. Expression levels of the three isoforms differ in AD: MTI and MTII expression is increased in AD [256], stimulated probably by the presence of free radicals, metal ions, and cytokines [256–258], whereas MTIII levels are decreased, notably in the temporal cortex [259]. MTIII shows extracellular growth inhibitory activity that antagonizes the ability of AD brain extract to stimulate survival and neuritic sprouting [260]. By a not fully understood mechanism, the extracellular Zn_7MTIII can protect neurons from toxic Aβ, through an interaction of free Cu(II) and Cu(II) bound to Aβ [257,261]. Accordingly, MTIII, but not MTI and MTII, have been reported to protect neuronal cells from Cu(II)-amyloid toxicity [257,258]. Similar mechanisms have been observed also for α-Syn and PrPSc proteins [258]: *in vitro* studies have demonstrated interaction of Zn_7-MTIII and Cu(II)-containing α-Syn and PrPSc indicating that Zn_7MTIII can be neuroprotective via copper transfer [257].

6. Biochemical evidence: APP/Aβ and copper transport

As expected from their major presence, APP and the APP-like proteins APLP1 and APLP2, serve natural roles in neurons. APP-knockout reduces synaptic proliferation [262]. Knockout of both APP and APLP2 simultaneously is lethal and indicates an important role of APP/APLP2 [263,264] with a partial ability of mutual compensation [265]. The proteins are particularly important for synaptic function and affects synaptic plasticity [266–269] and are essential for hippocampus function and spatial learning of mice [270].

Aβ also seems to have normal functions in neurons [271–274]. Nanomolar concentrations tend to be neurotrophic, whereas micromolar concentrations as seen in cell assays are toxic [273,275]. The existence of a therapeutic window of Aβ may explain observations of both loss- and gain of function in AD, depending on study conditions [273,276,277]. Inhibition or depletion of $A\beta_{40}$ by secretase inhibitors or antibodies is lethal to neurons [278]. Aβ depletion has also been shown to impair neuronal activity in mice [271]. More specifically, Aβ monomers seem to protect against copper- and iron-induced toxicity [272] and extracellular Aβ increases vesicle release probability in hippocampal synapses [274]. Together with the findings for APP, these findings point to an important role of APP/Aβ at the synapses.

Most of the proteins central to AD, including APP, Aβ, α-secretase, and the matrix metalloproteases that mainly degrade Aβ, have copper or zinc sites [14,279]. APP has multiple established metal binding sites [182,280–282] (Fig. 2A, 2B, and 2D). The large extracellular part of APP contains the copper-binding domain (CuBD) [141,144,283]. A Zn(II) site is located within residues 181–200 of APP [284]. In addition, several other Cu(II) and Zn(II) sites are present in the E2 domain of APP-like proteins [139,285].

APP can reduce bound Cu(II) to Cu(I) [286], suggesting that APP may aid in transporting Cu(I) across the membrane similar to other proteins that enable Ctr1 Cu(I) transport, which requires a copper (II) reductase to bind Cu(I); in analogy to this function of APP, Ctr1 is cleaved from the membrane upon high concentration of copper to prevent uptake of copper [287]. Noteworthy, APP ablation in APP knock-out mouse model facilitates massive copper increases in the liver (80%) and in the brain (40%) [288]. This perturbation of copper efflux linked to APP ablation can be associated

with an impairment of copper incorporation into copper proteins and the consequent increase of noncp-Cu that can reach the brain (up to 40%) [288] Previous data indicate that 3% of serum noncp-Cu crosses the BBB in living AD patients [289], and consistent findings indicate a direct interaction of copper with Aβ in the CNS [244,250,290].

Further evidencing a function of APP in copper transport, APP knockout in cultured neurons leads to reduced sensitivity to copper toxicity [291]. In addition, overexpression of APP can both lead to increased Cu(II) reduction and uptake in cells [292]; however, overexpression can also cause intracellular copper deficiency in mouse brains [293], indicating that APP, either directly or by its cleavage product Aβ, also contributes to copper export. Other studies have shown that copper export caused by APP can be reduced by copper-chelators such as clioquinol [294]. The specific copper-binding domain of APP reduces toxicity from copper exposure [295], indicating a sensing-transport function to maintain intracellular copper concentrations at adequate levels. Inhibition of copper transporters increase Aβ and decrease expression of Aβ-degrading zinc proteases [296].

Interestingly, this copper buffering function is regulated by calcium levels: APP cleavage is regulated by increases in calcium concentration [297]. Secreted APP fragments can protect the neurons against excitotoxicity and reduce intracellular calcium [298]. In contrast to its plausible role in reducing Cu(II), C-terminal APP instead increases intracellular calcium levels which causes glutamate-based excitotoxicity [299]. Thus the buffering effect of APP/Aβ on copper and calcium seems to be antagonistic. Copper exposure to aged mice leads to Aβ accumulation and down-regulation of low-density lipoprotein receptor-related protein 1 (LRP1) [244], which is a transporter of Aβ [300] that also controls APP trafficking and processing [301,302]. However, dietary copper can also reduce Aβ production [303], and copper deficiency directly changes APP processing and Aβ secretion [304].

Aβ is a genuine metal-binding peptide with a typical N-terminal metal-binding sequence including three histidines (His-6, His-13, and His-14 using Aβ numbering [59,305]. Only two of them (His-6 and either His-13 or His-14) are thought to bind Cu(II) at any instance, and several ligand environments of the predominantly square-planar complex exists depending on pH [59,113,305–307]. At low pH (~6), only one histidine may bind Cu(II) probably due to protonation of the other [308]. Metal ions such as Cu(II) and Zn(II) mostly form 1:1 complexes with Aβ [282,309–311]. The K_d for copper depends on the conditions of measurement but is approximately 10^{-10}–10^{-7} M [56,113,138,203,206,280,306,312–314], about 2–3 orders of magnitude more strongly binding than Zn(II), in accordance with the Irving-Williams series of metal ion binding [14,195].

Metal binding generally changes the aggregation tendency of Aβ, in some cases favoring fibrillar aggregates and in other cases oligomers [14,49,310,315–319]. The metal-rich fibrils resemble the senile plaques characteristic of AD [123,206]; however the Cu(II) bound states resemble early oligomers before aggregation, as estimated from thioflavin T assays [206,307]. The fibrils making up the senile plaques consist mainly of such extended sheets, packed with metal ions [17,123,320]. The coil-helix transition in low-dielectric environments (Fig. 1A–1C) provides a structural rationale for its membrane channel function [116,128,321,322]. Aβ is known to form metal ion channels in membranes [116,117,128,130,323–325]. Such interaction involves conformational transition from disordered to helix, as favored upon Cu(II) binding [326] and facilitated by membrane-like micelle environments [122]. In combination, these various facts provide the structural-chemical basis for the suggested function of Aβ as a naturally disordered Cu(II) transport peptide [60], in agreement with the biochemical evidence of a copper-APP/Aβ relationship cited above. Metal binding most likely increases the ability of the peptide to associate with membranes, since the hydrophilic charged N-terminal becomes helical and less charged upon metal binding (from −3 to −1 when binding a divalent metal ion) [327].

As mentioned, Aβ is toxic and aggregation-prone at micromolar concentrations in typical assays [328,329]. However, this concentration corresponds to typically one year of total production and is 1000-fold higher than the normal sub-nanomolar concentrations (10^{-10} M) of Aβ [330,331]. Thus, the threshold concentration range separating beneficial and harmful Aβ is 10^{-7}–10^{-9} M. Considering that Aβ typically forms 1:1 complexes with a K_d of Cu(II) of 10^{-10}–10^{-7} M, 2–3 orders of magnitude more strongly than Zn(II), one can expect synaptic Aβ to buffer the Cu/Zn ratio in the synapses at 1/1000–1/100 approximately; this ratio is arguably important upon Zn and Cu release during glutamatergic neurotransmission.

β-secretase (BACE1) initiates production of Aβ by cleaving its N-terminal from APP [332]. The transmembrane region of BACE1 contains a highly conserved copper binding site [333–335]. Overexpression of BACE leads to reduced SOD1 activity due to loss of intracellular protein-bound copper [334,335]. Similarly, copper exposure leads to increased APP expression and Aβ formation by action of a copper sensing protein (CUTA) that interacts with the β-cleavage site of APP [336]. Recent work suggests that BACE1 functions via trimer formation upon cystine bridge formation to compartmentalize copper within the cell [337].

Further support for an *in vivo* relationship between APP/Aβ and copper comes from chelation studies. A variety of metal chelators, typically large complex phenolic N- and O-donor systems such as clioquinol [338], coumarin [339] and benzylideneindanone [340] derivatives, and myricetin [341] reduce amyloid plaque formation [342], down-regulate APP expression and lower Aβ production with associated loss of intracellular loosely bound copper [343]. Strong metal chelators such as EDTA (estimated K_d ~10^{-19} M from $\log K_f = 18.8$) reduce γ-secretase activity and thereby, production of Aβ [344]. Metal chelators reduce Aβ levels in cultured cells [345,346] and in *Drosophila* expressing human Aβ [347], and at the same time such chelators can improve the cognition of mice [348].

7. Molecular mechanisms of copper toxicity in CuAD

The mechanisms relating copper translocation in compartments prone to the disease are both loss of function (loss of bound copper in enzymes involved in anti-oxidant stress and energy production) and gain of function (induced protein aggregation, toxic redox activity of Cu-complexes), and both processes eventually contribute to neuronal death [60,277]. Even though the detailed mechanisms of these complex processes are still elusive, the many data reviewed in this work suggest that they act in concert in some AD patients.

Recent work shows that noncp-Cu has 95% specificity in detecting AD patients affected by CuAD [219], characterized by higher frequency of rs1061472 and rs732774 of the *ATP7B* gene, associated with susceptibility to AD due to copper exposure [243]. These gene variants are functional single nucleotide polymorphisms that modify expression and properties of ATPase7B [227]. ATPase7B loads copper onto nascent ceruloplasmin in the hepatocyte and onto the glycophosphatidyl inositol (GPI)-ceruloplasmin expressed in the astrocyte, and slight defects in this process associated with *ATP7B* rs1061472 and rs732774 may explain the increased release of noncp-Cu in the blood [209] and decreases in ceruloplasmin expression and activity in both blood and CSF [135,187,191,217] and probably alterations in the expression of additional copper proteins of the brain [156].

Focusing first on the gain of toxic functions, when the pool of exchangeable copper increases in blood (as noncp-Cu) and in the brain, it may change the conformations of many intrinsically disordered proteins, as widely documented by Cu(II) binding leading to oligomer seeding and aggregation of Aβ [102,133,206,252,307,319,349,350]. Cu(II) may also engage in Fenton-like chemistry after reduction to Cu(I) in the reducing cell [14,351,352]. Overload of Cu(II)-Aβ molecules is also probable, particularly at the synapses where both are available at much higher than normal concentrations [53,159,274,353]. These complexes by themselves also have both redox-toxic and potentially beneficial effects [181,199,206,314,354] that are so far not well understood in context, and separating the negative and positive effects of Cu(II)-Aβ (and other metal-Aβ complexes) is of major importance [60,277]. Efforts to modulate the aggregation tendency and redox toxicity of both amyloids alone and Cu(II)-Aβ complexes are accordingly of major current interest [74,75,355–359].

One underlying systemic variable of neurons that could potentially unify the risk factors of metal ion and protein dyshomeostasis is thus energy. The brain uses half of its energy for glutamatergic signaling and perhaps 30% of the remaining energy for protein synthesis and degradation. Cognition and memory are defined by energy requirements unique to the brain and are much more expensive in humans than rodents, ranging from 34% in rodents to 74% in humans [360,361]. The energy production decreases and the energy cost of maintenance grow monotonously with aging, providing a direct rationale for the age trigger of disease [362–364], with energetic abnormalities and energy efficiency (diabetes risk factors) being hallmarks of AD [365–367].

The catalytic reduction of molecular oxygen (O_2) to water (H_2O) by CCO generates the electrical gradient used by the mitochondria to create ATP, and mitochondrial dysfunction and associated quality control, which occurs upon impaired energy production, is a hallmark of AD and other neurodegenerative diseases [363,368–370]. If copper is lost from the bound functional pools, mainly from CCO, this energy production will be impaired, such that energy surplus for cognition may ultimately be affected. At the same time, noncp-Cu can increase stress of misfolded proteins that in turn can increase the cost of turnover [371,372]. Aβ's role in this vicious circle may possibly be to restrain and buffer the increased levels of oxidative stress and the disruption of the ionic gradients due to copper dysfunction (Fig. 3B).

8. Analogies to other Cu-related diseases

WD is the paradigmatic disease of noncp-Cu overload in tissues and organs caused by failure of copper incorporation into ceruloplasmin in the hepatocyte [196,240]. Upon *ATP7B* mutations noncp-Cu increases in the liver, brain, and the cornea of the eye (Kayser-Fleischer rings), producing oxidative stress and tissue damage [135,373]. As a consequence WD has a broad spectrum of symptom presentation. About one third of cases present with liver disease, another third with neurological manifestations, and the last third involves psychiatric and behavioral abnormalities [196,374–380]. In WD brains, the labile component of Cu is increased and deposition occurs particularly in the basal ganglia, associated with gliosis, neuronal loss [381] and features that resemble Parkinson's disease [382].

Mass screening in Korea suggests that WD may be much more common than previously estimated and reach 1:3000–1:1500 cases [377,383]. In line with this, a recent study reported an unexpected rate of about 1 in 40, higher than the 1:90 previously reported, of *ATP7B* heterozygote mutation carriers in UK, yielding a prevalence of 1 per 7000 inhabitants [377,384]. The large discrepancy between the estimated genetic prevalence of WD based on *ATP7B* mutation frequency and the number of clinically diagnosed WD patients, which is significantly lower, motivates the hypothesis that *ATP7B* mutations may increase the likelihood of other neurological or mental disorders, e.g. AD. In line with this hypothesis, some *ATP7B* mutations have been identified in AD patients [385]. Multiple *ATP7B* variants modify copper homeostasis and the risk of the disease, and at least in a percentage of patients, specific loci of the *ATP7B* (primarily K952 and R832) contribute to the global susceptibility of developing AD (CuAD) [386]. *ATP7B* Genetics appears to drive a large part of the association between copper abnormalities and brain development and function. The BREATHE project highlights an interaction between specific *ATP7B* variants (including *ATP7B* R832) and alterations of basal ganglia structure and its function in the brain [387] (Table 1). Multiple *ATP7B* gene variants apparently interplay with diet or life style to modify susceptibility and disease phenotype, and could thus provide correlations to nutrition during aging, when the copper requirements are reduced relative to other phases of life [388].

9. Combining the facts into a simple model of AD due to copper dyshomeostasis

The many studies of the role of copper at the synapse suggest that APP can play a role in reducing Cu(II) to Cu(I) to enable Ctr1 function, and that ATP7A/B can facilitate copper translocation into vesicles, which can then be released at the synaptic cleft as part of the glutamatergic transmission that occurs also in the hippocampus at the pyramidal CA1 synapse [159,167,174] (Fig. 3A). In this model PrP and APP can serve as buffer proteins that control [Cu(II)] within the synaptic space, where it can reach micromolar concentrations and is subject to major variations [166,389]. The threshold binding strength of these Cu(II) ions at the synapse is of the order of 10^{-9} M, which is, incidentally, also the typical neuronal concentration of Aβ, which also tends to localize at synapses [353,390]. Aβ binds copper preferably with 1:1 stoichiometry [199,203,305,391] and interacts with the membranes to form calcium channels [115,116,128,324].

The evidence summarized above strongly suggest that copper dyshomeostasis can interfere with this basic synaptic process in at least a subset of AD patients. We define this dyshomeostasis as a change from strongly bound synaptic copper pools ($K_d < 10^{-9}$ M) to loosely bound pools ($K_d > 10^{-9}$ M) over time, with a correlated excess noncp-Cu in the blood. The resulting change in the proposed pathogenic ratio $[Cu](K_d > K_d^c)/[Cu](K_d < K_d^c)$ would make it more difficult for the copper buffer proteins to restore copper pools. A concomitant loss of functional bound copper in copper proteins will impair SOD1 activity and ATP synthesis by CCO, causing energy depletion and oxidative stress (Fig. 3B). The disruption of copper homeostasis at the synapse can affect the synaptic strength: Application of physiologically relevant concentrations of 1 μM $CuCl_2$ in rat hippocampal slices inhibited long-term potentiation, which is one of the basic processes of learning and memory [392]. Similar pathology is observed in rats fed with a high copper diet [393] or injected with copper [394].

A previous model of copper involvement in AD [240] held that the neurovascular unit has a primary role in regulating the transport of copper from the blood to the interstitial space in the brain. The concentration of Cu in the interstitial space is primarily controlled by the astrocytes and the other cells of the neurovascular unit [155]. ATPase7B and ATPase7A and Ctr1 have primary control over Cu levels (Fig. 3A). Specific variants in the *ATP7B* gene (e.g. rs1061472 and rs732774, but additional variants may exist) affect the expression and function of the ATPase7B pump [57,136,227] (Fig. 3B). Overtime, *ATP7B* dysfunction may increase noncp-Cu in serum plasma and reduce the copper loaded into copper proteins

(including transporters) and enzymes, as exemplified by reduced ceruloplasmin specific activity [135,187,191,217]; in the brain these processes can correspond to copper depletion and probably a general loss of function of copper proteins including ATPase7B, CCO, SOD1, DBH, and MAO [156]. In particular, DBH catalyzes the formation of noradrenaline from dopamine. Midbrain catecholamine diffuse neurotransmitter systems have broad effects that affect the entire cerebral cortex. Apart from the disturbed equilibrium of the neurotransmitters (dopamine, noradrenaline, serotonin) which almost certainly would affect brain function, increased levels of dopamine may contribute to oxidative stress via autoxidation and MAO activity, generating H_2O_2 that can directly react with noncp-Cu in Fenton-type reactions producing the extremely reactive hydroxyl radical (OH**.**) [14,395].

The many data indicating that the natural APP/Aβ function relates to copper (and possibly zinc) transport at the synapses put the amyloid hypothesis in close and logic relationship to the model described above. Since the APP/Aβ system aids copper transport at the synapses, the disruption of Aβ levels by PSEN or APP mutations would contribute to a disturbance of the copper homeostasis, as indicated in Fig. 3B [60,277,396]. In this model, APP and PSEN mutations, the main genetic risk factors of familial early-onset AD, cause disease by a partial loss of function that manifests at high age when the overall copper homeostasis is already stressed. We also note that mutations in SOD1 cause Amyotrophic lateral sclerosis [397–399], a severe motor neuron disease which we speculate, because of its many clinical similarities to AD, share some of the pathways shown in Fig. 3B, notably those relating to copper-constituted functional SOD1.

10. Conclusions

From the evidence collected it is clear that copper dysfunction pertains to a percentage of AD patients, a condition we call here CuAD. This copper dysfunction can explain a fraction of the global susceptibility for AD. In addition to the pathological and clinical evidence, there is also substantial biochemical support for a strong link between copper status and APP/Aβ, with current evidence pointing toward a function in copper transport across neuronal membranes, either directly or indirectly, as a support of Ctr1. Thus the main hallmark of AD, Aβ, is a proficient copper binding peptide with a very broad range of data now documenting how copper binds and modifies Aβ structure, aggregation, and toxicity.

For the subset of AD associated to copper dysfunction, causes relate to four processes that can be summarized as follows: i) increased frequency of specific *ATP7B* variants, ii) increased levels of noncp-Cu, and iii) decreased specific activity of ceruloplasmin; iv) decreased protein-bound copper into the brain, such as ceruloplasmin, ATPase7B, MT, CCO, SOD1, and DBH [400–403]. These processes can influence each other but standing alone they can still increase the risk of the disease. They can trigger a cascade of processes that involve neurotransmitter alteration, oxidative stress, and disruption of energy production, eventually leading to cell death. Large population studies and meta-analyses support the proposed pathways shown in Fig. 3.

We suggest that the transition from bound functional copper to "free" chelatable noncp-Cu is defined by a location-dependent threshold binding constant for pathogenicity which is approximately $K_d^c \sim 10^{-12}$ M in blood and $K_d^c \sim 10^{-9}$ M in synapses, where stronger binding represents innocent functional copper and weaker binding represents, at least to some extent, the redox-active gain-of toxic function. A large reduction potential of the Cu(II) concomitant with access to reductants such as ascorbate produces the Cu(I) state that may undergo Fenton-like reactions [404]; it is quite interesting that Cu(II)-Aβ, which is also a weakly bound copper-system, can do the same [202,405]. At oxidative conditions hydroxyl radicals are not formed to a great extent [352] but in the reducing environment of neurons they are likely to do so, as well as reacting with sulfur groups of proteins [404]. This threshold represents the shift form specific (controlled and contained) to nonspecific binding in physical chemical binding assays [14,193]. The K_d of Aβ for copper is 10^{-9} M which is strong enough to bind and sequester the "free" copper but weak enough to release it after transport. Copper binding to Aβ changes its conformation and increases its hydrophobicity, probably thus enabling interaction with membranes. Mutations in PSEN1 and PSEN2 that cause FAD change the production of Aβ peptides which may affect the specific transport capacity of copper-Aβ across the neuronal membrane [60].

Finally, we would like to emphasize that Professor Brewer, based on epidemiological and demographic data that are completely orthogonal to the chemical, genetic and clinical data presented here, has put forward a theory that a subset of sporadic AD can be caused by increased copper exposure. He reaches this suggestion based on data from developed and industrialized countries with and without copper piping in a historical context and from toxicological studies of the Aβ pathology induced by normal copper exposure in drinking water [406–408]. Given that the biochemical mechanisms would be similar to those described here and thus strongly documented, we suggest that this possible contribution to sporadic AD is explored further.

Conflicts of interest

KPK declares that he has no commercial or noncommercial conflicts of interest relating to this work. RS is Chief Scientific Officer of IGEA Pharma N.V. and has some shares in Canox4drug and IGEA Pharma N.V., but does not receive monetary compensation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ccr.2019.06.018.

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CellPress REVIEWS

Review

Copper and Zinc Dysregulation in Alzheimer's Disease

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Alzheimer's disease (AD) is one of the most common forms of dementia. Despite a wealth of knowledge on the molecular mechanisms involved in AD, current treatments have mainly focused on targeting amyloid β (Aβ) production, but have failed to show significant effects and efficacy. Therefore, a critical reconsideration of the multifactorial nature of the disease is needed. AD is a complex multifactorial disorder in which, along with Aβ and tau, the convergence of polygenic, epigenetic, environmental, vascular, and metabolic factors increases the global susceptibility to the disease and shapes its course. One of the cofactors converging on AD is the dysregulation of brain metals. In this review, we focus on the role of AD-related neurodegeneration and cognitive decline triggered by the imbalance of two endogenous metals: copper and zinc.

Highlights

Alzheimer's disease (AD) is a widespread neurodegenerative condition that affects approximately 50 million people worldwide. Costs for disease management are predicted to reach US$1 trillion in 2018 and are expected to double by the year 2030.

Around the world, one new AD case is diagnosed every 3 s. No disease-modifying strategies are available. The AD epidemic calls for a full investigation of novel disease mechanisms and targets.

The current AD drug development pipeline has centered around the amyloid cascade hypothesis. The approach has not paid off, and it is now time to recognize the multifactorial nature of the disease and expand the spectrum of intervention. A more effective approach should also consider the possibility of patient stratification in different subtypes and targeted interventions.

The dysregulation of metal ions in the brain is emerging as a comorbidity factor in subsets of patients with AD. Dysregulation of copper (Cu) is found in a subgroup of patients with AD and negatively shapes the course of the disease.

Brain zinc participates in AD pathogenesis by affecting amyloid metabolism, but the metal can also positively modulate synaptic functioning and neurotrophic signaling, as well as help to restore copper homeostasis.

The Changing Landscape of Alzheimer's Disease

AD is one of the most common forms of dementia. Despite a wealth of knowledge on the disease mechanisms, no disease-modifying drugs are available, and AD treatment is limited to symptomatic intervention. The overall failure of the therapeutic attempts tested so far suggests that a critical reappraisal of the AD paradigm is required.

The leading hypothesis on AD pathogenesis has been framed within the 'amyloid cascade' construct [1]. According to the model, the disease is set in motion by the damaging interaction of **Aβ peptides** (see Glossary) with hyperphosphorylated **tau proteins** (p-tau). The cascade has been the target of many therapeutic attempts, but this amyloid-centered approach has mostly failed so far [2], thereby prompting a critical reconsideration of the role of amyloid in AD [3,4]. Recent findings have shown that signs of brain amyloidosis, although necessary, are not sufficient to promote sporadic AD [5]. Aβ deposition, assessed with positron emission tomography (PET) and amyloid-sensitive radioligands (e.g., florbetapir, florbetaben, flutemetamol, and the Pittsburgh compound B), is a useful marker for predicting the clinical course of the autosomal-dominant AD forms, which account for ∼5% of AD cases. However, amyloid-PET is inconclusive in sporadic AD [6] because 20–40% of cognitively competent older individuals exhibit abnormal amyloid deposition [5,7]. Furthermore, many cognitively normal subjects are found to show postmortem signs of amyloid burden that fulfill the pathological criteria for AD, and 25–30% of patients clinically assessed as having probable AD lack brain amyloidosis [5,8].

Given these amyloid-related uncertainties, a more rational approach toward AD is to consider the disease as a multifactorial disorder. AD, rather than an amyloid-driven monolithic entity, is a complex process in which, along with Aβ and tau, the convergence of many polygenic, epigenetic, environmental, vascular, and metabolic factors increases susceptibility to the

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disease and shapes its pathogenic course [3]. In that regard, it is now commonly accepted that the AD brain, in addition to Aβ and tau-related hallmarks, also exhibits signs of oxidative stress, metal ion imbalance, mitochondrial dysfunction, neuroinflammation, metabolic deficiencies, and macro- and microvascular alterations.

Over the past two decades, a growing body of evidence, including *in vitro* and *in vivo* preclinical studies, meta-analyses, large epidemiology population studies, as well as **'omics**' evidence, has indicated that one of the potentially modifiable risk factors for dementia is the age-related imbalance of metal homeostasis. Copper (Cu) and zinc (Zn) are essential biological metals found at high micromolar concentrations in cortical tissue and released in free ionic forms upon neural activity. Compelling evidence indicates a strategic role for Cu and Zn dysregulation in the AD brain, a phenomenon recapitulated by the 'metal hypothesis' of AD [9]. The hypothesis does not assume toxicological exposure to the metals, but rather a failure of endogenous regulatory mechanisms that leads to functional deprivation in some cell compartments, and toxic excess in others.

Cu Regulation in Physiology and Disease

Cu is an essential transition metal that acts as a catalyst for a plethora of biological processes (see [10] for an extensive review). The processes of Cu absorption, distribution, and storage are finely regulated in mammals (Figure 1) and the derangement of these processes has important detrimental systemic effects [11]. Dietary or acquired Cu dysregulation results in severe neurological manifestations, as found in **Menkes' disease (MD)** or Wilson's disease (WD; Box 1). MD is a rare genetic disorder in which mutations in genes encoding the Cu-transport protein ATP7A lead to Cu deficiency and promote abnormalities of the central nervous system (CNS). WD is a rare inborn error of Cu metabolism that leads to the toxic build-up of the metal in tissues and organs, hepatic failure, and neurodegeneration [12,13]. Cu pool intervention, by supplementation in MD or by reducing Cu adsorption in WD, modifies the course of these diseases.

The liver acts as the critical organ controlling Cu homeostasis (Box 1). The correct handling of Cu is regulated by the activity of two classes of protein: (i) cuproenzymes that use Cu as a cofactor; and (ii) Cu trafficking proteins that transport the metal. However, Cu shows functional duality. Cu, when not used for vital catalytic functions, takes part in Cu(II)/Cu(I)-driven redox cycles and generates hydroxyl radicals ($^{\bullet}OH$) through the activation of cyclic Haber Weiss and Fenton reactions [14]. It is well established that Cu that is not bound to **ceruloplasmin** (nCp-Cu) is associated with this redox-mediated toxicity. nCp-Cu comprises 5–10% of the plasma Cu and is in dynamic equilibrium with albumin, peptides, and amino acids. nCp-Cu permeates tissues and organs, including the brain, where it constitutes the 'labile' Cu pool [15].

The activity of ATP7B (an ATPase that, in hepatocytes, controls Cu loading into nascent ceruloplasmin [Cp]) is critical for maintaining the body levels of Cu. In AD, the reduced activity of ATP7B is associated with increased nCp-Cu levels (reviewed in [16]; Figure 1), while mutations in both copies of the gene gives rise to WD [12,13].

The neurobiological activity of Cu is complex. Cells overloaded with Cu accumulate the metal in mitochondria [17], and alterations of mitochondrial Cu participate in neurodegenerative processes and apoptotic signaling [18,19]. Cu elevations have also been observed in lysosomes, the organelles involved in autophagy, a critical mechanism in the context of AD because impairment of the autophagy and/or lysosome pathways results in the detrimental accumulation of Aβ and tau adducts [20,21]. In addition, Cu is involved in glial apoptosis, and its synaptic

dysregulation promotes memory and learning dysfunctions [19]. Cu affects synaptic functions because the metal is released from Cu-containing vesicles in the cleft of excitatory synapses [22]. Once released, Cu acts, post synaptically, as a high-affinity blocker of the *N*-methyl-D-aspartic acid receptor (NMDAR), modulating NMDAR functions as well as the activity of the glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), thereby producing a marked inhibition of glutamate-mediated neurotransmission [22].

Cu and Amyloid Dysmetabolism

Cu significantly affects the amyloid cascade, where it interacts with the amyloid precursor protein (APP) through a Cu-binding domain [23] (Box 2). Increased Cu concentrations colocalize within Aβ and neurofibrillary tangles, and regulate APP expression [9,24,25]. The interaction of Aβ peptides with Cu and other metals has been proposed to promote gain-of-function activities that are instrumental in the development of Aβ neurotoxicity [26] (Box 2). In this context, the Aβ-mediated 'Cu toxicity' originates from the modulation of reduction-oxidation (redox) cycles, an activity that, through production of H_2O_2, generates oxidative stress [27], leading to the formation of Aβ oligomers and their precipitation within plaques as well as lipid peroxidation [27]. In preclinical AD models, dietary ingestion through drinking water of small amounts of Cu double the plasma concentrations of nCp-Cu and also affect Aβ production [28]. The breakdown of Cu homeostasis also participates in the formation of Aβ advanced glycation end-products (AGEs), toxic molecules that are found in the AD brain and in patients with diabetes [29]. Aβ glycation with sugars generates free radical superoxide anions (O_2^-) that, by reacting with Cu, are converted into hydroxyl radicals (OH) and contribute to AD-related oxidative stress [30]. More recently, a loss-of-function model of Cu-related toxicity has been proposed (Box 2). The model is based on the defective capability of the APP-Aβ complex to handle Cu, thereby leading to the extrusion of excess Cu across membranes [31].

Cu in Cognitive Impairment

Substantial evidence shows that changes in Cu levels in the serum, plasma, cerebrospinal fluid (CSF), and brain of patients with AD are associated with the occurrence of cognitive deficits and the transition from different stages of the AD-related spectrum [32]. Recent meta-analyses [33–36] indicated that patients with AD fail to maintain a physiological balance and distribution of the metal and show increased Cu levels in the serum [33–35] along with an overall Cu depletion in the brain [36] (Table 1). These studies revealed the presence of a composite pattern of Cu dysregulation where selective plasmatic increases of nCp-Cu [34] occur along with serum decreases of Cu bound to proteins [37]. These studies pinpoint the importance of the nCp-Cu specific pool rather than the bulk Cu concentrations. The measurement of nCp-Cu is not devoid of technical conundrums [38]. Limitations of the indirect Walshe's index have been partially resolved by new methods that allow direct measurements of nCp-Cu levels (reviewed in [39]). A recent study demonstrated a commonality between a subset of patients with AD and patients with WD, namely that both sets showed increased blood levels of nCp-Cu (Box 1) [40].

Increased levels of loosely bound Cu are found in soluble fractions of the AD cerebral cortex [41]. Furthermore, in the AD brain, the progressive increase of the nCp-Cu pool is consistent with the parallel presence of an expanded pool of exchangeable nCp-Cu in the blood [34,42]. In patients with AD, several clinical studies have demonstrated that increased levels of nCp-Cu correlate with the appearance of changes in electroencephalography (EEG)-recorded electrical brain activity, signs of brain atrophy, pathological CSF levels of Aβ and tau protein, and more severe clinical courses (reviewed in [43]). The association between altered Cu metabolism and AD is further supported by genetic data indicating that the increased frequency of specific

Glossary

Amyloid β (Aβ): protein fragments derived from the proteolytic and amyloidogenic cleavage of the amyloid precursor protein (APP). The peptides are the primary constituent of senile plaques.

Ceruloplasmin: a 151-kDa protein with ferroxidase activity responsible for Cu transport in the bloodstream.

Excitotoxicity: neuronal death mechanisms activated by the overstimulation of the excitatory neurotransmitter glutamate.

Matrix metalloproteinases (MMPs): a class of Zn-containing endopeptidases involved in an array of biological functions, including dendritic spine remodeling and maturation of neurotrophic molecules, such as BDNF.

Memantine: a US Food and Drug Administration (FDA)-approved drug used to treat AD. The drug works as a low-affinity blocker of the ionotropic glutamatergic receptor NMDA.

Menkes' disease (MD): a genetic disorder caused by mutations of the gene encoding the Cu-transporter ATP7A and leading to Cu deficiency.

Metallothioneins (MTs): a class of low-molecular-weight proteins enriched in cysteine residues and that bind endogenous and exogenous metals.

Metal-protein-attenuating compounds (MPACs): a class of drugs used to disrupt the damaging interaction between Aβ and some metal ions, Zn and Cu in particular, and to restore the phasic tonic activities exerted by these metals at the synaptic cleft.

Necrosis, apoptosis, and autophagy: forms of cell death triggered by different endogenous and/or exogenous stimuli and executed by specific and unique molecular pathways.

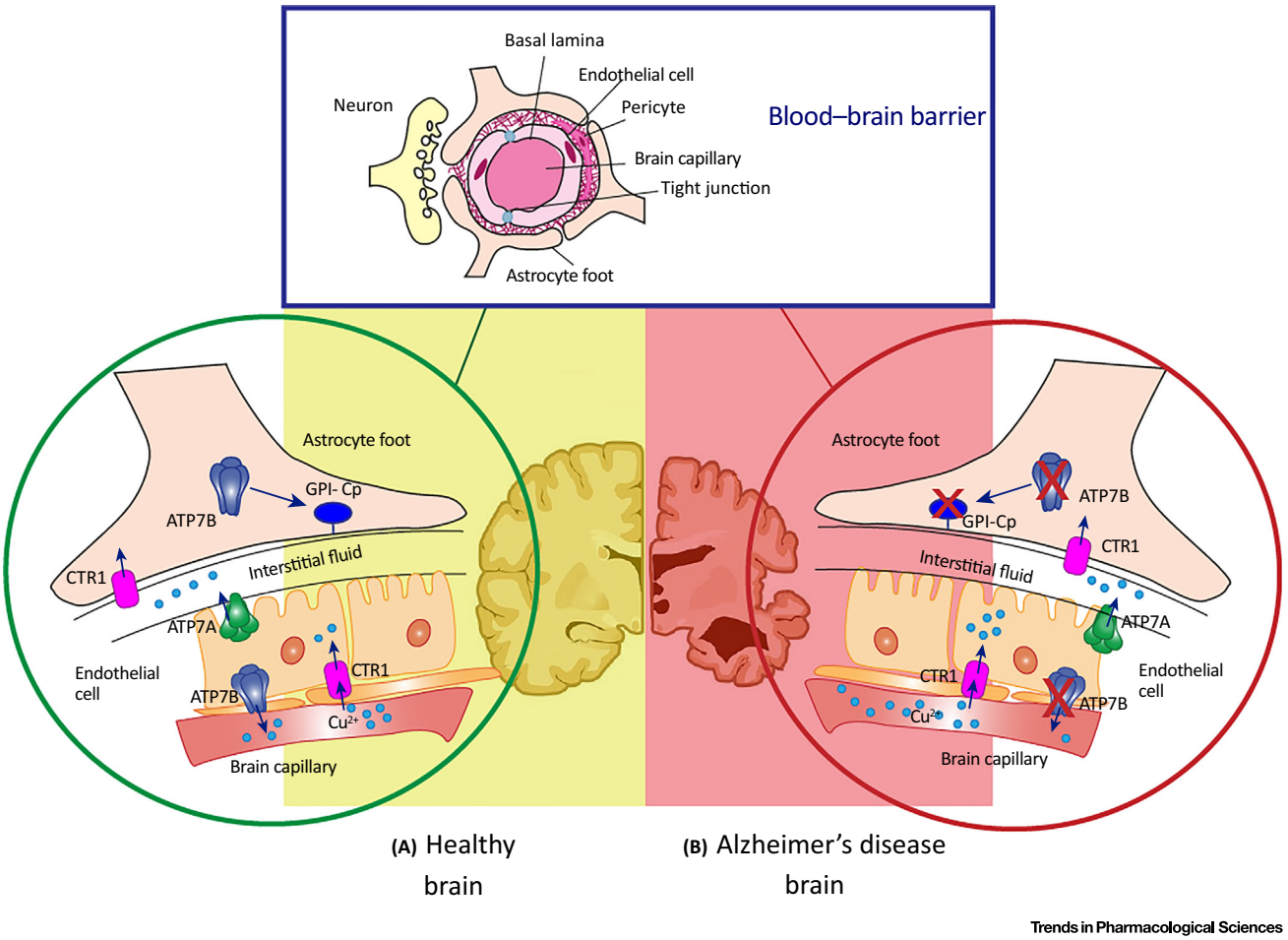
Omics: disciplines (i.e., genomics, proteomics, metabolomics, or ionomics) characterizing the function and interaction of single groups of molecules (i.e., genes, proteins, metabolites, or ions, respectively) in health and disease-related conditions.

Reactive oxygen and nitrogen species (ROS and RNS): unstable, extremely reactive chemical species containing oxygen or nitrogen. ROS

variants of the *ATP7B* gene is associated with an enhanced risk for AD (odds ratio from 1.63 to 5.16) [16]. Therefore, it has been proposed that gene variants in the region encoding the ATP7B transmembrane and A domains act as risk factors for AD [16].

Increased levels of nCp-Cu are also found in patients with mild cognitive impairment (MCI) [44,45]. High levels of nCp-Cu are observed during the very early stages of cognitive complaints and account for a faster rate of conversion to AD. In a 6-year survey period, 50% of MCI patients showing high levels of nCp-Cu were found to convert to AD [32]. These results are in line with a previous 5-year follow-up cohort study that indicated that increased total Cu levels and decreases in nonheme iron (Fe) provide a Cu:nonheme Fe ratio that can be used to single out patients with MCI that develop AD [44]. Elevated serum levels of nCp-Cu

and RNS not only have a crucial role as signaling molecules, but are also cytotoxic when dysregulated.
Tau proteins: a group of proteins physiologically involved in microtubule stabilization. When hyperphosphorylated, tau proteins aggregate into neurofibrillary tangles and promote neurodegeneration.
Zn transporters (ZnTs): a class of proteins that facilitate Zn transport from the cytosol into the lumen of intracellular organelles or allow its extrusion outside of the cell.



Trends in Pharmacological Sciences

Figure 1. Copper (Cu) Physiology and Pathology in the Brain. The neurovascular unit (NU; blue-outlined box) has a critical role in the regulation of brain Cu. The figure depicts Cu-related trafficking occurring at the critical intersection offered by the blood–brain barrier (BBB) and the NU. The CTR1 transporter controls the Cu exchange from the bloodstream and endotelial cells and astrocytes. In parallel, the two transporters, ATP7A and ATP7B, promote the metal extrusion from endotelial cells to the bloodstrean (ATP7B) or the interstitial fluid (ATP7A). Inside astrocytic feet ATP7B also promotes Cu loading into glycosylphosphatidylinositol-linked ceruloplasmin (GPI-Cp), thereby having an important role in keeping the intracellular Cu concentrations at bay [(A) green circle]. In the Alzheimer's disease (AD) brain [(B) red circle], evidence indicates that *ATP7B* polymorphisms, such as the K832R variant, negatively impact Cu loading into nascent GPI-Cp, thereby promoting damaging Cu accumulation inside astrocytic feet. Mutant ATP7B also affects Cu export from endothelial cells and contributes to Cu dysregulation.

Box 1. Wilson's Disease as a Model of Cu-Related Toxicosis

The liver is the primary organ for the control of Cu homeostasis. Cu is a highly reactive metal, safely delivered to target sites by Cu-trafficking proteins (also known as chaperones). WD is a paradigmatic disease for Cu toxicosis and accumulation. The disorder is characterized by a genetic defect in both copies of the *ATP7B* gene (which encodes a Cu-transporting ATPase controlling Cu homeostasis) located in the Golgi apparatus of liver and brain cells [12,13]. Defective ATP7B functioning leads to an expansion of the plasmatic Cu pool not bound to ceruloplasmin (nCp-Cu, also known as 'free copper'). nCp-Cu accumulates in the liver, brain, cornea (leading to the characteristic Kayser–Fleischer rings), and other organs of patients with WD, thereby producing oxidative stress and tissue damage. One-third of patients with WD exhibit signs of liver failure, another third neurological manifestations, and the final third behavioral abnormalities [112]. Patients with Cu-dysregulated AD or WD show commonality of some of the clinical features of these diseases, as well as Cu mislocation in the brain and serum, thereby suggesting neurodegenerative mechanisms that are shared between the two neurological conditions.

The Cu-AD connection has been empirically further substantiated by a case report of a patient who, although presenting with Kayser–Fleischer rings (a typical sign of WD), failed the diagnostic criteria for WD but showed cognitive impairment and neuroimaging features (Aβ-positive PET scan, ^{18}F-fluorodeoxyglucose [FDG]-PET hypometabolism, and brain atrophy) compatible with probable AD [113].

The precise mechanisms of the alterations in Cu compartimentalization that occurs with WD and in a subset of patients with AD are still not completely known. Cu trafficking is of particular importance in the critical milieu offered by the neurovascular unit (NU; blue box Figure 1 in main text). CTR1, ATP7Am and ATP7B actively participate in the physiological Cu transport occurring at the NU (green circle in Figure 1 in main text). ATP7B polymorphisms, such as the K832R variant, negatively impact these mechanisms, thereby increasing the Cu pool that is loosely bound to proteins and becomes available for citotoxic mechanisms occurring at the NU, ultimately affecting the brain Cu balance. Mutant ATP7B also affects Cu export from endothelial cells, thereby contributing to Cu dysregulation (red circle in Figure 1 in main text).

increase the susceptibility to AD approximately threefold [43]. Furthermore, patients with MCI and elevated nCp-Cu levels show a hazard rate of conversion to AD three times higher than MCI with normal nCp-Cu values [32]. Further support for the role of Cu in the modulation of cognition derives from large population studies demonstrating that alterations in Cu balance are associated with increased risk for cognitive decline [46,47]. Of note, Cp is also essential for Fe metabolism, another critical modulator of neurodegeneration [48]. The intersection between the two metals is relevant because Fe dyshomeostasis, similar to Cu mislocation, is a critical modulator of Aβ dysmetabolism and promotes cytotoxic effects that overlap with those triggered by Cu [48]. However, Fe dysregulation also triggers the unique phenomenon of ferroptosis, a mechanism with an important and emerging role in neurodegenerative processes and AD [48–50].

Cu Dysregulation as a Biomarker of a Subtype of Patients with AD

The evidence mentioned earlier suggests that, in a subset of patients, Cu abnormalities serve as a biomarker for the disease and have a role in its progression [51]. In recent years, direct methods to detect the toxic component of nCp-Cu associated with AD have been developed, and it was recently demonstrated that Cu dysregulation affects 50–60% of patients with MCI or AD [45,52]. The process appears to be specific in a subpopulation of AD who are carriers of selective *ATP7B* gene variants [53]. Further characterization of this Cu-related AD subtype revealed that this subset showed distinct EEG-derived brain activity alterations and neuroimaging features [51,54]. In analogy with WD, it is conceivable that, in the AD brain of a selected subset of patients showing increased levels of nCp-Cu, the mislocation of Cu promotes active mechanisms of neuronal toxicity. It is also possible that, in the larger group of patients with AD devoid of nCp-Cu alterations, collateral signs of Cu dysregulation are secondarily driven by the main molecular determinants (Aβ and tau) of the disease and have a minor, accessory, pathogenic role.

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Box 2. Cu, Zn, and Intrinsically Disordered Proteins

The 'amyloid cascade hypothesis' posits that aberrant accumulation of the Aβ peptide in the brain is the initial step in AD pathogenesis [1]. Aβ is a 37–43-amino acid peptide derived from the proteolytic cleavage of the amyloid precursor protein (APP), a synaptic membrane protein the physiological role of which remains elusive. The sequential processing of APP by β- and γ-secretases leads to the formation and release of Aβ peptides in the CNS extracellular space. Aβ belongs to the broad family of intrinsically disordered proteins (IDPs), a class of proteins with an undefined 3D structure that binds Cu and other metals. $A\beta_{40}$ and $A\beta_{42}$ are flexible peptides with small α-helix and β-sheet structures and are the two most abundant forms of Aβ. These proteins bind Cu and Zn [25,70]. The senile plaques of AD brains are highly enriched with Zn and Cu, suggesting that the two metals participate in Aβ dysmetabolism and neurotoxicity [69]. By contrast, sequestration within plaques of the two metals can reduce their bioavailability and prevent their physiological activities.

Two alternative models describe how Cu can affect Aβ neurotoxicity. In the gain-of-function model, Cu acts as a critical trigger for the Aβ-driven production of ROS. In that capacity, the metal, through its redox properties, can participate in the Aβ-mediated generation of H_2O_2. The loss-of-function model proposes that Aβ peptides act as protective buffers against the toxic availability of metals within the brain [31]. According to this model, Aβ works as a physiological chelator that exports excess Cu across membranes, thereby protecting neurons from Cu overload. The defective Cu chelation may also lead to the loss of soluble, metal-binding, functional Aβ monomers, which, in turn, aggregate in toxic oligomers and then in fibrils.

With some analogies with Cu, Zn also participates in Aβ dysmetabolism. Zn binding to Aβ in the synaptic cleft prevents the tonic and phasic physiological modulation of excitation and inhibition exerted by Zn on glutamatergic and GABAergic receptors (see also Box 3) [58]. Several lines of evidence indicate that Aβ sequesters the chelatable Zn that is released upon synaptic activity. In addition, Aβ can compete for Zn and detach the cation from the catalytic site of synaptic metalloenzymes (i.e., MMPs), thereby impairing their enzymatic activity at the cleft. Although Zn is not a redox metal, the cation can indirectly promote the oxidative stress found in AD because the metal is a central player in excitotoxicity-driven generation of damaging reactive oxygen species (ROS). Glutamate- and Aβ-driven as well as the Zn-dependent ROS production trigger further Zn mobilization from intracellular pools (i.e., MTs) and activate a feed-forward cascade involving a mixture of **necrosis, apoptosis,** and **autophagy** [58].

Zn Regulation in Physiology and Disease

Zn is found in structural or labile forms in the CNS. In close analogy with Cu, structural Zn is tightly bound to proteins and peptides, and acts as a crucial component for proper protein folding or as the catalytic/co-catalytic element required for the activity of many enzymes [55]. Labile, 'free' Zn is either stored in the lumen of intracellular organelles, such as mitochondria, lysosomes, endoplasmic reticulum (ER), and Golgi apparatus, or bound to **metallothioneins (MTs)**, a class of **reactive oxygen species** (ROS)- and pH-sensitive metal-binding proteins [56]. Under physiological conditions, cytosolic Zn concentrations are kept in the picomolar to the low nanomolar range [57], and the mobilization of this free Zn serves as a second messenger for intracellular signaling. However, a surge in Zn levels (in the nanomolar range) promotes neurotoxicity [58]. The tight control of Zn levels is achieved by the concerted activity of a series of **Zn transporters (ZnTs)**, 'Zrt-, Irt-related' proteins (ZIPs) as well as by mobilization from intra- and extracellular Zn stores and binding proteins [59]. Pathological conditions, such as transient global ischemia, status epilepticus, brain trauma, and **excitotoxicity**, build up the metal concentrations in neurons through its trans-synaptic influx. Additional accumulation of intraneuronal Zn occurs upon the ROS- and pH-driven mobilization from intracellular pools, such as MTs, mitochondria, lysosomes, and ER [58]. These intraneuronal Zn rises are critical to set in motion **necrosis**, **autophagy**, and **apoptosis** [58].

The role of Zn, and mitochondrial dysfunction in triggering and shaping pathways of neuronal death has been extensively investigated [58,60,61]. Zn enters mitochondria upon a steep electrochemical gradient (Δp), accumulates within the organelles, and potently disrupts their function, ultimately leading to irreversible organelle damage [58,61,62]. Zn triggers the permeabilization of the mitochondrial membrane through the activation of the mitochondrial

Table 1. Meta-Analyses and ROC Studies Evaluating Essential Metals in AD[a]

Study authors	Statistical analysis	Metal	Tissues sample sources	No. of studies analyzed	AD cases/controls analyzed	Meta-analysis trend	*P* value	Area under ROC[c] curve[d]	*P* value	Refs
Wang *et al*.	Meta-analysis	Fe	Serum	18	1035/1273	No change	0.086			[35]
		Zn	Serum	17	862/1705	Decrease	<0.001			
		Cu	Serum	27	1768/2514	Increase	<0.001			
Schrag *et al*.	Meta-analysis	Fe	Brain	14	206/251	No change	0.76			[36]
		Zn	Brain	10	166/118	No change	0.291			
		Cu	Brain	7	115/123	Decrease	0.0003			
Squitti *et al*.	Meta-analysis	nCp-Cu	Serum/plasma	10	599/867	Increase	<0.001			[34]
		Cu	Serum/plasma	27	1393/2159	Increase	<0.001			
		Adj-Cp Cu[b]	Serum/plasma	14	879/1712	Increase	0.006			
Li *et al*.	Meta-analysis	Fe	Serum	25	1379/1664	Decrease	0.001[e]			[33]
		Zn	Serum	22	1027/1949	Decrease	0.0005			
		Cu	Serum	35	2128/2889	Increase	<0.0001			
Du *et al*.	Meta-analysis	Mn	Serum	10	551/1134	Decrease	0.015			[104]
Paglia *et al*.	ROC	Mn	Serum		34/40			0.89	<0.001	[105]
		Vn	Serum		34/40			0.83	<0.001	
		Cu	Serum		34/40			0.70	<0.001	
		Mn/Cu	Serum		34/40			0.93	<0.001	
Squitti *et al*.	ROC	nCp-Cu	Serum		89/147			0.755	<0.001	[52]
Lopez *et al*.	ROC	Cu	Serum		36/33			0.676	0.015	[106]

[a]Abbreviations: AD, Alzheimer's disease; Cu, copper; Fe, iron; Mn, manganese; nCp-Cu, copper not bound to ceruloplasmin; Vn, vanadium; Zn, zinc.
[b]Adj-Cp Cu, adjusted Cu concentration method [38].
[c]ROC, receiver operating characteristic curve indicates the diagnostic ability of a binary classifier system.
[d]AUC, the area under the curve. ROC curves depict the likelihood of a given test to be excellent, good, or worthless. The accuracy of the test depends on how well it discriminates, within a group, in this case patients with AD versus healthy subjects. The accuracy is measured by the AUC of the ROC curve. An AUC = 1 represents a perfect test, a value of 0.5 represents a worthless test, and values in the 0.7–0.8 range are considered to be fair.
[e]After the exclusion of two studies due to heterogeneity in the analyzed cohorts.

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permeability transition pore (MPTP), a key and conserved effector of cell death. MPTP opening promotes the release and/or production of proapoptotic factors, such as cytochrome *c*, apoptosis-inducing factor (AIF), and ΔN-Bcl-X_L [62,63]. These Zn-dependent adverse effects on mitochondrial functioning and energy production may contribute to the deficient energetic balance found in the AD brain [64].

Zn can also target many cytosolic pathways to promote cellular demise, including the activation of apoptotic signaling cascades, the modulation of plasma membrane channels, and the depletion of metabolic substrates [58]. Unlike Cu, Zn is not a redox metal but, nevertheless can indirectly promote oxidative stress through the activation of ROS- and **reactive nitrogen species** (RNS)-generating enzymes, such as 12-lipoxygenase, NADPH oxidase, and the neuronal isoform of nitric oxide synthase (nNOS) [58]. These pathways promote a deadly convergence in which $O_2^{\bullet}-$ and $NO^{\bullet}$ lead to the formation of neurotoxic $ONOO^-$ (peroxynitrite). Of note, Zn-driven ROS and RNS generation promotes further Zn release from intracellular redox-sensitive stores, likely MTs, thereby exacerbating the Zn dyshomeostasis in a self-perpetuated feed-forward loop [58,65].

Zn, the Amyloid Cascade, and Beyond

Changes in Zn levels occur in AD and affect amyloid-dependent and -independent mechanisms critical for the disease progression [26] (Boxes 2 and 3). Studies of postmortem AD brains have shown variations in levels of proteins and transporters involved in Zn homeostasis [66]. These processes correlate with the disease progression and severity of cognitive impairment [67]. Clinical studies assessing levels of serum Zn in patients with AD resulted in a heterogeneous set of results [33,68]. Meta-analyses support the notion that Zn is decreased in the disease (Table 1). However, CSF assays or postmortem brain tissue analysis failed to provide a definitive answer on the amount of Zn found in AD [36]. The liaison between Zn and Aβ dysmetabolism dates back to the early 1990s, when the 'Zn hypothesis for AD' was formulated, a concept that was later framed within the AD 'metal hypothesis' [9,26].

Ex vivo observations have confirmed that Zn is highly enriched within senile plaques [69]. Recent evidence indicates a complex interplay between Zn and Aβ. Genetic and pharmacological manipulations show that Aβ peptides sequester Zn and deplete synapses from the

Box 3. Cu and Zn in Synaptic Plasticity

Synaptic plasticity is the molecular correlate of learning and memory. The process is finely regulated by the concerted activity of a plethora of positive and negative modulators (i.e., ionotropic and metabotropic receptors, second messengers, protein kinases, phosphatases, etc.). Zn and Cu participate in the shaping of synaptic plasticity [22,58]. Zn is stored within vesicles at the terminals of some, but not all, glutamatergic synapses. The cation is particularly abundant in strategic locations such as the hippocampal Mossy Fiber (MF)–CA3 synapse contacts, which is a key circuit for memory-related processes. The cation is also released at the Schaffer collateral (SC)–CA1 synaptic contacts. Once in the cleft, synaptically released Zn reaches postsynaptic terminals and interacts with, and modulates, an array of receptors. Zn exerts an adverse effect on NMDARs and $GABA_AR$, and, to a lesser extent, AMPARs [58]. The cation also activates a metabotropic G-coupled Zn^{2+}-sensing receptor (ZnR), which in turn promotes an intracellular signaling cascade that is involved in synaptic plasticity [96]. More recently, Zn has emerged as a critical contributor to the shaping of neurotrophic signaling [84,85,88,94]. The cation is required for MMP-dependent maturation of BDNF (see Figure 2 in main text). Moreover, Zn acting as MMP cofactor affects the dendritic spine and synapse remodeling through the degradation of the extracellular matrix, a critical step for spine enlargement and structural plasticity. The Cu activity at the synapse overlaps with Zn [22,58]. In fact, Cu is released from presynaptic vesicles in an action potential- and calcium (Ca)-dependent manner. Once in the cleft, the cation exerts an adverse effect on NMDARs, AMPARs, $GABA_AR$, and voltage-gated Ca channels (VGCCs). The synaptic Cu effect negatively modulates plasticity and long-term potentiation (LTP). Intriguingly, Cu, through interference with MMP-9 activity via mechanisms that remain unknown, has also been described, *in vitro*, as a modulator of the BDNF/TrkB neurotrophic axis.

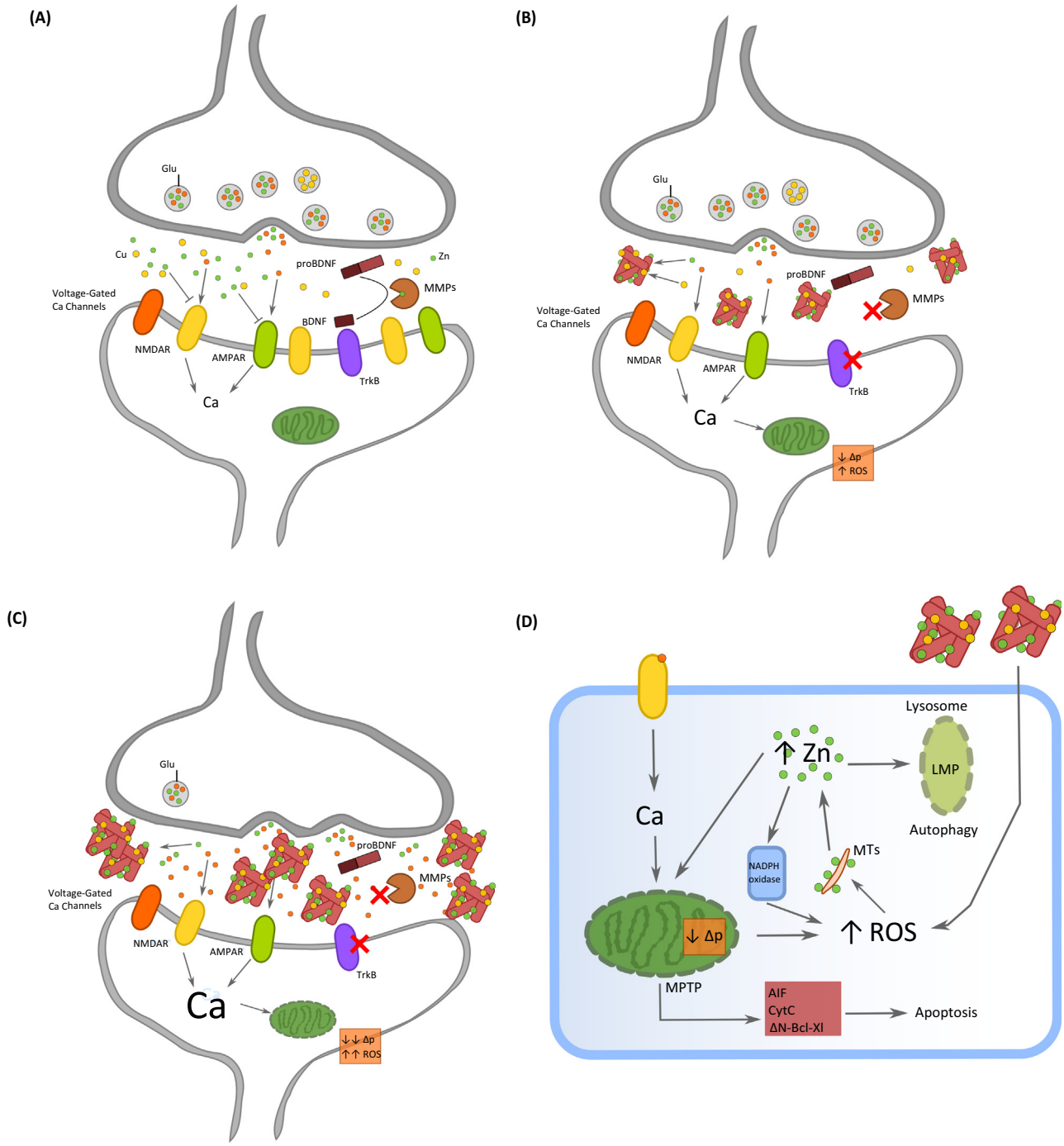

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Figure 2. Pleiotropic Activities of Copper (Cu) and Zinc (Zn) in Shaping Synaptic Plasticity and Neuronal Death. The figure illustrates a glutamatergic synapse upon (A) physiological conditions, (B) amyloid beta (Aβ)-driven synaptic deficits, or (C) in a synergistic Alzheimer's disease (AD) model, in which Aβ converges with excitotoxicity to promote synaptic failure and neuronal death. (A) Cu and Zn are released, along with glutamate, upon synaptic activity. In the cleft, the two metals modulate excitatory neurotransmission by inhibiting the activity of the ionotropic receptors *N*-methyl-D-aspartic acid receptor (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR). Synaptic Zn also shapes the proper functioning of the neurotrophic brain-derived neurotrophic factor (BDNF)/TrkB axis because the metal is an essential cofactor for the activity of matrix metalloproteinases (MMPs). MMPs are a class of extracellular enzymes that promote the

(Figure legend continued on the bottom of the next page.)

metal physiological activities, including the tonic-phasic modulation of NMDARs or its activity as a cofactor for **matrix metalloproteinases (MMPs)** [9,58,70] (Figure 2; Boxes 2 and 3). The notion that synaptic Zn deprivation contributes to AD-related dysfunction is corroborated by the AD-like phenotype observed in aging ZnT3-knockout (KO) mice [71], a transgenic model that is devoid of presynaptic vesicular Zn. Recent preclinical findings provided evidence that Zn may also modulate tau pathology by a phosphorylation-independent pathway, in which Zn directly binds the protein and promotes its aggregation [72] and/or a pathway in which elevations of intra- and extraneuronal Zn trigger aberrant tau phosphorylation by inhibiting the protein-phosphatase 2A (PP2A) [73] and activating tau phosphorylating kinases (i.e., GSK-3β, ERK1/2, and cJunK) [74,75]. These mechanisms ultimately lead to a net increase in phospho-tau adducts that may exacerbate tau pathology in AD.

Vascular Disorders, Cu, Zn, and Excitotoxicity

The failures of most AD clinical trials aimed at targeting Aβ and the growing skepticism towards a dogmatic interpretation of the 'amyloid hypothesis' have prompted a more complex and synergistic view of the disease mechanisms [3]. For instance, cerebrovascular pathology is present in most patients with late-onset AD [76]. Vascular-related white matter lesions, alterations of the neurovascular unit, and cortical microinfarcts are common comorbidity features, especially in the oldest patients [77]. Cu and Zn can modulate these processes.

As for Cu, a series of longitudinal cohort studies has indicated the association between the risk for vascular diseases or mortality and altered levels of Cu and Cp [78]. Studies have also revealed an increased likelihood for vascular diseases related to Cu [79,80].

By contrast, Zn is an essential player in excitotoxicity (a damaging mechanism therapeutically addressed in patients with AD with the NMDAR blocker **memantine**), which represents a critical point of convergence between vascular-dependent dysfunctions and Aβ and tau pathology [81]. Historically, excitotoxicity has been described as a process driven by the overactivation of glutamatergic receptors, especially NMDARs, an injurious cascade of events that triggers aberrant elevations in cytosolic calcium (Ca) and Ca-dependent generation of ROS/RNS, mitochondrial dysfunction, and increased potassium channel activity, ultimately leading to the activation of necrotic, proapoptotic, and autophagy-related pathways [82]. A substantial body of evidence now supports the notion that dysregulation of brain Zn is an essential contributor to the process. Evidence accumulated over the past 30+ years has demonstrated that the alteration of the brain Zn homeostasis is not only an accomplice, but also a coleading actor in the glutamate-driven murder case [60,83].

Yin and Yang of Brain Zn: Modulation of Neurotrophic Signaling

Similar to Cu, Zn has dual activities. As mentioned earlier, Zn is not only a potent modulator of amyloid metabolism, but also a regulator of neurotransmission, neurotrophism, and

maturation of proBDNF in mature BDNF. mBDNF binds with high affinity to the TrkB receptor and activates neuroprotective and/or plasticity-related signaling. Cu positively modulates this cascade by favoring MMP activity. (B) In AD, Aβ sequesters Cu and Zn, thereby blocking the physiological processes described in (A). Decreased Cu and Zn levels in the cleft can alter glutamatergic neurotransmission and promote synaptic deficits. Amyloid can remove Zn from synaptic enzymes, including MMPs, thereby leading to impaired BDNF-related signaling. (C) Aβ can act in synergy with glutamate to exacerbate the synaptic damage and neuronal loss. In the proposed model, the Aβ-driven sequestration of Cu and Zn is paralleled by the excessive synaptic release of glutamate and the two metals. In this scenario, the reduced Cu- and Zn-mediated inhibition of glutamate receptors coupled with decreased neurotrophic signaling potentiates the neurotoxic effects associated with the glutamatergic overdrive. (D) Reactive oxygen species (ROS) generated from Aβ-Cu and calcium (Ca) influx through ionotropic glutamatergic receptors and promote the intraneuronal released of Zn because the cation is mobilized in a ROS- and reactive nitrogen species (RNS)-dependent manner from metallothioneins (MTs). In the cytosol, Zn impairs mitochondrial functioning, promotes the release of proapoptotic factors, and induces autophagy by favoring lysosomal membrane permeabilization (LMP) and the release of lysosomal proteases.

synaptic plasticity (Box 3). Supporting the notion of a complex regulation of brain functions by the metal, Zn supplementation has been found to positively affect neurotrophic signaling and, in particular, the activation of the brain-derived neurotrophic factor (BDNF)-TrkB axis [58,84,85] (Figure 2; Box 3). Neurotrophins are a class of proteins involved in neuronal survival, growth, and plasticity. Defective neurotrophic signaling has been associated with several neurodegenerative disorders, including AD [86]. Zn supplementation counteracted the decrease of BDNF levels in a murine AD model by promoting the Zn-dependent and MMP-mediated maturation of inactive proBNDF to its mature and functional form [85]. Moreover, in preclinical murine models, brain Zn deficiency or chelation, if occurring upon development, have been linked to reduced levels of synaptic plasticity-related proteins and the presence of cognitive impairment [71,87,88]. Of note, in contrast to mature BDNF, proBDNF promotes impairment in synaptic transmission and participates in neuronal death-related processes. proBDNF release inhibits GABAergic transmission [89], a finding that, along with the inhibitory effects of Aβ oligomers on BDNF maturation [90], provides a novel mechanism for the altered neuronal excitability observed in AD models and patients with AD [91]. Thus, a Zn-dependent defective BDNF maturation may have significant consequences for brain functioning. In that context, the Zn-mediated modulation of MMPs, BDNF, and TrkB signaling may critically impinge on the course of neurodegenerative processes and symptoms. Furthermore, genetic or pharmacological enhancement of MMP activity, and MMP-9, in particular, reduces cognitive decline in AD models by decreasing Aβ oligomers [85,92]. Thus, the Zn-mediated increase in MMP activity may

Table 2. Preclinical and Clinical Evidence for Zinc Supplementation

Study authors	Zinc formulation	Animal study or AD[a] cohort	Dose	Duration	Outcome	Refs
Phase I clinical trials in animal studies						
Corona *et al*.	Zinc sulfate	3 × Tg-AD mice (Aβ- and tau-pathology)	30 mg/l in tap water	11–13 months	Improved spatial memory, mitochondrial functioning, and neurotrophic signaling; reduced Aβ and tau pathology	[85]
Harris *et al*.	Zinc acetate	Tg2576 mice (Aβ-pathology)	2 g/l	6 months	Reduced Aβ pathology; no behavioral changes	[107]
Flinn *et al*.	Zinc carbonate	CRND8/E4 (Aβ-pathology and APOE4 risk factor)	10 mg/l in tap water	4 months	Negative effect on cognition and Aβ pathology	[108]
Phase I and II clinical trials in humans						
Constantinidis	Zinc bis-DL-hydrogen aspartate	10 patients	3 × 50 mg/day	3–12 months	Improved memory, understanding, communication, and social interaction	[109]
Potocnik *et al*.	Zinc methionine	4 patients	2 × 10 mg/day	1 year	MMSE and ADAS-Cog improvements	[110]
Brewer	reaZinc	42 patients	150 mg/day	6 months	Post-hoc analysis in patients aged 70 years and older revealed statistically significant improvement in in ADAS-Cog and CDR-SOB scores and near-significant results in MMSE scores	[103]
Maylor *et al*.	Zinc gluconate	387 healthy older individuals	30 mg/day	6 months	A significant treatment × time interaction for aspects of CANTAB; long-term benefits unclear	[111]

[a]Abbreviations: Aβ, amyloid β; AD, Alzheimer's disease; ADAS-Cog, Alzheimer's disease assessment scale-cognitive subscale; CDR-SOB, clinical dementia rating scale; CANTAB, Cambridge neuropsychological test automated battery; MMSE, mini-mental state examination.

have a dual beneficial effect. The process not only promotes BDNF maturation, but also participates in the clearance of small Aβ adducts, thereby favoring the production of nontoxic Aβ byproducts [93]. Manipulation of the Zn-MMP-BDNF axis also affects synaptic plasticity and may contribute to the appearance of neuropsychiatric symptoms [88,94]. Zn deficiency may alter the levels of proBDNF and affect plasticity, and has been found to correlate with the onset of depressive symptoms, while Zn supplementation has antidepressant effects obtained mainly through the potentiation of BDNF signaling [95]. Also worthy of further exploration is the role of Zn as a neurotransmitter through the activation of its metabotropic receptor mZnR/GPR39 [96]. The receptor, by enhancing the inhibitory tone or reducing neurotransmitter release, acts as a negative modulator of neuronal activity [96], a mechanism potentially involved in the hyperexcitability observed in AD [91].

Targeting Cu and Zn Dysregulation

The issue of metal dysregulation has been the target of therapeutic attempts. The use of **metal-protein-attenuating compounds (MPACs)** provides a complex array of actions that are at the same time potentially beneficial or detrimental and remain a matter for debate [97]. Broad chelation of Cu and/or Zn can not only positively modulate the beneficial solubilization of amyloid plaques [9], but also promote the detrimental sequestration of the two cations at the synaptic cleft, thereby negatively affecting neurotransmission as well as the neurotrophic axis [88].

Clioquinol (CQ) and PBT2, two MPACs, have been used with the aim of restoring Zn and Cu homeostasis in preclinical AD models as well as in patients with AD. In preclinical AD models, the two MPACs have been found to be effective in reducing Aβ and tau pathology as well as to decrease the cognitive deficits of the animals [98,99]. CQ and PBT2 have also been used in two clinical trials and reported to show beneficial effects on AD-related molecular biomarkers and cognition [100,101]. A PBT2-based Phase II trial, the IMAGINE trial, conducted on a small cohort of patients with prodromal or mild AD, was at first thought to have produced only a small reduction in hippocampal atrophy in the treated group [49]. A further revision of the results revealed that the lack of major beneficial cognitive effects was likely due to a suboptimal randomization of the study participants, their variable outlook in terms of amyloid load, as well as the small size of the study cohort. These findings reinforce the need for more stringent and personalized patient recruitment criteria to clarify the therapeutic potential of this class of MPACs. For instance, the use of a recently developed MPAC-derived radioligand opens the possibility to dissect the critical issue of the regional regulation of these two metals and help the more selective recruitment of study subjects [102]. In that regard, so far, MPAC trials have not been designed to include patients with clear signs of Cu dysregulation, thereby critically neglecting a subset of patients who could have benefitted the most from this therapeutic approach. Thus, a more promising approach is offered by selectively targeting subsets of patients with AD and/or MCI showing signs of nCp-Cu dysregulation. Preclinical and clinical evidence indicates that Zn supplementation can be a feasible and cost-effective line of intervention to restore the brain Cu balance (Table 2). Zn supplementation, a mainstay in the treatment of patients with WD, diminishes nCp-Cu levels by affecting Cu absorption through an increased mucosal block in the gut. In a small-sized AD cohort, the use of Zn at 150 mg/day was found to induce a negative body Cu balance, thereby possibly reverting the Cu compartmentalization and distribution from the blood to organs and tissues, including the brain [103]. Zn may also positively affect neurotrophic signaling and synaptic plasticity (Box 3). To date, only a few small clinical studies have evaluated the effects of Zn supplementation in AD (Table 2). Thus, large-scale trials are urgently needed.

Concluding Remarks and Future Perspectives

The changing landscape of AD opens new opportunities. In line with the multifactorial nature of the disease, growing and robust evidence indicates that further exploration of the AD-related metal dysregulation offers valuable therapeutic avenues. The use of MPACs or any other line of intervention aimed at targeting biometals faces essential challenges (also see Outstanding Questions). Further tinkering will be required to engineer compounds that can only act within a narrow dynamic range of concentration, and show selectivity of action in preferred regional and/or subregional localizations or target selected cellular population. A neurocentric bias has so far neglected the study of metal balance in critical cells involved in the control of the neurovascular unit and inflammation, such as astrocytes, microglia, and pericytes. The timing of intervention also appears to be critical. The potential of these new therapeutic approaches strongly depends on a conceptual leap forward and the willingness to move away from the reductionist assumption that there is only one AD and a 'silver bullet' to treat it. In line with a more up-to-date interpretation of the disease status, including AD as the result of the joint dysfunction of several health-related networks, targeting metal dysregulation may be a not-for-all therapy, but rather an option for selected subtypes of patients with AD who are more prone to metal imbalance.

Outstanding Questions

Is there only 'one' AD or is the condition instead the personalized, individual-specific, convergence of the network disequilibrium of many health-related systems and networks?

The one-size-fits-all therapeutic approach has failed. Would it be more useful to target selected subgroups of patients identified by specific molecular and/or genetic pedigrees?

Will nanotechnological advances allow the targeting of metal imbalance in selected cellular subpopulations as well as subcellular localizations?

When is the most appropriate time to approach the issue of metal imbalance?

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Disclaimer Statement

S.L.S. is a minor shareholder (<US$ 5000) in PRANA Ltd. R.S. is the inventor of a Cu-related kit for AD diagnosis and an equity owner of IGEA Research Co (3% shares) and Canox4drug SPA (3%) without monetary compensation.

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Excess of serum copper not related to ceruloplasmin in Alzheimer disease

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Abstract—*Objective:* To assess the role of serum copper in relation to ceruloplasmin and other peripheral markers of inflammation in Alzheimer disease (AD). *Methods:* The authors studied serum levels of copper, ceruloplasmin, and transferrin, as well as total peroxides, antioxidants, and other peripheral markers of inflammation in 47 patients with AD, 24 patients with vascular dementia (VaD), and 44 healthy controls. Biochemical variables were related to the patients' and controls' clinical status. *Results:* The authors found that copper ($p < 0.001$), peroxides ($p = 0.026$), and ceruloplasmin ($p = 0.052$) were increased and TRAP was decreased ($p = 0.006$) in patients with AD, while no other markers of inflammation were altered. The calculation of the ratio between copper and ceruloplasmin suggested the presence in the serum of AD patients, but not of VaD or normal controls, of a large pool of non-ceruloplasmin-bound copper. *Conclusions:* Changes in the distribution of the serum copper components, consisting of an increase of a copper fraction not explained by ceruloplasmin, seem to be characteristic of Alzheimer disease and may be implicated in the pathogenesis of the disease.

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Trace metals such as zinc, copper, and iron, and oxyradicals are reactive species involved in the neurodegeneration of Alzheimer disease (AD).[1,2] Both iron and copper can catalyze Fenton's reactions, generating a flux of reactive oxygen species that can damage functional and structural macromolecules.[3] The toxicity of beta-amyloid (Aβ) may also depend on copper-mediated free radical generation, and on copper- and zinc-induced deposition of amyloid plaques, as demonstrated by in vitro studies and in animal models of AD.[3-6] We recently reported a perturbation of copper homeostasis specific to AD patients, consisting of elevation of serum copper levels and increased activity, in red blood cells, of the copper enzyme superoxide dismutase.[7-10]

Dysfunction of redox status and trace metals homeostasis could be related to an inflammatory response. Significant changes in copper absorption, transport, metabolism, or excretion do occur in inflammation, where plasma copper levels rise, along with levels of the acute phase copper-protein ceruloplasmin, the main carrier of fasting serum copper.[11] Ceruloplasmin is in fact increased due to an augmented rate of its hepatic synthesis and secretion.[11,12] Much evidence supports the presence, in AD, of an inflammatory component leading to brain tissue damage, possibly through activated glia.[13] In addition there is yet somewhat controversial evidence of a concomitant inflammatory response in the general circulation of patients with AD.[14,15]

In order to assess the role of peripheral markers of redox trace metals in a putative inflammatory response in AD, we studied serum levels of copper, transferrin, total hydro- and lipoperoxides, and antioxidants, as well as a number of peripheral markers of inflammation, including ceruloplasmin. In addition, in order to identify changes most specific to patients with AD, we compared the results of the same assays in patients with vascular dementia (VaD).

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Methods. Forty-seven patients with AD (mean age 75.6), 44 cognitively normal individuals (mean age 71.1), and 24 patients with VaD (mean age 77.4) were included in the study (table 1); the groups were slightly different for sex and age, thus all statistical analyses were covaried for these two confounders. Both the AD patient sample, consisting of individuals with a clinical diagnosis of probable AD (National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association criteria),[16] and VaD patients, who met National Institute of Neurological Disorders and Stroke–Association Internationale pour la Recherche en l'Enseignement en Neurosciences criteria,[17] as well as brain MRI criteria suggestive of microangiopathic (small vessel) pathology,[18] had a Mini-Mental State Examination (MMSE)[19] score of 25 or less and were mild to moderate. AD and VaD patients underwent neurologic, neuroimaging (MRI or CT), and extensive neuropsychological evaluation, as well as routine laboratory tests. Duration of disease since onset of symptoms was 27 months (range 6 to 96) for AD and 24 (range 6 to 72) for VaD. The control group consisted of elderly volunteers with no

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Table 1 *Characteristics of the study groups*

	Controls	AD patients	VaD patients	Overall significance
No. of subjects	44	47	24	
M/F	24/20	12/35	13/11	$\chi^2 = 10.471$; $df = 1$; $p = 0.005$
Age, y, mean (SD)	71.1 (11)	75.6 (7.7)	77.4 (7.0)	$F(2,112) = 4.624$; $p = 0.012$
APOE ε4 frequency, %	5.3	19.3	16.6	$\chi^2 = 7.106$; $df = 2$; $p = 0.035$
MMSE, mean (SD)	28.1 (1.3)	18.6 (4.7)	22.7 (3.6)	$F(2,103) = 112.25$; $p < 0.001$
Disease duration, mo, median (min–max)		33 (12–120)	24 (8–72)	Mann-Whitney $U = 191.5$, $p = 0.398$

AD = Alzheimer disease; VaD = vascular dementia; MMSE = Mini-Mental State Examination.

clinical evidence of neurologic or psychiatric disease. Twelve patients with AD and five patients with VaD had either poor quality MRIs or contraindications to the examination (e.g., metal prostheses); therefore, a CT scan was done to exclude other treatable causes of dementia. Six patients with AD and one patient with VaD did not complete the neuropsychological test battery. All controls underwent a neurologic examination and MMSE, yet only 16 agreed to undergo extensive neuropsychological testing and 12 consented to MRI.

Criteria for exclusion of both patients and controls were conditions known to affect copper metabolism and biologic variables of oxidative stress (e.g., diabetes mellitus, inflammatory diseases, Hodgkin's disease, recent history of heart or respiratory failure, chronic liver or renal failure, malignant tumors, alcohol abuse), on the basis of past medical history and screening laboratory tests. These tests included complete blood count, erythrocyte sedimentation rate (ESR), cholesterol, triglycerides, ferritin and fibrinogen levels, serum protein electrophoresis, renal function (creatinine, BUN), fasting glucose, electrolytes, vitamin B12, folic acid, and uric acid levels; thyroid function tests (thyroxin, triiodothyronine, and thyrotropin levels); liver enzymes (SGOT and SGPT), cardiac enzymes (CPK, LDH), bilirubin levels; serology for syphilis; and urine analysis. Patients and controls with abnormal values for thyroid, liver, kidney, or cardiac functions were also excluded from the study.

The study was approved by the local Institutional Review Board and all participants or legal guardians gave written, informed consent.

Biochemical and molecular investigations. Sera from fasting blood samples were collected in the morning and rapidly stored at −80 °C. Serum copper concentration was measured following the method of Abe et al. (Randox Laboratories, Crumlin, UK),[20] and by an A Aanalyst 300 Perkin Elmer atomic absorption spectrophotometer equipped with a graphite furnace with platform HGA 800.[8] Measurements of biologic variables of oxidative stress are described in detail elsewhere.[21] Briefly, hydro- and lipoperoxide content was assessed by d-ROMs test (Diacron, Italy) and expressed in arbitrary units (U.CARR), 1 U.CARR corresponding to 0.08 mg/100 mL of hydrogen peroxide.[22] Normal range was between 230 and 310 U.CARR.[21,22] Total Radical Trapping Antioxidant capacity or TRAP was assayed by the TAS kit (Randox Laboratories), based on published methods.[21,23] The serum reference range is 1.30 to 1.77 mmol/L.[21,23] Transferrin and ceruloplasmin were measured by immunoturbidimetric assays (Roche, Diagnostic, Germany) utilizing a goat anti-human transferrin antibody in TRIS/HCl buffer,[24] and a rabbit anti-human ceruloplasmin antibody in phosphate buffer.[25]

All biochemical measures were automated on a Hitachi 912 analyser (Roche Diagnostics) and performed in duplicate. *APOE* genotyping was performed according to established methods.[26] Serum protein electrophoresis was carried out by means of the Beckman Paragon SPE agarose system with densitometric quantitation of the protein fraction (Analis, Namur, Belgium). Interleukin-1 β (IL-1β) and tumor necrosis factor α (TNFα) from serum were assessed with ELISA methods (DRG Instruments GmbH, Germany) based on appropriate and validated sets of monoclonal antibodies.[27,28]

Cognitive evaluation. Neuropsychological testing included the MMSE,[19] the Mental Deterioration Battery (MDB),[29] the Digit Span,[30] and the test of Corsi as described elsewhere.[8,30]

MRI evaluation. Brain MRI was performed using a 1.5 Tesla superconductor magnet. The imaging protocol consisted of axial T2 W double spin echo (SE) sequences and T1 W SE images in axial, coronal, and sagittal planes, with 5 mm slice thickness and intersection gap = 0.5 mm. MR images were evaluated by two experienced neuroradiologists blinded to the patients' diagnoses or laboratory results, approaching total agreement (95%). Atrophy and white matter changes lesions were graded following standardized visual rating scales on plain MRI.[31-33] The degree of medial temporal lobe atrophy was evaluated with a ranking procedure and validated by linear measurements of the medial temporal lobe including the hippocampal formation and surrounding spaces occupied by CSF, following standardized criteria (five-point rating scale of medial temporal lobe atrophy [MTA]).[31] Generalized brain atrophy (ventricular and sulcal atrophy) was rated as present (1) or absent (0; global atrophy). The visual rating scale of white matter changes included the anatomic distribution as well as the severity of the lesions. Based on anatomic distribution, a distinction was made between areas of periventricular hyperintensities (PVH) (caps and rims) and deeper hyperintensities (including frontal, parieto-occipital, and temporal white matter hyperintensities [DWMH]; basal ganglia hyperintensities [BGH]; and infratentorial hyperintensities [ITH]). Large vessel cortical infarcts in the anterior, posterior, and medial cerebral artery were also evaluated. Presence of mass lesions and lobar hemorrhages were exclusion criteria from the study.[32,34]

Ultrasonographic examination of the cerebral vessels. The carotid and vertebral arteries were studied by means of color coded duplex ultrasonography (7.5 MHz probe; Acuson, Aspen) according to standardized criteria.[35] Detailed description of the sonographic procedures has been previously reported.[8] Particular attention was paid to detect both presence and degree of stenosis of atherosclerotic plaques in the carotid and vertebral arteries. The intima and media thickness (IMT) from the distal portion of the CCA was investigated in detail in each patient according to previous studies.[36] Intracranial vessels were also examined by transcranial Doppler (TCD Multidop T TCD- DWL, Germany), according to previously described methods.[37]

Statistical analyses. AD and VaD patients and controls were described in terms of main demographic, genetic, and cognitive characteristics and statistically compared with analysis of variance (ANOVA) F, χ^2, or Mann–Whitney tests. Both age and sex were considered as covariates in all parametric statistical analyses.

In order to assess the role of the copper and the other biologic variables in separating patients with AD, VaD, and healthy subjects, discriminant analysis was performed. Of note is that copper values were entered after logarithmic transformation. The analysis involved a linear combination of the independent variables that discriminates between the a priori defined groups, minimizing the misclassification rates. The findings of the discriminant analysis were verified by means of percentages of correct classification (both in the whole sample and after eliminating one case at a time according to the leave-one-out procedure).

The relationship between copper and ceruloplasmin was studied by means of polynomial regression analysis. This technique allowed us to choose the appropriate model, and, subsequently, two copper measures were calculated: copper explained and copper not explained by ceruloplasmin.

Eta-squared (the proportion of the dependent variable variance

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Table 2 *Serum copper, peroxides, TRAP, and transferrin concentrations in healthy controls, patients with AD, and patients with VaD*

	Controls, n = 44	AD patients, n = 47	VaD patients, n = 24	ANOVA
Copper, μmol/L	12.6 (2.5)	17.2 (5.9)	12.9 (2.3)	$F(2,108) = 13.025$, $p < 0.001$
Ceruloplasmin, mg/dL	26.5 (4.7)	30.1 (5.9)	26.8 (5.6)	$F(2,95) = 3.057$, $p = 0.052$
Peroxides, U.CARR	299 (68)	348 (64)	329 (56)	$F(2,109) = 3.779$, $p = 0.026$
TRAP, mmol/L	1.39 (0.14)	1.28 (0.14)	1.32 (0.17)	$F(2,108) = 5.391$, $p = 0.006$
Transferrin, g/L	2.7 (0.4)	2.5 (0.5)	2.7 (0.6)	$F(2,104) = 1.318$, $p = 0.272$

Values are mean (SD). Based on an analysis of covariance model with age and sex as covariates.

TRAP = Total Radical Trapping Antioxidant capacity; AD = Alzheimer disease; VaD = vascular dementia; ANOVA = analysis of variance.

accounted for by the differences among groups) was also used as association measure.

Finally, correlation analyses between biologic variables and neuropsychological scores (Pearson's r), and MRI atrophy and vascular measurements (Spearman's rho) were performed.

A p value less than 0.05 was considered significant in all statistical analyses, except when a correction for multiple comparisons was appropriate. Tukey's test was used when the homogeneity of variances could be assumed. When the variances were statistically heterogeneous, the Games-Howell procedure was applied. In order to avoid redundancies, the p values of the ANOVA (differences between controls, AD, and VaD) are reported in table 2 while the p values of the pairwise comparisons (adjusted for multiple comparisons) are reported in the text.

We used the SPSS 11.5 for Windows statistical software package (SPSS Inc., Chicago, IL).

Results. Patients and controls differed for age, controls being younger than patients with VaD (Tukey's $p = 0.019$) and patients with AD (age overlap provided a Tukey's p of 0.054) (see table 1). The groups were not balanced for sex, since the proportion of women in the AD group was higher (76% vs 46% both in VaD and controls). AD and VaD patients did not differ for disease duration. The three groups differed for MMSE, as revealed by all pairwise comparisons (Tukey's $p < 0.001$). No differences for fasting glucose, vitamin B12, folic acid and uric acid levels, ESR, bilirubin levels, cholesterol, triglycerides, complete blood count, and hypertension among groups were evident. *APOE* ϵ4 allele was more frequent in both AD and VaD patients.

Table 2 summarizes the data of copper, ceruloplasmin, peroxides, TRAP, and transferrin in patients with AD, VaD, and controls. Although the results on copper shown here were obtained by the colorimetric method,[23] those obtained by atomic absorption spectrophotometry assays were superimposable (intraclass correlation coefficient for consistency = 0.89, $p < 0.001$). Copper ($p < 0.001$) and peroxides ($p = 0.008$) were increased in patients with AD compared to controls (see table 2). Peroxides were higher in patients with VaD vs controls, though the difference did not reach significance ($p = 0.058$).

TRAP was always lower in both groups of patients in comparison to controls ($p = 0.004$ for AD and $p = 0.036$ for VaD). The difference in ceruloplasmin content between AD and controls was 2.730 (95% CI: lower bound = −0.130, upper bound = 5.591), and that between VaD and controls was 0.003 (95% CI: lower bound = −3.011, upper bound = 3.061; $p = 0.987$). However, after correcting for age and sex, the increase of ceruloplasmin observed in patients with AD only approximated significance ($p = 0.052$). None of the oxidative and trace metal species measured correlated with age, except for transferrin, which was inversely correlated ($r = -0.20$, $p = 0.037$).

Discriminant analysis was applied to assess the potential implication of indices of copper metabolism in AD. In particular, we developed a model to identify which biologic (copper, ceruloplasmin, peroxides, and TRAP) and demographic variables (age and sex) could discriminate among patients with AD, patients with VaD, and controls. This procedure automatically identified two functions as linear combinations of the biologic variables. Function 1 was obtained combining three biologic variables high in the AD group—that is, copper, peroxides, and ceruloplasmin—that clearly separated AD patients from healthy controls (see figure E-1, Function 1 on the *Neurology* Web site at www.neurology.org). The second linear combination (Function 2) provided by the discriminant procedure was due almost exclusively to the TRAP contribution to the statistical model. This function distinguished patients with VaD from controls, TRAP being lower in the VaD group.

As a whole, this discriminant analysis was adequate in differentiating patients with AD from controls (correct classification of 80%, cross-validated, 73%), but not in separating patients with VaD from controls (correct classification rate of 69%, cross-validated, 59%). The discriminant power between AD and VaD could be improved, however, by adding MRI indices of white matter changes and atrophic burden (correct classification rate of 90%, cross-validated, 79%). This analysis could not be applied to controls because of insufficient neuroimaging data. The most valuable variables in group discrimination were DWMH and copper. DWMH alone could discriminate AD from VaD with a classification rate of 77%, reaching 84% when copper levels were included in the analyses. The contribution of other variables was small (approximately 6%) and the total correct classification rate approached 90%.

In order to address the disequilibrium between the measured biologic markers, two ratios were calculated: copper:transferrin and copper:ceruloplasmin. ANOVA [$F(2,106) = 19.53$; $p < 0.001$] and post hoc Games-Howell comparisons indicated that the copper:transferrin ratio was higher in AD vs controls and VaD ($p < 0.001$; figure 1). Comparisons of copper:ceruloplasmin ratios with ANOVA [$F(2,94) = 5.91$; $p = 0.004$] and post hoc Games-Howell comparisons confirmed higher values in patients with AD ($p = 0.028$ vs controls, $p = 0.015$ vs patients with VaD). Comparisons between controls and patients with

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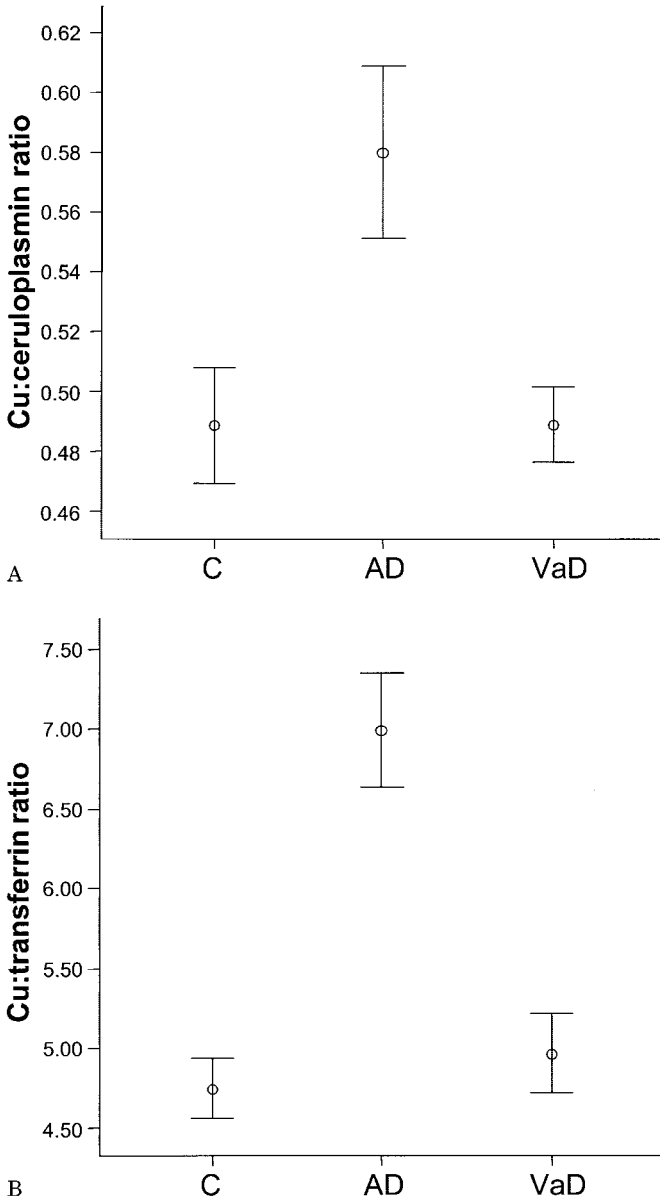

Figure 1. Mean values (±1 SEM) of copper:ceruloplasmin ratio (A) and copper:transferrin ratios (B).

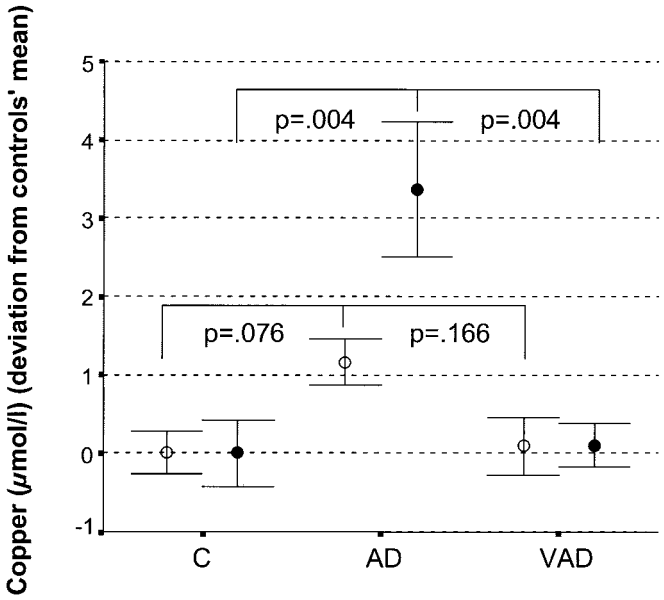

Figure 2. Mean values (±1 SEM) of copper explained by ceruloplasmin (open circle) and copper not explained by ceruloplasmin (filled circle) in the three groups of subjects. Data are expressed in terms of deviations from controls' means. Eta-squared resulted equal to 0.06 (p = 0.056) for copper explained by ceruloplasmin and 0.14 (p = 0.001) for copper not explained by ceruloplasmin. Post hoc Tukey's comparisons vs Alzheimer disease are reported.

VaD showed similar values for both ratios ($p = 0.953$ and $p = 0.999$; see figure 1).

In order to explain the physiologic relationship between ceruloplasmin and copper we applied both lowess nonparametric and polynomial regression models to data obtained from the control group. After eliminating control subjects with abnormal influence statistics, the simple linear regression provided high goodness-of-fit. Pearson's r resulted equal to 0.75, indicating that about 56% of copper variability could be explained by ceruloplasmin. We calculated that, in healthy subjects, for each 10 points of ceruloplasmin levels increase, copper is expected to increase by 3.28 points (95% CI = 2.15 to 4.41). On the basis of this physiologic relationship, we computed two copper measures: the first, copper explained by ceruloplasmin, corresponds to the theoretically expected level of copper, given a ceruloplasmin level; the second, copper not explained by ceruloplasmin, corresponds to the residual copper with respect to the regression line determined in controls. When both measures of copper levels were entered in the discriminant analysis, copper not explained by ceruloplasmin was able to differentiate AD from both VaD and controls (0.610, highest correlation with canonical discriminant function 1). Copper explained by ceruloplasmin, instead, did not contribute to the discriminant model (correlation coefficients = 0.05 with canonical discriminant function 1 and = 0.08 with canonical discriminant function 2). The higher discriminant power of copper not explained by ceruloplasmin is illustrated in figure 2, where data were normalized with respect to the controls' mean.

No inflammatory processes were determined to be specific to AD in our patient sample, as the markers of inflammation we analyzed did not differ among the three groups (see table E-1 on the *Neurology* Web site at www.neurology.org).

Elevated copper and oxidative stress inversely correlated with performance on most neuropsychological measures, namely immediate and delayed recall (Rey's Auditory Verbal Learning Test), Phonemic Verbal Fluency, Copy Drawing, Copy Drawing with Landmarks, and Raven's Progressive Matrices (table 3), confirming what was previously reported.[8] No correlation was instead present between cognitive testing and physiologic indices of peripheral inflammation, namely albumin, ESR, IL-1β, TNFα, and serum α1, α2, and γ fractions (results not shown). Correlation analysis from the Doppler examination revealed that no patient with AD had abnormal flow velocity in the cerebral arteries examined by Duplex sonography and TCD (results not shown). Moreover, none of the biochemical variables assayed correlated with ultrasound indicators of cerebrovascular burden, namely pres-

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Table 3 *Correlation between outcomes of neuropsychological examination and copper, ceruloplasmin, peroxides, TRAP, and transferrin*

Neuropsychological test	Copper (n)	Ceruloplasmin (n)	Peroxides (n)	TRAP (n)	Transferrin (n)
MMSE	r = −0.335; p = 0.003; (79)	−0.355; p = 0.002 (73)	−0.359; p = 0.001 (80)	0.249; p = 0.027 (79)	−0.099; p = 0.392 (77)
RAVLT Immediate Recall	r = −0.154; p = 0.187 (75)	r = −0.163; p = 0.181 (69)	r = −0.072; p = 0.534 (76)	r = 0.191; p = 0.100 (75)	r = 0.010; p = 0.930 (73)
RAVLT Delayed Recall	r = −0.222; p = 0.056 (75)	r = −0.249; p = 0.039 (69)	r = −0.238; p = 0.038 (76)	r = 0.277; p = 0.016 (75)	r = −0.048; p = 0.688 (73)
Digit span	r = −0.048; p = 0.684 (75)	r = −0.192; p = 0.114 (69)	r = −0.083; p = 0.476 (76)	r = −0.028; p = 0.812 (75)	r = −0.138; p = 0.245 (73)
Corsi Test	r = −0.154; p = 0.196 (72)	r = −0.139; p = 0.261 (67)	r = −0.240; p = 0.041 (73)	r = 0.014; p = 0.904 (73)	r = −0.287; p = 0.016 (70)
Immediate Visual Memory	r = −0.240; p = 0.042 (72)	r = −0.237; p = 0.056 (66)	r = −0.166; p = 0.161 (73)	r = 0.019; p = 0.873 (72)	r = −0.051; p = 0.677 (70)
Raven's Progressive Matrices	r = −0.288; p = 0.014 (72)	r = −0.319; p = 0.009 (66)	r = −0.324; p = 0.005 (73)	r = 0.085; p = 0.480 (72)	r = −0.082; p = 0.501 (70)
Verbal Fluency	r = −0.309; p = 0.007 (75)	r = −0.252; p = 0.037 (69)	r = −0.167; p = 0.150 (76)	r = 0.164; p = 0.159 (75)	r = 0.052; p = 0.665 (73)
Sentences Construction	r = −0.256; p = 0.038 (66)	r = −0.147; p = 0.259 (61)	r = −0.103; p = 0.406 (67)	r = 0.015; p = 0.904 (67)	r = −0.110; p = 0.388 (64)
Copy Drawing	r = −0.336; p = 0.005 (69)	r = −0.187; p = 0.140 (64)	r = −0.219; p = 0.068 (70)	r = −0.087; p = 0.473 (70)	r = −0.081; p = 0.516 (67)
Copy Drawing with Landmarks	r = −0.292; p = 0.016 (68)	r = −0.301; p = 0.016 (63)	r = −0.360; p = 0.002 (69)	r = 0.074; p = 0.548 (69)	r = 0.027; p = 0.830 (66)

r = Pearson correlation coefficient. Significant at the 0.005 level (two-tailed, after Bonferroni's adjustment). TRAP = Total Radical Trapping Antioxident capacity; MMSE = Mini-Mental State Examination; RAVLT = Rey's Auditory Verbal Learning Test.

ence of carotid plaque or stenosis, except for the IMT values that correlated with *APOE* ϵ4 genotype (Spearman rho = 0.358; $p < 0.005$). Analyses of MRI data showed that presence of supratentorial atrophy (enlarged sulci, widened lateral and third ventricles) and atrophy of the temporal lobes (reduction in hippocampal volume) was prominent in AD subjects, while white matter changes and other deep-seated hyperintensities were more prevalent in VaD (see table E-2). Control subjects' values were consistent with those found in normal aging. Among MRI measurements and serum oxidative-trace metals values, there was a negative correlation between isolated MTA and TRAP (Spearman rho = −0.451, p = 0.001). Deep-seated ischemic changes, namely BGH, correlated with *APOE* ϵ4 genotype (Spearman rho = −0.251, p = 0.049). Correlation analyses of ϵ4 allele status with the biologic variables of oxidative stress and trace metals for all participants showed that presence of the *APOE* ϵ4 allele correlated only with lower TRAP concentrations, [t(99) = −2.91, p = 0.004].

Discussion. The results of this study show that the portion of serum copper unexplained by ceruloplasmin, as calculated on the ratio between ceruloplasmin and copper levels in healthy controls, can discriminate subjects with AD from both normal conrols and subjects with VaD better than copper explained by ceruloplasmin. This finding demonstrates an alteration of the ratio between copper and ceruloplasmin in this disease. Ceruloplasmin, an α-2 globulin with ferroxidase and copper transport functions, is a marker of both plasma copper status and inflammation.[12,38] We had previously reported a relative elevation of total serum copper in AD,[8] although levels were within the usual reference range for copper of 10 to 22 μmol/L.[20] Also another study analyzing indices of copper metabolism in AD showed that the ceruloplasmin-copper relationship probably represents the key issue in interpreting in vivo copper studies on AD,[39,40] rather than absolute serum copper levels, making less relevant the discrepancy among studies that did[8,41] or did not find differences in serum copper levels in AD.[42,43] Normally, over 90% of human serum copper is considered to be tightly bound to ceruloplasmin,[44] even though some authors[11] suggested a value closer to 60% for ceruloplasmin-bound copper, similar to our study. The rest of the copper would be distributed among transcuprein (12%), albumin (12%), aminoacids (e.g., histidine), and small molecular weight complexes (0.5 to 5%), named exchangeable component. Studies on transgenic mouse models show that copper can bind and interact with APP (or Aβ), which has been proposed to function as a copper/zinc metalloprotein, part of a failing metal homeostatic mechanism in AD.[45,46] In particular, copper-binding APP has been hypothesized to represent a means for removing excess copper from brain tissue.[45,47,48] The excess of serum copper we estimated in AD could be explained by an efflux from cortical cells, as also proposed by some authors,[47] which would also explain why the

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rise observed is mainly due to copper unbound to ceruloplasmin, rather than the bound fraction biosynthesized, in fact, in the liver. This hypothesis is coherent with the APP-/- knockout mouse model, where absence of APP, proposed to balance cell copper concentration, is causative of the 40% increase in brain copper observed. Alternatively our results could be described by a failure of copper incorporation into APP in the liver.[45] In the APP-/- mouse model copper increases by 80% in the liver and 40% in the brain, while apparently no serum variations are found.[45] This situation closely resembles Wilson's disease, a degenerative condition where copper metabolism abnormalities are key to the pathogenesis, even though serum copper levels are within the normal range. In this disease, in fact, the micronutrients-associated fraction—that is, the exchangeable serum copper component—is extremely elevated.[49] In Wilson's disease copper is bound to small molecules that can easily reach organ tissues and cross the blood–brain barrier, which normally functions as an effective mechanism to control redox metals brain tissue levels.[50,51] In studies on the distribution of copper among serum components in patients with cancer, variations in the four serum fractions were found and some patients with very high level of low-molecular weight copper were described.[11] The increase in ceruloplasmin unexplained copper calculated in our patients with AD is far from the unbound copper estimated in Wilson's disease, yet it approaches the levels described in patients with cancer and it could be toxic, partaking in Aβ-mediated toxicity of AD, as it can easily cross the blood–brain barrier.[51] In addition, our findings are coherent with the proposed activity of APP in balancing copper concentrations.[45,46] A recent report showed that ingestion of low concentrations of copper (2 μm) added to drinking water markedly impairs biometals homeostasis and increases brain parenchymal Aβ in rabbits fed cholesterol-supplemented diets.[52] The specific increase in total serum copper in our patients with AD was in the micromolar range, yet some authors have suggested that even increases in the nanomolar range could have an impact on AD pathogenesis.[47] Although we could not look, in vivo, for direct evidence of a toxic effect or a change in brain Aβ burden, we developed a statistical approach to assess the potential implication of copper in the pathogenesis of AD. Our aim was to estimate the specificity of the biologic indices of copper metabolism and oxidative stress in discriminating patients with AD from healthy and demented controls, and correlating them to the most relevant clinical characteristics of the disease. According to the discriminant analysis model, when patients with AD are compared to controls the biologic variables of trace metals and oxidative stress can correctly classify 80% of subjects. When the model is applied to AD and VaD populations and the MRI indices are included in the canonic function, they can discriminate 90% of patients. Indeed, a correlation between Aβ burden in CSF and copper in the serum is also suggested by preliminary evidence from our laboratory (manuscript in preparation).

While copper discriminates patients with AD from both controls and patients with VaD, the increase in peroxides seems to be reflective of a pro-oxidant status caused by an increase in redox copper. The decrease of TRAP, which is aspecific and is seen in both patients with AD and patients with VaD, may either reflect a primary defective antioxidant mechanism, conferring susceptibility to all dementias, or, on the contrary, may be secondary to brain tissue degeneration.

The ϵ4 frequencies of our patients with AD (19.3%) and healthy elderly controls (5%), much lower than frequencies in Northern Europe and the United States, are similar to those reported by studies documenting a dramatic decrease of the ϵ4 frequency from Northern to Southern Europe, and even from Northern to Southern Italy.[53,54] In addition, our patients with VaD show an ϵ4 frequency relatively comparable to the patients with AD, as previously reported in other Italian samples.[55,56] The overlap in *APOE* alleles frequencies in Italian patients with VaD and patients with AD, in addition to a geographic variation in the influence of this genotype over AD susceptibility, may reflect the partial neuropathologic and pathogenetic overlap between the two nosologic categories in general, as also documented by the ongoing debate over the adequacy of the international criteria used to distinguish AD from VaD.[57,58]

Much evidence suggests a role of inflammatory processes in AD pathogenesis, as well as a concomitant peripheral inflammatory response.[59] The concomitant increase in our patients with AD of the copper fraction explained by ceruloplasmin, a marker of inflammation, suggests that at least in part our findings could relate to general inflammatory mechanisms, despite the lack of significant differences in other peripheral markers of inflammation.

Our main finding is the probable existence in AD of an excess of copper not bound to ceruloplasmin, a serum component that might play a role in Aβ-mediated toxicity to the brain.[51] This serum copper component should also be sought in other neurodegenerative disorders. A study evaluating the reliability of copper in discriminating AD from Parkinson disease is currently in progress, and will help determine whether copper-mediated pathogenetic mechanisms are specific to AD.

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Value of Serum Nonceruloplasmin Copper for Prediction of Mild Cognitive Impairment Conversion to Alzheimer Disease

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Objective: Meta-analyses show that nonbound ceruloplasmin (non-Cp) copper (also known as free or labile copper) in serum is higher in patients with Alzheimer disease (AD). It differentiates subjects with mild cognitive impairment (MCI) from healthy controls. However, a longitudinal study on an MCI cohort has not yet been performed to assess the accuracy of non-Cp copper for the prediction of conversion from MCI to AD during a long-term follow-up.
Methods: The study included 42 MCI converters and 99 stable MCI subjects. We assessed levels of copper, ceruloplasmin, non-Cp copper, iron, transferrin, ferritin, and *APOE* genotype. A multiple Cox regression analysis—with age, sex, baseline Mini-Mental State Examination, *APOE4*, iron, non-Cp copper, transferrin, ferritin, hypercholesterolemia, and hypertension as covariates—was applied to predict the conversion from MCI to AD.
Results: Among the evaluated parameters, the only significant predictor of conversion to AD was non-Cp copper (hazard ratio = 1.23, 95% confidence interval = 1.03–1.47, $p = 0.022$); for each additional micromole per liter unit (μmol/l) of non-Cp copper, the hazard increased by ~20%. Subjects with non-Cp copper levels >1.6μmol/l had a hazard conversion rate (50% of conversion in 4 years) that was ~3× higher than those with values ≤1.6μmol/l (<20% in 4 years). The rate of conversion was similar between *APOE4* carriers and noncarriers ($p = 0.321$), indicating that the non-Cp copper association was independent of *APOE4*.
Interpretation: Non-Cp copper appears to predict conversion from MCI to AD. These results encourage healthy life style choices and dietary intervention to modify this risk.

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Amnestic mild cognitive impairment (MCI) is characterized by memory impairments that do not yet warrant the diagnosis of dementia, and it is considered a precursor of Alzheimer disease (AD), due to the high rate of progression from MCI to AD. Sensitive cognitive tests, coupled with cerebrospinal markers of AD (ie, β-amyloid [Aβ] 1–42 peptide and tau proteins), have been tested as predicting markers of the conversion from MCI to AD.[1–3] The cause of AD appears to be closely related to aggregation and deposition within the brain of the Aβ peptide in oligomers and plaques and of tau proteins in neurofibrillary tangles, as has been claimed in the

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"amyloid cascade," which is the most popular Alzheimer hypothesis.[4] This hypothesis has taken many forms, as new details about the disease emerge. For example, in addition to the Aβ oligomers and toxic tau aggregates, oxidative damage, aberrant inflammation, or impaired energy metabolism have been included among the pathogenic pathways involved in the disease cascade. Recently, researchers have discovered an important role played by iron, copper, and zinc metabolism in the pathogenesis of AD.[5–9] These metals are essential for life, but they become dangerous when their regulatory mechanisms fail. We recently demonstrated that, by combining information from copper and iron studies, it is possible to sensitively and specifically distinguish AD patients from healthy controls and subjects with other forms of dementia.[10]

It has been reported that the homeostasis of these metals is already altered in MCI, regarding the serum copper portion not bound to ceruloplasmin. Nonbound ceruloplasmin (non-Cp) copper increased in MCI subjects, and its levels were in-between those of healthy controls and those of AD patients.[11]

Recent studies have indicated that the alteration of copper homeostasis (non-Cp copper normal values are 0–1.6μmol/l)[12] is one of the pathogenic mechanisms of AD. This suggests that the diet–gene interplay is at the basis of the copper phenotype of sporadic AD.[13]

In a 5-year follow-up study,[14] researchers demonstrated that total copper and the ratio of copper/nonheme iron clearly distinguished progressive MCI subjects from cognitively stable MCI subjects.[14] In this study, we have explored the hypothesis that a deregulation of the non-Cp copper pool in serum could be a determinant of the variability of the cognitive decline in those MCI subjects converting to AD.

Subjects and Methods

Subjects were recruited at the Department of Neuroscience of the Fatebenefratelli Hospital, Isola Tiberina, Rome, and at the Memory Clinic of the IRCCS Istituto Centro San Giovanni di Dio, Fatebenefratelli, Brescia, Italy, using a common standardized clinical protocol.[11] Local institutional ethics committees approved the study. All participating subjects gave written informed consent to be included in the study, 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.

Assessments and evaluations of the participating subjects occurred at baseline visit and at 6-month intervals. The visit at which the subject exhibited a clinical worsening sufficient to fulfill AD criteria (National Institute of Neurological and Communicative Diseases and Stroke–Alzheimer's Disease and Related Disorders Association)[15,16] was considered the time of AD conversion, and consequently the subject's follow-up period was calculated as the time difference of this visit from the baseline; if the subject did not convert, the MCI diagnosis remained until the end of the study. The inclusion and exclusion criteria for amnestic MCI were based on previous seminal studies.[17–20] Briefly, criteria were as follows: (1) objective memory impairment on neuropsychological evaluation, as defined by performances $\geq$1.5 standard deviation below the mean value of age- and education-matched controls for a test battery including Busckhe–Fuld and Memory Rey tests revealed at screening; (2) confirmation of the memory impairment in the subsequent baseline visit (baseline maximum duration: 3 days, to collect fasting blood samples); (3) normal activities of daily living as documented by the history and evidence of independent living; and (4) a clinical dementia rating score of 0.5. The exclusion criteria for MCI included: (1) mild AD; (2) evidence of concomitant dementia, such as frontotemporal form, vascular dementia, reversible dementias (including pseudodepressive dementia), fluctuations in cognitive performance, and/or features of mixed dementias; (3) evidence of concomitant extrapyramidal symptoms; (4) clinical and indirect evidence of depression, as revealed by Geriatric Depression Scale scores >13; (5) other psychiatric diseases, epilepsy, drug addiction, alcohol dependence, and use of psychoactive drugs, including acetylcholinesterase inhibitors or other drugs that enhance cognitive functions; and (6) current or previous uncontrolled or complicated systemic diseases (including diabetes mellitus) or traumatic brain injuries.[11]

Additional exclusion criteria were conditions known to affect copper metabolism and biological variables of oxidative stress on the basis of past medical history and screening laboratory tests; this is reported in detail elsewhere.[21]

We also excluded patients with a history of stroke, presence of focal neurological signs, ischemic lesions or severe corticosubcortical leukoencephalopathy on brain magnetic resonance scans, presence of atherosclerotic plaques and hemodynamically significant stenosis or occlusion in cerebral (neck and intracranial) arteries, or history of coronary artery disease (myocardial infarction, angina, coronary artery angioplasty, or bypass surgery) or cardiac arrhythmias (atrial flutter or fibrillation) and history of peripheral arteriopathy. Blood was drawn on the same day of the clinical interview.

Biochemical and Molecular Investigations

Patients had samples drawn at their baseline visit. Fasting blood samples were collected in the morning and the serum was quickly stored at $-80°$C for blind biochemical measurements.

We measured copper by spectrometry,[11] using an A Analyst 600 PerkinElmer (Waltham, MA) atomic absorption spectrophotometer.

We analyzed the ceruloplasmin with an immunoturbidimetry assay (Horiba ABX, Montpellier, France). To better circumstantiate the immunoturbidimetry assay used for non-Cp copper calculation, we determined the serum ceruloplasmin oxidase activity with o-diansidine dihydrochloride as a substrate spectrophotometrically. The methodological details are reported in a previous study.[22]

For the non-Cp copper calculation, we used values of ceruloplasmin obtained immunologically.[22] More precisely, for each serum copper and ceruloplasmin pair, we computed the amount of copper bound to ceruloplasmin (CB) and the amount of non-Cp copper, following standard procedures.[12] Briefly: CB = ceruloplasmin (mg/dl) $\times$ 10 $\times$ n; n = 0.0472 (μmol/mg); Non-Cp copper = absolute serum copper $-$ CB.[12] This calculation expresses non-Cp copper in μmol/l and is

based on the finding 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 mass atomic units (Cu molecular weight)/132kDa (Cp molecular weight)] × 100 = 0.3%; more details are available at http://www.j-alz.com/letterseditor/index.html#March2013). Thus, for a patient with a serum absolute (or total) copper concentration of 17.3μmol/l and a serum ceruloplasmin concentration of 33mg/dl, the bound copper concentration equals 33 × 10 × 0.0472 = 15.6μmol/l, and the non-Cp copper concentration is 17.3 − 15.6 = 1.7μmol/l.[12]

Measurements of the other biological variables of metals are described in detail elsewhere.[10] We used an immunoturbidimetry assay to analyze transferrin and ferritin levels, and ferene to analyze iron. All of these reagents were from the ABX Pentra series from Horiba ABX. All biochemical measures were automated on a Cobas Mira Plus (Horiba ABX) and performed in duplicate. *APOE* genotyping was performed according to standard methods.[23] Among the study population, 13 cases were not analyzed for *APOE*, because it was not possible to assess the genotype (due to insufficient DNA, n = 8; insufficient blood samples, n = 2; and polymerase chain reaction failure, n = 1).

Data Analysis

We started to recruit MCI patients in 2005 and followed up with them through 2013, observing an attrition rate of 10% per year. The minimum follow-up was 2 years, whereas the patient with the longest follow-up was observed for 6 years. To recognize as significant (at a 2-sided alpha level of 0.05) a hazard ratio (HR) of 2 between those patients with non-Cp copper >1.6μmol/l and those with non-Cp copper ≤1.6μmol/l (where the annual hazard rate was assumed to be ~5%), we planned to recruit at least 140 MCI subjects to reach an adequate power (0.8). We further assumed that the whole sample was equally distributed below and above 1.6μmol/l.

Because the aim of the study was to see whether and how the incidence of conversion to AD is related to non-Cp copper, we applied a multiple Cox regression analysis, taking into account demographic, clinical, and metal metabolism measures. To control the risk of overfitting, we limited the number of candidate predictors performing different regression analyses with different subsets. More precisely, to avoid multicollinearity, we refrained from entering correlated variables into the same subset (eg, because copper, ceruloplasmin, and non-Cp copper are linked through a linear function, we considered copper and ceruloplasmin to belong to one subset and non-Cp copper to another). In this way, the event per variable (EPV) ratio was maintained at >5. Nevertheless, EPV was not high and remained below the suggested rule of thumb of 10. Thus, the stability of our prognostic models could not be considered optimal.

Risk proportionality was checked through the conventional methods (dependency of partial residuals, log − log plots). The number of events different from conversion to AD and censoring was very low (5 patients had a diagnosis of vascular dementia during follow-up, and they were not considered in the analysis). Therefore, no attempt to apply a competing risk model was made.

To provide additional information potentially useful for clinicians, we entered non-Cp copper both as a continuous and as a dichotomous variable (predefined cutpoint = 1.6 μmol/l).

TABLE 1. Baseline Characteristics, MMSE Scores, and Metal Studies in the 141 Subjects with Mild Cognitive Impairment Participating in the Study

Characteristic	Value
Age, yr, mean (SD)	70.8 (8.1)
Sex, No. women [%]	66 [47]
Risk factors, %	
Hypertension	23
Hyperlipidemia	20
APOE ε4 frequency, No. [%]	43/128 [33.6]
Biological variables in serum at baseline, mean (SD)	
Copper, μmol/l	14.8 (2.9)
Cp, mg/dl	27.7 (5.2)
Cp activity, IU/l	105.2 (21.7)
Non-Cp copper, μmol/l	1.8 (2.2)
Iron, μg/dl	100.0 (63.3)
Tf, g/l	2.5 (0.7)
Ferritin, ng/ml	141.2 (145.9)
Cp/Tf ratio	12.4 (6.7)
Cognitive evaluation	
MMSE score, mean (SD)	27.2 (1.5)

Cp = ceruloplasmin; MMSE = Mini-Mental State Examination; SD = standard deviation; Tf = transferrin.

Results

Subjects' Description

The main baseline demographic, biochemical, and clinical features of subjects enrolled in the study are reported in the Table. The frequency of allele ε4 of the *APOE* gene (*APOE4*) was 33.6%. Seventy-eight subjects (55%) had non-Cp copper levels higher than normal (>1.6 μmol/l).[12]

Prediction of Cognitive Decline

Forty-two of 141 subjects (30%) converted to AD during the follow-up observational period. According to a Cox regression model—with sex, age, baseline MMSE, *APOE4*, iron, non-Cp copper, transferrin, ferritin,

hypercholesterolemia, and hypertension as covariates—(sex, age, and baseline MMSE were entered as forced terms to adjust for their association), the only significant predictor of conversion to AD was non-Cp copper (HR = 1.23, 95% confidence interval [CI] = 1.03–1.47, $p = 0.022$). For each additional μmol/l unit of non-Cp copper, the hazard increased by ~20%. None of the other variables entered in the model had probability values consistently >0.15. Because non-Cp copper is a derived measure, we tested the model by removing non-Cp copper and entering as candidate predictors both components (copper and Cp). No variable passed the entry criteria ($p < 0.05$).

When non-Cp copper was considered as a binary variable (with respect to the cutoff of 1.6μmol/l), its association was still significant (HR = 3.34, 95% CI = 1.21–9.24, $p = 0.020$). This indicated that the hazard rate of MCI subjects with non-Cp copper >1.6μmol/l was ~3× higher than of those with values ≤1.6μmol/l. As shown in the Figure, 1 of 5 subjects with high non-Cp copper values converted to AD within 1.5 years of baseline evaluation (compared to >4 years for patients with low non-Cp copper). That is, the first 20% of conversion occurred 2.5 years earlier in MCI subjects with high non-Cp copper. In addition, the median conversion time was ~4 years in the first group and was not estimable in the second.

To compare *APOE4* with non-Cp copper in predicting MCI conversion to AD, we classified our patients as carriers (of at least 1 copy) or noncarriers of the *APOE4* genotype. Among the 128 subjects analyzed, the rate of conversion was similar between *APOE4* carriers and noncarriers (HR = 1.4, 95% CI = 0.7–2.8, $p = 0.321$). The non-Cp copper association with AD conversion was independent with respect to the *APOE4* association, as indicated by the lack of interaction ($p = 0.989$), and by the confirmation of the significant association of non-Cp copper ($p = 0.036$) when *APOE4* was entered as a stratification variable.

The lack of a correlation between partial residuals and time ($r = 0.002$, $p = 0.990$) and the linearity and parallelism of a log − log plot indicated that the proportionality assumptions were not violated.

Discussion

The most important result of this study is that a deregulation of non-Cp copper has an association with the rate of clinical conversion from MCI toward AD full dementia in an MCI cohort that received clinical follow-ups for 6 years. Our current findings show that higher levels of non-Cp copper, in the very early stages of cognitive complains, could account for a faster rate of conversion to AD. This is in line with a previous study.[14] Our data

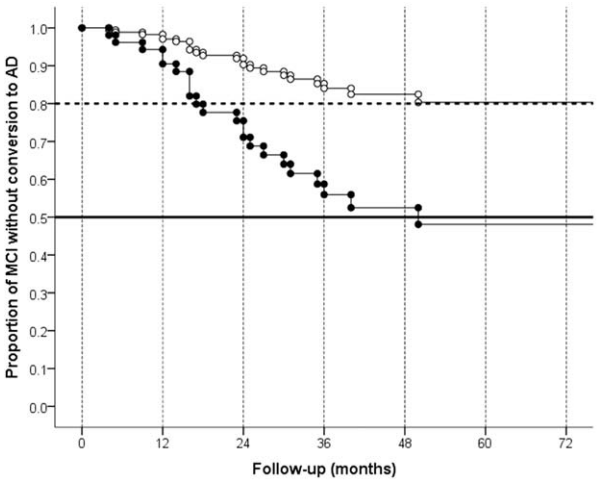

FIGURE 1: Probability of remaining at mild cognitive impairment (MCI) status during the follow-up, according to non-bound ceruloplasmin (non-Cp) copper levels (*open circles*, ≤1.6; *closed circles*, >1.6). Half of the patients in the group with high non-Cp copper values converted to Alzheimer disease (AD) after approximately 4 years. At approximately the same interval, <20% of low non-Cp copper MCI patients developed AD.

parallel those published in 2009,[22] demonstrating that non-Cp copper can predict the clinical worsening of an AD cohort followed for 1 year. Two seminal studies have been published on the predictive values of Aβ markers and tau proteins in cerebrospinal fluid (CSF) for prediction of AD conversion.[1,3] Non-Cp copper has been demonstrated to correlate well with these markers of AD in CSF.[24] These findings, along with the current results, suggest a link between metal abnormalities and Aβ cascade in the early stages of the clinical worsening. Our study has a longer observational period than those studies focusing on CSF markers.[1,3] This shows that, over a 4-year period, 50% of subjects with higher levels of non-Cp copper definitely declined to full dementia. Non-Cp copper detection in serum allows us to obtain useful information for the identification of MCI subjects at a higher risk for a rapid and pronounced conversion to AD by means of a simple blood test.

To estimate the risk posed by *APOE4* in MCI conversion to AD full dementia, numerous studies have been carried out, with results not always significant.[25] Recently, these studies have been evaluated in a meta-analysis,[25] demonstrating a final significant association between *APOE4* and MCI conversion to AD. More specifically, although 2 studies have demonstrated that *APOE4* leads to a greater decline in MCI subjects converting to AD,[26,27] an additional 6 studies have demonstrated that cognitive decline and progression to AD is not strongly related with *APOE4*.[19,28–32] Our current results are in line with the meta-analysis conclusions, as

we have observed an increase in the percentage of positive events among the *APOE4* carriers.

Our conclusion regarding the association of non-Cp copper with cognitive worsening is supported by a recent and elegant experimental study demonstrating that chronic exposure to even moderately elevated levels of copper can be dangerous and can be associated with AD pathology.[33] More precisely, extending previous findings pioneering a role of copper in AD etiology,[34,35] these authors have demonstrated that consuming 0.13mg/l of copper in drinking water for 90 days can double non-Cp copper plasma concentrations in wild-type mice.[33] Moreover, they demonstrated that non-Cp copper, which selectively regulates low-density lipoprotein receptor–related protein 1 (LRP1), decreased Aβ clearance across the blood–brain barrier in wild-type mice and increased Aβ production and neuroinflammation in a mouse model of AD.[33]

Evidence-based medicine supports the use of knowledge acquired studying populations of patients to guide decision making in clinical practice. Thus, intervention to control the risk of cognitive worsening due to non-Cp copper could be immediately adopted on the basis of 4 meta-analyses having already been published between 2011 and 2013,[36–39] along with a large epidemiological study, the Chicago Health and Aging Project,[40] published in 2006, that demonstrates the association of copper with worsening cognition. More precisely, the Chicago Health and Aging Project demonstrated a borderline correlation in a cohort of 3,718 subjects between copper intake and cognitive performance. Specifically, those subjects with a higher copper intake, along with a higher intake of saturated and unsaturated trans fats, experienced a cognitive decline equivalent to 19 additional years of aging.[40]

As a whole, this evidence supports the idea that changes toward a safer diet in those subjects with an ascertained copper dysfunction,[13] in conformity with the recommended dietary allowance (RDA) for copper (released by the Food and Nutrition Board of the Institute of Medicine of the National Academies, 2001), could be adopted immediately. Copper enters the body mainly through dietary intake (food = 75%, drinking water = 25%). RDA for copper is 0.9mg/day, whereas the tolerable upper intake level has been set at 10mg/day (Food and Nutrition Board of the Institute of Medicine of the National Academies, 2001). These values are quite impressive if one considers that 100g of liver contains 14mg of copper, with a mean rate of absorption of 30%. However, a scientific consensus will be finally and fully achieved only after a controlled clinical trial tests the proof of concept that a reduction in copper absorption can change the clinical history of cognitive decline.

We have previously performed a pilot clinical trial examining the efficacy of the copper chelator D-penicillamine in AD.[41] The study was terminated early because of ethical reasons, because 50% of the patients complained of moderate to severe adverse events. Although the D-penicillamine study was a disappointment from a clinical point of view, biochemical effects consisting of a decrease of H_20_2 concentrations, which together with copper acts as a mediator of free radical toxicity in AD, have been revealed.[6,7,41] The main important lesson to be learned from that study regards side effects of aggressive molecules, such as D-penicillamine, which strip and sequester copper from vital enzymes and proteins regulating processes essential for life and causing adverse events. Conversely, zinc therapy, as established in Wilson disease clinical care, is remarkably safer. More precisely, one of the biological effects of zinc in our body is to potentiate the decrease of copper absorption through the mucosal block. Zinc can induce a 25-fold increase of the expression of metallothioneins, which tightly bind atoms of copper and trap them into enterocytes that are eliminated through exfoliation with stools, thus preventing copper transfer into the blood. During therapy with a dosage of 150mg/day of zinc, body copper balance becomes negative, reverting copper compartmentalization and distribution from the blood to organs and tissues, including the brain. The screening of the MCI subjects on the basis of non-Cp copper serum concentrations is mandatory for personalized health care. Copper metabolic dysfunction is a condition exhibited only by a percentage of AD patients, estimated to be approximately 60% of AD patients and 50% of MCI subjects.

The present results demonstrate that copper phenotype is recognizable early in the disease course. Defining this disease phenotype provides interventions tailored to this specific AD profile. This will prevent ineffective and even potentially dangerous adverse events deriving from treating with zinc therapy AD patients not exhibiting the copper phenotype.

The considerations are as follow: (1) the tolerability of zinc; (2) a criterion of a non-Cp copper inclusion higher than normal reference values, which is mandatory for subject selection, as suggested by current data; (3) the size of the sample, which should be large enough to verify the hypothesis proposed; and (4) the choice of the MCI condition as the study population, allowing evaluation of the early stages of mental disabilities, which can more easily be reversed by the treatment. All of these considerations should be addressed in designing a new clinical trial that could provide a significant step forward beyond the D-penicillamine study.

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Authorship

R.S., R.G., and M.V. contributed to study design and manuscript preparation, drafting, and revising. M.S., L.B., and A.P. contributed to study design and data acquisition. R.S., M.V., M.M., and P.P. contributed to data analysis and interpretation. G.B., E.C., D.C., F.V., and P.M.R. contributed to patient recruitment, conceptualization of study design, and manuscript drafting.

Potential Conflicts of Interest

R.S.: patents, royalties given to Canox4drug; grants, Italian Ministry of Health, CNR Aging Project, Canox4drug; personal fees, Canox4drug.<zbmrule>

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Non-Ceruloplasmin Copper Distincts Subtypes in Alzheimer's Disease: a Genetic Study of ATP7B Frequency

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Abstract Meta-analyses show that serum copper non-bound-to-ceruloplasmin (non-Cp-Cu) is higher in patients with Alzheimer's disease (AD). *ATP7B* gene variants associate with AD, modulating the size of non-Cp-Cu pool. However, a dedicated genetic study comparing AD patients after stratification for a copper biomarker to demonstrate the existence of a copper subtype of AD has not yet been carried out. An independent patient sample of 287 AD patients was assessed for non-Cp-Cu serum concentrations, rs1801243, rs1061472, and rs732774 *ATP7B* genetic variants and the *APOE4* genotype. Patients were stratified into two groups based on a non-Cp-Cu cutoff (1.9 μM). Single-locus and haplotype-group analyses were performed to define their frequencies in dependence of the non-Cp-Cu group. The two AD subgroups did not differ regarding age, sex, MMSE score, or *APOE4* frequency allele, while they did differ regarding non-Cp-Cu concentrations in serum, allele, genotype, and haplotype frequencies of rs1061472 A > G and rs732774 C > T after multiple testing corrections. AD patients with a GG genotype had a 1.76-fold higher risk of having a non-Cp-Cu higher than 1.9 μmol/L ($p = 0.029$), and those with a TT genotype for rs732774 C > T of 1.8-fold ($p = 0.018$). After 100,000 permutations for multiple testing corrections, the haplotype containing the AC alleles appeared more frequently in AD patients with normal non-Cp-Cu [43 vs. 33 %; Pm = 0.03], while the haplotype containing the GT risk alleles appeared more frequently in the higher non-Cp-Cu AD (66 vs. 55 %; Pm = 0.01). Genetic heterogeneity sustains a copper AD metabolic subtype; non-Cp-Cu is a marker of this copper AD.

Keywords Alzheimer's disease · Copper · Ceruloplasmin · Non-ceruloplasmin-copper · Free copper · ATP7B

Introduction

Alzheimer's disease (AD) is the most common form of dementia in the elderly. It is the result of complex interactions of predisposing genes, biochemical variables, and environmental factors, including metals (copper, iron, and zinc primarily).

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Copper abnormalities in AD associate with oxidative stress, and both local (in the brain) and systemic metal dysfunction have been reported to contribute to the disease cascade [1, 2] in a pathway involving beta amyloid production and clearance [3], along with a strong enhancement of beta amyloid toxicity caused by the properties of copper oxidation-reduction [1, 4].

Blood copper can be differentiated into two pools. The larger pool comprises about 85–95 % of the total, encompassing copper covalently bound to the ceruloplasmin. The smaller pool comprises copper more loosely bound to albumin and various small molecules. This copper pool is called non-ceruloplasmin copper (non-Cp-Cu, or 'free' copper). Because of its relatively loose binding to the albumin and small peptide and amino acid pool in the blood, it is available to meet tissue needs in the body. If the non-Cp-Cu pool expands, the copper becomes toxic. This is what occurs in the case of Wilson's disease, the paradigmatic disease of non-Cp-Cu toxicosis, or accumulation [5, 6].

A number of studies have also demonstrated non-Cp-Cu involvement in AD [3, 7–9]. In living patients with AD, the non-Cp-Cu pool is larger than it is in the age-matched controls [10]. The size of the non-Cp-Cu pool correlates negatively with measures of cognition and cerebrospinal fluid markers [7, 11–13]. It correlates positively with the rate of cognition loss over time [14] and with the risk of patients converting from mild cognitive impairment—the precursor state to AD to full AD [8, 15, 16]. These studies tie copper toxicity closely to the pathogenesis of loss of cognition in AD [17, 18].

Recent meta-analyses confirm that metal abnormalities occur even at the systemic level [9, 19–22]. Zinc seems to be lower [22] and both copper and non-Cp-Cu are higher in the serum of AD patients than in healthy controls [23]. Recent genetic evidence has revealed a locus of susceptibility for AD in the gene that codes the master protein regulating copper excretion and the size of the non-Cp-Cu pool in serum: the *ATP7B* gene [24–27]. Mutations in the *ATP7B* gene typically cause Wilson's disease, which is an autosomal rare recessive disorder characterized by defective copper excretion [5, 6] and labile copper accumulation in the brain, even though the total brain copper amount is decreased [28].

In addition, it has recently been reported that AD shares many features with Wilson's disease, in terms of its etiology and potential treatment [1, 29, 30]. The existence of a subtype of AD [31], typified by the breakdown of copper homeostasis, is associated with *ATP7B* genetic variants [23]. In a previous study, we investigated the effect of certain *ATP7B* gene alleles on the size variation of the non-Cp-Cu pool, after the stratification of the subject sample into two classes based on a non-Cp-Cu cutoff [27].

Herein, we have carried out an independent replication study of rs1801243 A > C (exon 2), rs1061472 A > G (exon 10), and rs732774 C > T (exon 12) to verify whether the genetic distribution differs between the two AD groups as a result of stratification with a non-Cp-Cu cutoff of 1.9 μmol/L.

Material and Methods

Two hundred eight-seven AD patients (mean age 81.0) were included in the study. The AD patient sample consisted of individuals with a diagnosis of probable AD (NINCDS-ADRDA criteria [32, 33]) who had a Mini-Mental State Examination (MMSE) score of 25 or less [34]. Subjects were recruited at the Memory Clinic of the IRCCS Istituto Centro San Giovanni di Dio, Fatebenefratelli, Brescia, Italy.

The AD patients underwent neurologic neuroimaging (MRI or CT) and an extensive neuropsychological evaluation, as well as routine laboratory tests, to determine whether they had conditions known to affect copper metabolism and biological variables of oxidative stress (e.g., diabetes mellitus, inflammatory diseases, a recent history of heart or respiratory failure, chronic liver or renal failure, malignant tumors, or a recent history of alcohol abuse).

Local institutional ethics committees approved the study. All participating subjects gave written informed consent to be included in the study, 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.

Our study poses the working hypothesis that people with AD—whose non-Cp-Cu pool sizes have been altered—have a diverse distribution of rs1801243, rs1061472, and rs732774 *ATP7B* frequencies with respect to AD people who have a non-Cp-Cu within normal limits.

Non-Cp-Cu was measured with a new CE certified test (C4D test, 2012 CE certified test code n. 1211662 [35]). With respect to the normal reference values 0–1.6 μmol/L reported for non-Cp-Cu, as calculated with the Walshe formula [36], we used a value of 0–1.9 μmol/L herein, which was estimated in a cohort of 250 healthy subjects [35]. The upper reference limit (95 %) for the healthy population has been set to 1.90 μmol/L (its 90 % CI is equal to 1.78–2.06 [35]). To test this hypothesis, we then considered a cutoff of 1.9 μmol/L and divided the AD patients into two subgroups: a "high non-Cp-Cu" AD group, comprising 176 patients who have non-Cp-Cu > 1.9 μmol/L) and a "normal non-Cp-Cu" AD group, comprising 111 AD patients with non-Cp-Cu ≤ 1.9 μmol/L. Then, we analyzed the informative single nucleotide polymorphisms (SNPs) in these two AD groups in terms of allele, genotype, and haplotype distributions. A second step of the study sought to test whether the *ATP7B* variants under study could modulate the size of the non-Cp-Cu pool.

Biochemical and Molecular Investigations

Fasting blood samples were collected in the morning. Serum was separated and quickly stored at −80 °C for the blind biochemical measurements of the non-Cp-Cu pool by the C4D test [35] performed at Biofordrug Srl, Spin-off, Università degli studi di Bari "A. Moro." Briefly, 100 μL of serum was

charged on a solid exclusion chromatography column (ultra-high molecular weight polyethylene) and eluted at a flow rate of 200 μL/min. Only the fraction containing non-Cp-Cu was collected into a micro-plate reader 96/wells, and the fluorescent probe was added at a final concentration 10.5 M and then read in Victor3 fluorimeter at 380-nm excitation and 495-nm emission wavelengths.

Genotyping

Genomic DNA was extracted from whole blood using a method based on an organic deproteinization reagent [37]. The *ATP7B* SNPs were genotyped with one SNaPshot assay. Firstly, 200 ng of DNA of every samples was amplified in the GeneAmp® PCR System 9700 (Applied Biosystem) in a Reaction Buffer, 4 mmol/L $MgCl_2$, 0.16 mmol/L dNTPs, 0.4 μmol/L primers (Table 1), and 0.5 U Taq (BIOLINE) in 25 μL of total volume. Polymerase chain reaction (PCR) conditions were the initial denaturation and activation step of 5 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 60 °C (rs1801243) or 62 °C (rs1061472 and rs732774), and 30 s at 72 °C, as well as a 5-min final elongation step at 72 °C.

The same PCR conditions were used to amplify the *APOE* fragment, adding 2.5 μl of DMSO for each reaction and setting a 60 °C melting temperature. Both *APOE* SNPs were genotyped in a single SNaPshot assay. The SNaPshot analyses were performed according to the manufacturer's instructions, with specific primers (Table 1) and were evaluated on an ABI3100xl Genetic Analyzer. Electrophoresis results were analyzed using GeneMapper® ID Software v4.0.

Statistical Analysis

Demographic and clinical characteristics in our patient sample were described either in terms of mean ± standard deviation (SD), if quantitative, or in terms of proportions. The student's *t* test and the chi-square (χ^2) test were used to compare the characteristics of high non-Cp-Cu AD and normal non-Cp-Cu AD groups using the package SPSS 21.0. The estimation of power to detect an association between *ATP7B* and the "copper group" was evaluated using the Power of Genetic Analysis (PGA) package. The minimum detectable effect with odds ratios (OR) was calculated in a general (codominant) model, based on an alpha of 0.05 and a copper abnormality prevalence (in terms of non-Cp-Cu values higher than normal) equal to 5 % in the general population. With this sample size, we have an 80 % power to detect an OR of 1.5 if the risk allele frequency is ≥30 %.

Allele and genotype frequencies and the Hardy-Weinberg equilibrium were evaluated using Plink (Plink v1.07; http://pngu.mgh.harvard.edu/~purcell/plink/ [38]) and SPSS 21.0. The differences in the genotype distributions between high non-Cp-Cu AD and normal non-Cp-Cu AD groups were assessed through the χ^2 test. To account for multiple tests, we used the Single Nucleotide Polymorphism Spectral Decomposition (SNPSpD) program to correct the significance threshold, taking into account the linkage disequilibrium (LD) between SNPs [39]. The experiment-wide significance thresholds required to keep the type I error rate at 5 % are 0.03. So, this threshold has been taken into account to claim the statistical significance of the results after multiple testing corrections.

Logistic regression was used to calculate ORs and 95 % confidence intervals (CI) for the association between *ATP7B* SNPs and non-Cp-Cu AD groups. Because the mode of inheritance was unknown, different genetic models were considered: dominant (one copy of the allele is sufficient to increase the disease risk) and recessive (two copies of the allele are necessary to increase the disease risk).

The distribution of haplotypes was compared in normal and high non-Cp-Cu AD groups with a χ^2 test in HaploView [40] and Plink [38]. Permutation tests were used to correct multiple testing errors with 100,000 simulations. ORs and 95 % CIs were computed for each haplotype and compared to the most common haplotype with Plink. Haplotypes with frequencies greater than 1 % were considered.

Univariate ANOVA analyses were applied to determine the differences in terms of non-Cp-Cu values among AD patients

Table 1 PCR primers

PCR Primers	Forward	Reverse
ATP7B-rs1801243	5′ AGCTCCTAGGGGTTCAAAGT 3′	5′ CAAGGAAAGTTTGCAGGATT 3′
ATP7B-rs1061472-rs732774	5′ TAACAGCTGGCCTAGAACCT 3′	5′ TGAATAATTAAAGCCCAGTGAA 3′
APOE-112-158	5′ CCAAGGAGCTGCAGGCGGCGCA 3′	5′ GCCCCGGCCTGGTACACTGCCA 3′
SNaPshot Primers	Forward	Reverse
ATP7B-rs1801243		5′ AGCTCTGAGTTCTTCTGGGCTAATTACAG 3′
ATP7B-rs1061472	5′GTGCAGCGGGGCGATATCGTCA 3′	
ATP7B-rs732774		5′ CAAGGCATTCAACTTACAGGAAAGTAT3′
APOE-112	5′ACTGCACCAGGCGGCCGC 3′	
APOE-158		5′ ATGCCGATGACCTGCAGAAG 3′

stratified for *ATP7B* genetic variants. Post hoc comparisons were calculated through the Fisher's least significant difference (LSD) test.

Results

The main demographic and clinical characteristics of the patients participating in this study are reported in Table 2. One hundred seventy-six AD patients with high non-Cp-Cu and 111 AD patients with normal non-Cp-Cu AD resulted after the stratification of the sample in two groups on the basis of the non-Cp-Cu cutoff. The two AD subgroups clearly differ in terms of their non-Cp-Cu status, while they did not differ in terms of age, sex, MMSE score, or *APOE*4 frequency allele, even though normal non-Cp-Cu AD group had an increased frequency of the *APOE4* allele without reaching the statistical threshold ($p = 0.092$; Table 2).

We selected a set of genetic markers as informative SNPs of the *ATP7B*'s genetic structure validated in some previous studies [23, 25, 41].

All the genotype frequencies of *ATP7B* SNPs in this AD population were within the ranges previously reported in the data available on http://hapmap.ncbi.nlm.nih.gov [26, 27, 41].

Allele frequency distribution of rs1061472 A > G (exon 10) and rs732774 C > T (exon 12) *ATP7B* SNPs differed between high non-Cp-Cu AD and normal non-Cp-Cu AD individuals, while rs1801243 A > C (exon 2) did not (Table 3). Specifically, for rs1061472 A > G (exon 10) substitution, the risk allele G had a frequency of 65.6 % in the high non-Cp-Cu AD individuals and 55 % in the normal non-Cp-Cu AD group ($p = 0.010$; Table 3). For rs732774 C > T (exon 12), the risk allele T had a frequency of 66.2 % in AD individuals with high non-Cp Cu AD and a frequency of 56.3 % in the normal non-Cp-Cu AD group ($p = 0.017$; Table 3). Also, genotype frequencies for both rs1061472 A > G and rs732774 C > T differed between the two groups (Table 3). Specifically, the GG rs1061472 A > G genotype was 43 % in high non-Cp-Cu AD individuals, while it was 30 % in normal the non-Cp-Cu AD group, ($p = 0.029$; Table 3). Moreover, the TT genotype for rs732774 C > T (exon 12) had a frequency of 44 % in the high non-Cp-Cu AD group, as opposed to 30 % in normal non-Cp-Cu AD individuals ($p = 0.018$; Table 3).

To verify whether *ATP7B* variants adequately explain the increased risk of having non-Cp-Cu values higher than normal in AD, linear regression models were performed assuming the copper group as the dependent and the *ATP7B* SNPs as the independent variables. A significant association between rs1061472 A > G (exon 10) and rs732774 C > T (exon 12) and the non-Cp-Cu groups was revealed, with the recessive model better fitting the genetic distributions between the two groups. Specifically, AD patients with a GG genotype had a 1.76-fold higher risk of having a non-Cp-Cu higher than 1.9 μmol/L (OR 1.76, 95 % CI 1.059–2.908; $p = 0.029$). Conversely, AD patients with a TT genotype for rs732774 C > T (exon 12) had a 1.8-fold higher risk of having a non-Cp-Cu higher than 1.9 μmol/L [OR = 1.838; 95 % CI 1.110–3.44; $p = 0.018$].

After the single-locus analysis, we performed haplotype-group association analyses to investigate whether *ATP7B* haplotypes frequencies differ in terms of the dependence of the 'non-Cp-Cu group' factor. We also aimed to determine the percentage of the risk of having copper abnormalities (Table 4). rs1801243 A > C (exon 2), rs1061472 A > G (exon 10), and rs732774 C > T (exon 12) were in LD, with a high association (Fig. 1), confirming previous reports [26] and data reported for HapMap populations. The haplotype block with the three SNPs was 24 kb, while the one with the SNPs rs1061472 A > G (exon 10) and rs732774 C > T (exon 12) was less than 1 kb and had a higher association (Fig. 1).

After 100,000 permutations for multiple test corrections, the three SNP haplotype blocks containing the AAC alleles showed a statistical difference between the two AD groups, with the AAC haplotype appearing more frequently in AD patients with normal non-Cp-Cu (43 vs. 33 %; Pm values are obtained by 100,000 permutation multiple tests correction,

Table 2 Characteristics of the AD groups stratified for non-Cp-Cu

	AD patients, high non-Cp-Cu	AD patients, normal non-Cp-Cu	Significance of the comparison between groups (p)	Whole AD group
Number of subjects	176 (61.3)	111 (38.7)		287
Sex [*n* of F] (F%)	118 (67)	76 (68.5)	$p^1 = 0.80$	194 (67.6)
Age [years] mean (SD)	80.7 (6.9)	81.5 (6.8)	$p = 0.34$	81.0 (6.8)
APOE4 allele frequency [%]	24.7	31.5	$p^1 = 0.092$	27.4
Carriers of at least one *APOE4* allele [*n*] (%)	74 (42)	58 (52)		132 (46)
MMSE [score] mean (SD)	11.9 (7.5)	11.5 (6.7)	$p = 0.59$	11.7 (7.2)
Education [years] mean (SD)	6.2 (3.6)	6.2 (3.4)	$p = 0.97$	6.2 (3.5)
Non-Cp-Cu [μmol/L] mean (SD)	2.5 (0.5)	1.6 (0.3)	$p < 0.0001$	2.1 (0.6)

p indicates *t* test significance, and p^1 indicates χ^2 test nonparametric test significance

Table 3 Allele and genotype distribution of *ATP7B* variants AD groups stratified for non-Cp-Cu

ATP7B genetic variant	AD patients, high non-Cp-Cu ($n=176$)	AD patients, normal non-Cp-Cu ($n=111$)	p value (χ^2)	OR (95 % CI) p value
rs1801243 A > C				
Allele A, *n* (%)	161 (45.7)	117 (52.7)	$p=0.10$	
Allele C, *n* (%)	191 (54.3)	105 (47.3)		
AA, *n* (%)	36 (20)	27 (24)	$p=0.15$	1.25 (0.71–2.20); $p=0.44$[a]
AC, *n* (%)	89 (51)	63 (57)		
CC, *n* (%)	51 (29)	21 (19)		1.75 (0.98–3.11); $p=0.057$[b]
rs1061472 A > G				
Allele A, *n* (%)	121 (34.4)	100 (45)	$p=0.01$	
Allele G, *n* (%)	231 (65.6)	122 (55)		
AA, *n* (%)	20 (11)	22 (20)	$p=0.037$	1.93 (0.99–3.73); $p=0.051$[a]
AG, *n* (%)	81 (46)	56 (50)		1.76 (1.06–2.91); $p=0.029$[b]
GG, *n* (%)	75 (43)	33 (30)		
rs732774 C > T				
Allele C, *n* (%)	119 (33.8)	97 (43.7)	$p=0.017$	
Allele T, *n* (%)	233 (66.2)	125 (56.3)		
CC, *n* (%)	20 (11)	19 (17)	$p=0.047$	
CT, *n* (%)	79 (45)	59 (53)		1.61 (0.82–3.18); $p=0.17$[a]
TT, *n* (%)	77 (44)	33 (30)		1.84 (1.11–3.44); $p=0.018$[b]

A p value < 0.03 is considered significant according to multiple test correction, according to single nucleotide polymorphism spectral decomposition (SNPSpD)

[a] Dominant Model (wt/mut + mut/mut vs wt/wt)

[b] Recessive model (mut/mut vs wt/mut + wt/wt)

Pm = 0.03; Fig. 2). It was the same considering the two SNP haplotype blocks, with the AC haplotype appearing more often in AD patients with normal non-Cp-Cu (43 % vs. high non-Cp-Cu 34 %; Pm = 0.024).

Furthermore, the three SNP haplotype blocks containing the risk alleles at each SNP locus (CGT) were differently distributed between the two AD groups, even though they did not reach the statistical threshold (53 vs. 45 %; Pm = 0.07; Table 4). However, when considering the two SNP haplotype blocks, the GT haplotype was significantly different; the GT haplotype was more frequent in higher non-Cp-Cu AD than in normal non-Cp-Cu AD group [66 % vs. normal non-Cp-Cu 55 %; OR 1.57; 95 %CI (1.1–2.5); Pm = 0.01; Table 4; Fig. 2].

A second aim of the study was to investigate whether the *ATP7B* variants under scrutiny could modulate the size of the non-Cp-Cu pool. We classified all the AD under study as carriers or non-carriers of at least one allele of each *ATP7B* risk allele. The groups were compared for non-Cp-Cu by ANOVA separately. While rs1801243 A > C (exon 2) had no effect on the size of the non-Cp-Cu pool, carriers of at least one G in rs1061472 A > G (exon 10) and of one T in rs732774 C > T (exon 12) had increased values of non-Cp-Cu (Table 5). Furthermore, the size of the non-Cp-Cu pool differed at the significance threshold ($p=0.05$) when considering classification, since the genotype, both homozygous GG (LSD post hoc comparison, $p=0.017$) and heterozygous AG ($p=0.033$) at rs1061472 A > G (exon 10), had higher values of non-Cp-Cu than homozygous AA (Table 5). For rs732774 C > T, the size of the non-Cp-Cu pool differed regarding genotype distribution (not reaching the significance threshold, $p=0.062$), and the risk genotype homozygous TT had an increased size of the non-Cp-Cu pool that was greater than the homozygous CC (LSD post hoc comparison, $p=0.019$).

We compared the size of the non-Cp-Cu pool in the AD patients stratified for the *ATP7B* risk haplotypes identified. Specifically, we divided the entire AD population into classes that depended on the number of copies (0, 1, 2) of the GT haplotype carried. ANOVA revealed that the three AD classes differed in terms of non-Cp-Cu values [$F(2284)=3.64$, $p=0.048$]. Carriers of the GT haplotype in a single [138 AD carriers, non-Cp-Cu mean (SD) = 2.16 μmol/L (0.6); $p=0.035$] or in a double copy (107 AD carriers, 2.2 μmol/L (0.61); $p=0.016$) had higher values of non-Cp-Cu than non-carriers [42 of AD patients non-carries, 1.94 μmol/L (0.46), LSD post hoc comparison].

Stratification for *APOE*ε4 frequency revealed that carriers of at least one *APOE*ε4 allele had a lower age of disease onset ($p=0.011$) and a higher familiarity ($p=0.037$), but they did

Table 4 Frequency and associations between haplotypes of *ATP7B* and copper dyshomeostasis in AD

	rs1801243 (A>C)	rs1061472 (A>G)	rs732774 (C>T)	AD patients High Non-Cp-Cu	AD patients Normal Non-Cp-Cu	p value	OR (95% CI)	Pm value
ATP7B Haplotypes	C	G	T	0.53	0.45	Reference	Reference	0.07
	A	A	C	0.33	0.43	**0.026**	1.55 (1.06 - 2.27)	**0.03**
	A	G	T	0.12	0.09	0.71	0.90 (0.50 - 1.61)	0.61
	C	A	C	0.010	0.011	0.93	1.09 (0.18 - 6.62)	1
		G	T	0.66	0.55	Reference	Reference	**0.01**
		A	C	0.34	0.43	**0.015**	1.57 (1.09 - 2.24)	**0.024**
		A	T	0.006	0.018	0.13	3.75 (0.67 - 21.15)	0.26

After 100,000 permutations for multiple test corrections, four haplotypes with a frequency higher than 1 % resulted from the *ATP7B* rs1801243 (A > C), rs1061472 (A > G), and rs732774 (C > T) in linkage disequilibrium considering a three SNP haplotype block analysis. When considering a two-SNP haplotype block [from rs1061472 (A > G) to rs732774 (C > T)], three haplotypes resulted (gray). Pm values are obtained by 100,000 permutation multiple (Pm) for multiple tests correction. A p value < 0.03 is considered significant according to multiple test correction, according to single nucleotide polymorphism spectral decomposition (SNPSpD)

not differ for *ATP7B* alleles or haplotypes (consistently $p > 0.2$). Even though not reaching the statistical threshold, the *APOE4* carries had lower levels of non-Cp-Cu ($p = 0.061$), reflecting the result of the different distribution of the *APOE4* allele in the two AD groups stratified on the basis of non-Cp-Cu cutoff ($p = 0.092$; Table 2).

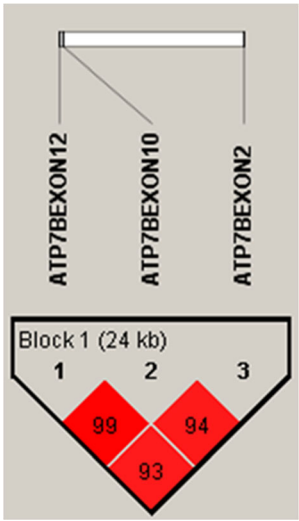

Fig. 1 rs1801243 A > C (exon 2), rs1061472 A > G (exon 10), and rs732774 C > T (exon 12) were in linkage disequilibrium (LD), with a high association among them

Discussion

The main result of this study is that single-locus and haplotype-group associations for rs1061472 A > G (exon 10) and rs732774 C > T (exon 12) *ATP7B* SNPs differed in AD patients who were stratified into two groups on the basis of a non-Cp-Cu cutoff, after multiple test corrections. These results demonstrate that the two AD groups were genetically different in terms of their *ATP7B* variant distributions. As was extensively discussed in a previous paper [23], to prove the existence of a subtype of a disease, four different strategies should be taken into account. Among them, the identification of a specific biomarker that distinguishes the cases, classifying them into sub-groups that are then compared for demographic, clinical, genetic, or biological variables, is definitely a suitable strategy. The current study has explored this exact hypothesis [23]. The results demonstrate the existence of a high copper subtype of AD. In the same line, even though not reaching the statistical threshold, the heterogeneity in terms of *APOE4* allele distribution between the two AD groups sustains our working hypothesis. Moreover, this study has provided an independent replication of the results of a previous investigation of ours, in which we compared AD patients and healthy controls [27].

As a multifactorial disease, the sporadic late onset form of AD has a complex heritability, and the genes ascertained to be associated with AD account for only a small proportion of the

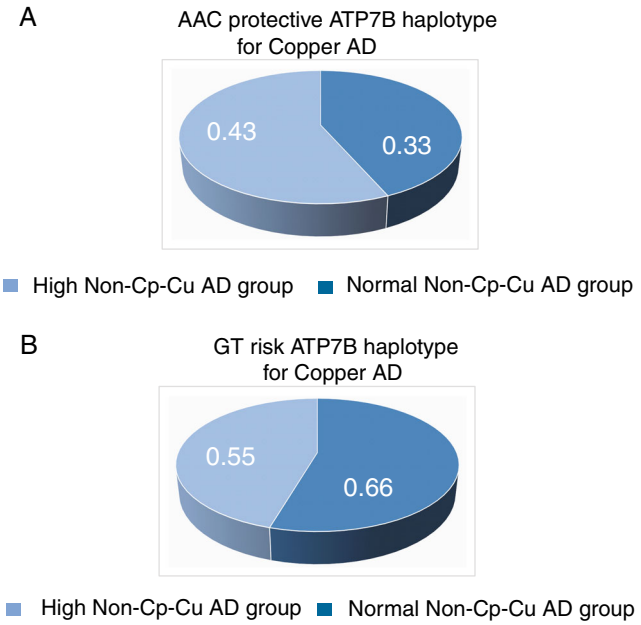

Fig. 2 Haplotypes with increased or decreased risk for copper Alzheimer's disease (AD). The pie charts show the frequency distribution (percentage) of the ATP7B haplotypes between the high non-Cu-Cp AD group (dark blue) and the normal non-Cp-Cu AD group (light blue). **a** Protective haplotype: the AAC haplotype appears more frequently in AD patients with normal non-Cp-Cu (43 vs. 33 %; Pm values are obtained by 100,000 permutation for multiple test correction, Pm = 0.03). **b** Risk haplotype for copper AD: the GT haplotype appears more frequently in the higher non-Cp-Cu AD than in normal non-Cp-Cu AD group [66 % vs. normal non-Cp-Cu 55 %; OR 1.57; 95 % CI (1.1–2.5); Pm = 0.01) (color figure online)

genetic contribution to AD. While the role of *APOE4* has been established, and we confirm in the current dataset an effect on the age of onset and familiarity, the involvement of the *ATP7B* gene is still under investigation. Previous genetic association studies demonstrated that *ATP7B* contributes to global susceptibility to AD [25–27, 42]. In line with previous investigations, about 60 % of AD patients evaluated in the current study had values of non-Cp-Cu higher than normal [43]. These AD patients had a diverse frequency of some common *ATP7P* SNPs (Table 3), and the results of the second facet of this study (Tables 4 and 5) confirm that they have effects on the size of the non-Cp-Cu pool, which in turn has been demonstrated to have a close link to the disease onset and progression [8, 13, 14].

We have demonstrated that, even after multiple test corrections, the haplotype AAC was more frequent in the normal non-Cp-Cu AD, engendering a protective effect on the probability of having higher levels of non-Cp-Cu, and that the haplotype with the GT risk alleles was more frequent in high non-Cp-Cu AD patients (Table 4); their probability of having a non-Cp-Cu pool larger in size than normal increased by approximately 60 %.

While the rs1801243 (exon 2) does not seem to be associated with increased levels of non-Cp-Cu pool, rs1061472 A > G (exon 10) and rs732774 C > T (exon 12) appear to be strongly associated with each other and with the copper AD. They also have a significant effect on the size of the non-Cp-Cu pool.

Table 5 *ATP7B* modulation of the size of the non-Cp-Cu pool in AD patients

ATP7B genetic variant	No. of AD carriers (%)	Non-Cp-Cu (μmol/L) mean (SD)	*p* value (χ^2)
rs1801243 A > C			
Non-carriers of C allele	63 (22)	2.06 (0.55)	$F(1285) = 1.262$; $p = 0.262$
At least one C allele	224 (78)	2.16 (0.61)	
AA genotype	63 (22)	2.06 (0.55)	$F(2284) = 0.742$; $p = 0.48$
AC genotype	152 (53)	2.15 (0.61)	
CC genotype	72 (25)	2.19 (0.61)	
rs1061472 A > G			
Non-carriers of G allele	42 (14.6)	1.94 (0.46)	$F(1285) = 5.854$; $p = 0.016$
At least one G allele	245 (85.4)	2.17 (0.61)	
AA genotype	42 (14.6)	1.94 (0.46)	$F(2284) = 3.022$; $p = 0.05$
AG genotype	137 (47.7)	2.16 (0.61)	
GG genotype	108 (37.7)	2.19 (0.61)	
rs732774 C > T			
Non-carriers of T allele	39 (13.6)	1.94 (0.48)	$F(1285) = 5.080$; $p = 0.025$
At least one T allele	248 (86.4)	2.17 (0.61)	
CC genotype	39 (13.6)	1.94 (0.48)	$F(2284) = 2.814$; $p = 0.062$
CT genotype	138 (48.1)	2.15 (0.61)	
TT genotype	110 (38.3)	2.20 (0.61)	

A *p* value <0.03 is considered significant according to multiple test correction, according to single nucleotide polymorphism spectral decomposition (SNPSpD). Carriers of the risk allele/genotype at each SNP were compared for non-Cp-Cu levels

Allele frequency distributions reported in our current and past studies [26, 42] are in line with those reported in internet-available databases [1000 genomes on NCBI, Hap-map (available on http://hapmap.ncbi.nlm.nih.gov)] and in previous investigations [41]. In the population Cauc1 (31 individuals), Cauc2 (60 unrelated controls), and Cauc3 (66 unrelated controls) in NCBI, the frequency of the allele G of rs1061472 is 58, 50, and 64 %, respectively, and on average, it is very close to 54 %. This conforms to what we found previously, when analyzing a sample of 230 healthy control subjects [26] and is close to the frequency of 51 % reported for the CEU population (90 subjects) of the HapMap project [41]. The frequency of this allele in AD patients increases to 61 %, as reported in our previous study analyzing a population of 285 AD patients [26] and has been confirmed in the current AD dataset (61 %). It is worth noting that the stratification for copper abnormalities that we have applied in this study revealed an increased frequency, reaching 65 % in the high non-Cp-Cu AD and 55 % in the AD patients without signs of copper dysfunction. Similarly for rs732774 C>T (exon 12), the risk allele T has a frequency of 59, 50, and 65 % in Cauc1, Cauc2, and Cauc3, respectively. On average, it is close to 52 %, which corresponds to what we found in our previous study on 230 healthy control subjects [26]. This frequency is very close to the frequency of 51 % reported for the CEU population of the HapMap project [41]. The frequency of this allele in AD patients was 59 % in our previous study [26] and 61 % in the current one, which becomes 66 % when considering the sole high non-Cp-Cu AD, indicating an AD heterogeneity and the value of the stratification of AD patients on the basis of copper abnormalities.

As was previously showed [8, 26], at difference from reported in the past for total serum copper [2], the study in this AD population confirmed no significant correlation between the size variation of the non-Cp-Cu pool and the *APO*E genotype, suggesting independent pathogenic pathways for *ATP7B* and *APOE*. Moreover, even though it did not reach the statistical threshold, the *APOE4* allele appeared more frequently in the normal copper AD (Table 2), suggesting the heterogeneity of the two AD populations even in terms of *APOE4* distribution.

The *ATP7B* gene codes for a copper-pump in the Trans Golgi network of the hepatocyte, which controls copper excretion through the bile and copper incorporation into nascent ceruloplasmin [44]. More specifically, based on findings on the *ATP7B* mutation in inbred LEC rats that develop Wilson's disease, the ATP7B copper pump has been demonstrated to alter copper transport at more than one cellular site, influencing copper entry into lysosomes and copper excretion into the bile. Moreover, the ATP7B copper pump loads copper into nascent ceruloplasmin, accounting for the pleiotropic effects of the gene on copper homeostasis [44, 45].

Although guidelines report that Wilson's disease is an established monogenic disorder that acts upon *ATP7B* mutations, heterogeneity in the clinical phenotype has been observed, even among patients harboring mutations that affect the mutant protein similarly (e.g., different truncation mutations in the same region of the gene or exactly the same set of mutations in two or more sibs, as reviewed in [23, 46]). A direct effect of diet on the severity of the biochemical lesion and the clinical phenotype is shown by studies in LEC rats demonstrating an early and rapid onset of fulminant hepatitis for a high-copper dietary regimen but long-term survival for a low-copper dietary regimen. In addition to diet, recent observations sustain evidence that modifying loci can modulate the phenotype resulting from the aberration in the *ATP7B* gene. Two of the candidate genes that might play the role of a modifier locus are the copper chaperones ATOX1 [47] and MURR1 (renamed COMMD1) [48]. All these factors have an effect on the severity of the copper homeostasis breakdown and impact the clinical phenotype and age of onset of the disease. As a result, the diagnosis of Wilson's disease depends on a number of clinical and copper signs [49]. In fact, a variety of clinical and biochemical signs can refer to Wilson's disease: hepatic disturbances as well as neurological, psychiatric, hematological, renal, or osteochondrosis symptoms [17]. The age of onset varies from childhood to adulthood, and late-onset Wilson's disease (over 70 years of age) has been documented [17]. About 20 % of people with Wilson's disease have a psychiatric presentation, and cognitive impairments have been described as seriously disturbing people with advanced or untreated Wilson's disease, often unrecognized patients, presenting, generally, executive function complaints [17].

For a second facet of our current study, we demonstrated that the specific *ATP7B* gene variants under investigation effect the size of the non-Cp-Cu pool. Diet and modifier genes corroborate these effects in individuals exhibiting a susceptibility for non-Cp-Cu abnormalities.

On the one hand, the significant differences in terms of allele, genotype, and haplotype frequencies of the *ATP7B* genetic variants under investigation demonstrated a genetic heterogeneity of the two AD populations generated by the use of the biomarker (C4D test) typifying the copper subtype of AD. On the other hand, a 10 % difference in the distribution of the *ATP7B* gene variant frequencies puts forward the existence of additional loci in the same gene, as well as on potential modifier genes, along with a relevant effect of diet. As a matter of fact, the *ATP7B* gene is a large gene (more than 500 Wilson's disease mutations and 800 SNPs have been reported), and in a previous study, we extensively demonstrated a LD association with the loci under investigation in the current study, with unknown spots within the gene region coding the transmembrane domains of the ATP7B ionic pump (Block 1 rs732774-rs1061472 [26]). These still elusive and probably rare loci in the *ATP7B*, along with those in *ATOX1* and *COMMD1* genes, can considerably impact the probability of having copper AD (reviewed in [2]).

The involvement of *ATP7B* in AD onset [18] has been questioned. Some researchers argue that, when homozygous, carriers of *ATP7B* mutations show a huge increase in their

labile circulating copper pool (as exemplified by Wilson's disease). Specifically, an author claimed [18] that Wilson's disease patients could be generally considered to be free from detectable complaints in cognition: at most, they have behavioral problems which can give the impression of cognitive loss, but it is not real cognitive loss [18]. However, *ATP7B* homozygotes are treated (or die while they are still young), and the copper overload is eliminated, thus annulling the effect of the *ATP7B* gene on the risk of cognitive complaints. Conversely, heterozygotes have a mild copper overload that lasts a lifetime, and this can affect cognition in the elderly [2, 8–10, 13, 14, 17, 18]. As demonstrated for other genes associated with monogenic forms of Parkinson's disease (e.g., GTP-Ch1, Parkin, PINK1, Dardanin, SMPD19), we posit that *ATP7B* specific loci in the recessive autosomal mendelian trait cause a monogenic disease with a low penetrance (Wilson's disease); heterozygosity in individuals can be a risk factor for the sporadic form of copper AD.

A limit of our study is the size of the sample. A larger patients' sample would have allowed us to reach the significant threshold for the difference of the two AD groups in terms of *APOE4* frequency. An additional limitation pertains to the fact that our results remain informative of the presence of hidden hot spots for AD susceptibility in specific *ATP7B* gene regions and provide no information about the potential effects of modifier genes and diet. A dedicated DNA sequencing study to identify these mutations is in progress.

This line of evidence, along with findings from meta-analyses [9, 19–21], large population studies [50–52], in vitro models of copper toxicity in the beta amyloid disease cascade [1, 4], and animal models of the disease [3], supports biomarker science applied to copper AD. Biomarker science sets the stage for developing drugs that can be used to take control of disease-causing molecular pathways before clinical symptoms develop. For example, a drug, along with suggestions to the public for disease prevention aimed at correcting risk factors, as recently reported [53], would delay the onset of AD by some years and save billions in direct health costs [54].

The potential benefits for copper AD also appear enormous in terms of precision medicine, which can take advantage of the experience acquired in Wilson's disease. However, a controlled clinical trial testing the "proof of concept" that a reduction in copper absorption can change the clinical history of AD cognitive decline would grant a full scientific consensus (in progress).

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Compliance with Ethical Standards

Conflict of Interest Authors declare nothing to disclose, and Rosanna Squitti is consult for Canox4drug, SpA

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Excess of nonceruloplasmin serum copper in AD correlates with MMSE, CSF β-amyloid, and h-tau

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Abstract—*Objective:* To assess whether serum copper in Alzheimer disease (AD) correlates with cognitive scores, β-amyloid, and other CSF markers of neurodegeneration. *Methods:* The authors studied copper, ceruloplasmin, total peroxide, and antioxidants levels (TRAP) in serum; β-amyloid in plasma; and copper, β-amyloid, h-tau, and P-tau in the CSF of 28 patients with AD and 25 healthy controls, in relation to clinical status. *Results:* Serum copper ($p < 0.0001$), peroxides ($p = 0.002$), a copper fraction unexplained by ceruloplasmin ($p < 0.0001$), and CSF h-tau ($p = 0.001$) were increased in AD, whereas serum TRAP ($p = 0.03$) and CSF β-amyloid were decreased ($p < 0.0001$). Plasma β-amyloid increased with age in healthy controls ($r = 0.6$; $p = 0.05$). CSF markers of AD correlated with serum copper variables. CSF copper was partially dependent on the serum copper fraction unexplained by ceruloplasmin ($t = 2.2$, $p = 0.04$). CSF β-amyloid seemed to be related to serum copper ($r = -0.46$; $p = 0.002$). Mini-Mental Status Examination scores correlated positively with β-amyloid ($r = 0.46$, $p = 0.002$) and inversely with copper unexplained by ceruloplasmin ($r = -0.45$, $p = 0.003$). *Conclusions:* The authors' results confirm the existence of changes in copper component distribution, particularly the copper fraction unexplained by ceruloplasmin and support the hypothesis of a β-amyloid and copper connection in Alzheimer disease.

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Metals, such as zinc, copper, and iron, and oxidative stress are likely involved in the neurodegeneration of Alzheimer disease (AD).[1-5] Both iron and copper can catalyze a flux of reactive oxygen species that can damage functional and structural macromolecules in the brain.[3,5] The toxicity of β-amyloid and tau proteins may depend on copper-mediated free radical generation and on copper- and zinc-induced deposition of amyloid plaques.[6,7] β-Amyloid seems to be a high-affinity copper protein that binds copper when purified from AD brains.[7] Although the large body of in vitro and animal models supporting the role of copper in AD, only few in vivo studies on patients have been carried out so far. We previously reported that the ceruloplasmin-copper relationship, rather than absolute serum copper levels, could represent a key issue in interpreting in vivo copper findings in AD.[8-10] Even though a dysfunction in serum copper balance has been previously shown to correlate with a patient's clinical status, this has never been investigated in relation to other biologic and clinical markers of neurodegeneration in AD.[8]

In this study, we sought to estimate the correlation between the excess of serum copper not related to ceruloplasmin and documented candidate markers of neurodegeneration in AD, namely, CSF β-amyloid and tau proteins, as a means to verify the clinical significance of increased unexplained copper in AD; moreover, the relationship with other established neuropsychological markers of disease was also carried out in the same subjects.

Additional material related to this article can be found on the *Neurology* Web site. Go to www.neurology.org and scroll down the Table of Contents for the July 11 issue to find the title link for this article.

Methods. Twenty-eight patients with AD (mean age 71.4 years) and 25 elderly controls (mean age 70 years) were included in the study; the groups did not differ in sex and age (table 1). The AD patient sample consisted of individuals with a diagnosis of probable AD according to National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association criteria[11] and a Mini-Mental Status Examination (MMSE)[12] score of 25 or less. Patients with mild cognitive impairment (MCI) were excluded from the study. AD patients underwent neurologic, neuroimaging (brain MRI), and extensive

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Table 1 *Demographic characteristics of the investigated groups*

	AD patients	Controls	Significance
No. of subjects	28	25	
Sex, M/F	8/20	8/17	$\chi^2 = 0.07$*; $df = 1$; $p = 0.51$
Age, y, mean (SD)	71.4 (8.6)	70 (9.6)	$F_{1,50}$† = 0.33; $p = 0.57$
MMSE, mean (SD)	15.5 (6.2)	28.4 (1.2)	$F_{1,50}$† = 114.8; $p < 0.0001$

* Chi-square test; significant at the $p = 0.05$ level.

† Analysis of variance F test; significant at the $p = 0.05$ level.

neuropsychological evaluation as well as routine laboratory tests in order to exclude other types of dementia. The control sample consisted of 25 healthy volunteers with no clinical evidence of neurologic and psychiatric disease. Criteria for exclusion of both patients and controls were conditions known to affect copper metabolism and biologic variables of oxidative stress (e.g., diabetes mellitus, ongoing inflammatory diseases, recent history of heart or respiratory failure, chronic liver or renal failure, malignant tumors, and a recent history of alcohol abuse). We found no evidence of blood-brain barrier (BBB) dysfunction as estimated by the albumin ratio between CSF and serum (data not shown). Although a neuroimaging (MRI or CT) examination was done in all AD patients to exclude other treatable causes of dementia, for 16 of them and eight controls, an extensive MRI study was performed (data not shown). Briefly, volumetric analysis of the hippocampus was visually performed by scoring every patient according to the width of the choroid fissure and the temporal horn, following the criteria of a rating scale of temporal lobe atrophy previously reported (5-point rating scale of medial temporal lobe atrophy [MTA]).[8] MRI revealed that atrophy of the medial temporal lobes with a reduction in hippocampal volume (score 2 to 4) was prominent in AD, whereas control subjects' MTA visual rating scale values were 0 or 1.

Nine patients with AD did not complete the extensive neuropsychological test battery. All healthy controls underwent a neurologic examination and an MMSE, yet only few cooperated with extensive neuropsychological testing. An investigation of β-amyloid content in plasma was performed in 20 AD patients and 14 healthy controls whose ethylenediamine tetraacetic acid (EDTA) plasma sample was available. The subgroups did not differ in sex and age, and MMSE and biologic variables differences were confirmed (data not shown).

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

Biochemical and molecular investigations. Sera from fasting blood samples and CSF by lumbar puncture were collected in the morning, centrifuged, and rapidly stored at −80 °C until analysis. All participants underwent lumbar puncture; however, for four AD patients and five controls, the CSF sample was not sufficient for copper measurements. Copper concentration was measured following the method of Abe et al.[13] (Randox Laboratories, Crumlin, UK) and by an A Aanalyst 300 Perkin Elmer atomic absorption spectrophotometer equipped with a graphite furnace with platform HGA 800.[14] Measurements of biologic variables of oxidative stress are described in detail elsewhere.[14,15] Briefly, hydro- and lipoperoxide content was assessed by d-ROMs test (Diacron, Italy) and expressed in arbitrary units (U.CARR), with 1 U.CARR corresponding to 0.08 mg/100 mL hydrogen peroxide.[16] Normal range was between 230 and 310 U.CARR.[14-16] Total radical trapping antioxidant capacity, or TRAP, was assayed by the TAS kit (Randox Laboratories), based on published methods.[17] The serum reference range is 1.30 to 1.77 mmol/L.[15,17] Ceruloplasmin was measured by immunoturbidimetric assays (Roche Diagnostic, Germany) using a rabbit anti-human ceruloplasmin antibody in phosphate buffer.[18] CSF levels of total tau protein (h-tau), Thr 181 phosphorylated tau protein (P-tau), and β-amyloid 1-42 both in CSF and EDTA plasma were determined by ELISA (INNOGENETICS, Belgium).[19-21]

All biochemical measures from serum samples were automated on a Hitachi 912 analyzer (Roche Diagnostics) and performed in duplicate.

Cognitive evaluation. Neuropsychological testing included the MMSE,[12] and the Mental Deterioration Battery,[22,23] as described elsewhere.[15] The battery, which comprises seven neuropsychological tests investigating the functional efficiency of language, verbal memory, visual memory, logical reasoning, and constructional praxis areas of cognition, was compressed into three factors providing information of three main cognitive domains: factor 1 (visuospatial), factor 2 (verbal memory), and factor 3 (language).[24] Because these three factors were demonstrated to account for 75% of total variance, we lost only 25% of the total information provided by the complete battery and obtained a clear reduction of the variables to be evaluated.

Statistical analyses. Patients with AD and controls were described in terms of main demographic and cognitive characteristics and statistically compared with analysis of variance F and χ^2 tests.

A linear regression model between serum copper and ceruloplasmin, previously described,[8] was applied, and the two derived copper measures, copper "explained" and copper "unexplained" by ceruloplasmin, were calculated for each subject. Further linear regression models were calculated to evaluate CSF copper variations depending on the unexplained fraction of copper and to estimate serum copper dependence on β-amyloid changes in CSF.

Correlation analyses between biologic variables in the serum and in the CSF and between biologic variables and neuropsychological scores were performed (Pearson's r). As stated in the cognitive evaluation section, a data reduction was performed before applying statistical tests. A better cases-to-variables ratio could be obtained and, thus, a reduction of both type I (alpha inflation) and type II error (to not recognize a true difference or a true correlation). Therefore, exact p values were reported and no attempt to further control the alpha inflation was applied.

The statistical software package SPSS 13.0 for windows (SPSS Inc., Chicago, IL) was used for all analyses.

Results. Patients and controls did not differ in sex and age, but differed on the MMSE ($p < 0.0001$) (table 1). None of the oxidative and metal species measured correlated with age and sex (data not shown).

The data on copper, ceruloplasmin, peroxides, and TRAP in the serum of AD patients and controls is summarized (see table E-1 on the *Neurology* Web site at www.neurology.org). Although the results for copper shown here were obtained by the colorimetric method,[13] those obtained by atomic absorption spectrophotometry assay were superimposable (intraclass correlation coefficient for consistency = 0.9, $p < 0.0001$). Copper ($p < 0.0001$) and peroxides ($p = 0.002$) in serum were increased in AD patients compared to controls, whereas TRAP was decreased ($p = 0.03$).

To assess the copper-ceruloplasmin relationship in serum, we calculated a linear regression considering copper as the dependent variable in the control group (regression slope 0.51; 95% CI: 0.19 to 0.83). The estimated association was superimposable with the one reported in a previous study.[8] Thus, to further improve the accuracy of the copper-ceruloplasmin relationship, we re-estimated the regression on a larger set of healthy controls, consisting of 25 subjects recruited in the present study and 44 subjects enrolled in the previous one (see figure E-1).[8] The regression model, valid on average in the range of the healthy controls evaluated, was now y(μmol/L explained copper) = 4.4 × (μmol/L ceruloplasmin) + 4.5; and the ratio between the explained copper, calculated with this equation, and μmol/L concentration of ceruloplasmin was consequently 6.68 (0.3), mean (SD). Therefore, on average, in the healthy subjects analyzed, 1 μmol/L ceruloplasmin can be considered to account for 6.68 μmol/L explained copper. This value is consistent with the notion that ceruloplasmin contains six copper atoms per molecule.[25] Moreover, to further circumstantiate our concept of "explained copper," we compared the portion of copper bound to ceruloplasmin

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Table 2 *Markers of CSF in controls and AD patients: Mean (SD)*

	AD patients, n = 28	Controls, n = 25	ANOVA
β-Amyloid (ng/mL)	0.26 (0.11)	0.5 (0.25)	$F_{1,48} = 19.86$ $p < 0.0001$
h-tau (ng/mL)	0.48 (0.27)	0.27 (0.14)	$F_{1,49} = 11.68$ $p = 0.001$
P-tau (ng/mL)	0.05 (0.03)	0.06 (0.06)	$F_{1,48} = 1.004$ $p = 0.3$
CSF copper (μmol/L)	0.4 (0.14)	0.44 (0.15)	$F_{1,42} = 0.91$* $p = 0.35$

* Number of Alzheimer disease patients is 24 and the number of healthy controls is 20.

(CB), which can be calculated theoretically as follows[26]: B = n * ceruloplasmin mg/L; n = 0.0472 (μmol/mg), with the explained copper as we estimated. The mean value (SD) of the CB obtained in our control population was 12.4 (1.9) and did not differ from the mean value (SD) of the estimated explained copper 13 (1.3) (*t* test; $p = 0.061$). By subtracting the explained copper from the total serum copper, the unexplained copper value can be estimated (Cu unexplained = serum copper – Cu explained). The unexplained copper could thus be referred to as "nonceruloplasmin-bound copper" (NCC).[26-29] The explained and unexplained copper were calculated for each serum copper and ceruloplasmin pair. Both serum copper explained and unexplained by ceruloplasmin were higher in AD patients (explained, $F_{1,43} = 4.38$, $p = 0.04$; unexplained, $F_{1,43} = 19.09$, $p < 0.0001$). In an attempt to validate the concept of a copper fraction unbound to ceruloplasmin that could cross the BBB in AD, experiments were performed on two separate pools of sera from 20 AD patients and 20 controls that were filtered through a 10-kd cutoff membrane. Hard data revealed that in AD, the amount of copper measured in the ultrafiltrate by absorption spectroscopy was higher than controls. On these bases, according to published procedures,[30] a free copper/protein-bound copper (F/PB) ratio (%) can be obtained, which was 3.7-fold higher in AD than in controls (preliminary results).

In table 2 comparisons of the biologic variables evaluated in the CSF for the two groups of subjects are summarized. Copper values reported have been obtained by atomic absorption spectrophotometry. Levels of h-tau were increased ($p = 0.001$), whereas β-amyloid was decreased ($p < 0.0001$) in AD patients. CSF copper and P-tau did not differ between groups. Even if not reaching the significance threshold, a positive correlation between copper and β-amyloid, both measured in the CSF, could be evidenced in the AD population only ($r = 3.93$, $p = 0.064$).

The data set analyzed in table 2 (see also table E-1) was then elaborated to address correlations between biologic variables in the serum (total copper and total peroxides) and neurodegenerative AD markers in the CSF (β-amyloid, h-tau, and P-tau; figure and table 3). Serum copper correlated inversely with β-amyloid ($r = -0.35$; $p = 0.02$), a correlation driven by the controls ($r = -0.6$, $p = 0.02$) (figure, A), and positively with h-tau ($r = 0.43$; $p = 0.003$; figure, B) in the CSF. Total peroxides in the serum correlated with CSF h-tau ($r = 0.4$; $p = 0.03$; figure, C). A correlation of peroxides with copper was also found in serum ($r = 0.95$; $p < 0.0001$ figure, D). When correlations between β-amyloid and tau protein burdens in CSF and explained and unexplained copper in serum were taken into account, it appeared that they were driven by the serum copper unexplained fraction (table 3). Moreover, ap-

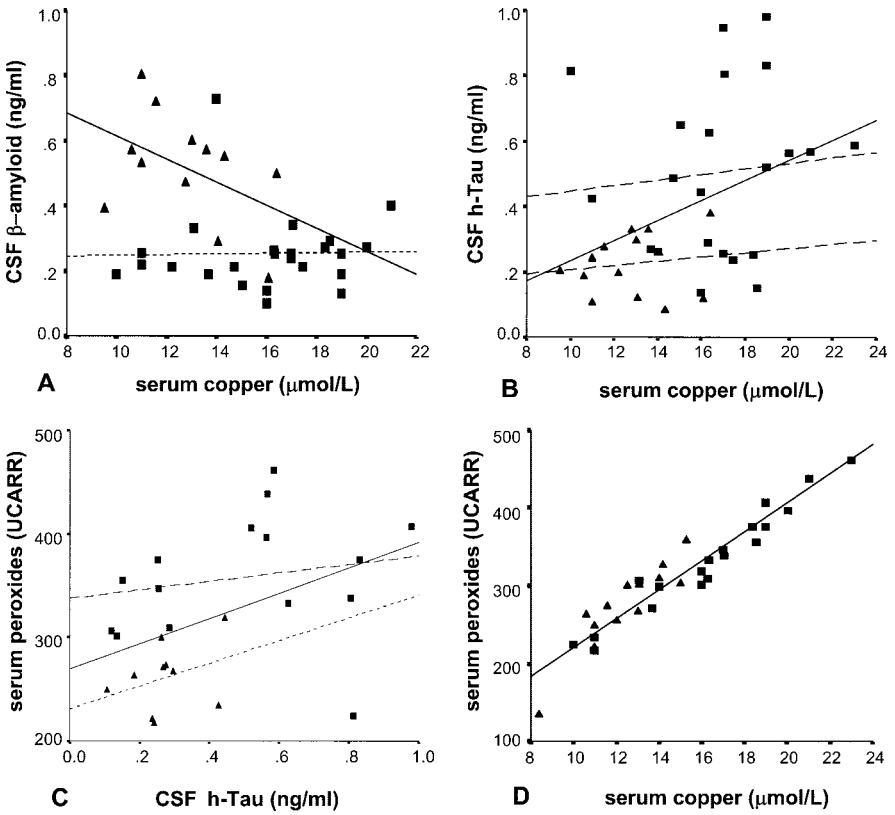

Figure. Scatter plots of the correlations between the biologic variables under study; AD patients (■) and controls (▲). (A) Serum copper inversely correlates with β-amyloid in the CSF (log-linearized correlation: r = −0.38; p = 0.04); (B) serum copper positively correlates with h-tau in CSF (r = 0.4; p = 0.03; (C) total serum peroxides correlate with CSF h-tau (r = 0.4; p = 0.02); (D) total peroxides strongly correlate with serum copper (r = 0.95; p < 0.01).

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Table 3 Correlations between copper explained and unexplained by ceruloplasmin in the serum and CSF markers of neurodegeneration

	CSF		
Serum	β-Amyloid (ng/mL) (n = 42)	h-tau (ng/mL) (n = 43)	P-tau (ng/mL) (n = 43)
Copper explained (μmol/L)			
r	−0.1	−0.21	−0.12
p	0.52	0.18	0.43
Copper unexplained (μmol/L)			
r	−0.46	0.49	−0.21
p	0.002	0.001	0.17

Pearson correlation coefficient (*r*) significant at the 0.05 level.

plying a regression model that assumes β-amyloid in the CSF as the independent variable and copper as the dependent one, an increment of copper depending on CSF β-amyloid decreasing amount in the CSF could be quantifiable only in healthy controls (linear regression model, $t = -2.6$, $p = 0.03$).

The relationship between CSF copper and the two serum copper fractions was also investigated. We found that copper in the CSF was positively associated only with the unexplained copper ($r = 0.41$; $p = 0.03$). To test the hypothesis of CSF copper variations depending on the unexplained fraction of copper, we developed a regression model that assumes the unexplained copper as the independent variable and the CSF copper as the dependent one. With this model, a unit micromolar increase in unexplained copper in the serum produces an expected 0.03-μM increase of copper in the CSF in AD patients (linear regression model, $t = 2.2$, $p = 0.04$). The same model did not reach significance in the controls ($t = 2.2$, $p = 0.06$).

Plasma β-amyloid positively correlated with age ($r = 0.6$; $p = 0.05$) in healthy controls; therefore, age was used as covariate in the statistical analysis. Plasma β-amyloid was lower in AD patients (0.03 ± 0.02 ng/mL) than in healthy controls (0.05 ± 0.02 ng/mL; $F_{1,31} = 6.42$, $p = 0.02$). The analysis of correlations between plasma β-amyloid and the other biologic variables under study revealed a positive association with CSF β-amyloid ($r = 0.42$, $p = 0.02$), driven by controls. In the AD group, a plasma β-amyloid increase corresponded to an increase in total serum copper ($r = 0.53$, $p = 0.029$) that could be ascribed to the fraction of copper unexplained by ceruloplasmin ($r = 0.54$, $p = 0.03$).

Correlations were also found between cognitive domain deficit measures and biologic variables (table 4). In particular, factor 1 (visuospatial ability) correlated with CSF copper ($r = -0.524$, $p = 0.05$, only 14 cases available for correlation, table 4). Higher scores of factor 2 (verbal memory) corresponded to higher β-amyloid concentrations in the CSF ($r = 0.43$, $p = 0.03$) and to lower levels of unexplained copper in the serum ($r = -0.45$, $p = 0.04$); when factor 2 was split into its neuropsychological components, higher Rey's Delayed Recall Test scores corresponded to higher soluble CSF β-amyloid concentrations ($r = 0.45$, $p = 0.023$) and to lower unexplained serum copper ($r = -0.52$, $p = 0.01$). It is worth noting that verbal memory, the function investigated by the Rey's Delayed Recall Test, is the main cognitive function impaired in AD. MMSE score was evaluated as an independent variable: results showed that a higher MMSE score corresponded to higher β-amyloid concentrations in both CSF ($r = 0.46$, $p = 0.002$) and plasma ($r = 0.48$, $p = 0.005$) and to lower copper unexplained by ceruloplasmin in the serum ($r = -0.45$, $p = 0.003$).

Table 4 Correlation of neuropsychological examination scores with copper, markers of neurodegeneration in CSF, and biologic variables of copper metabolism in the serum

	Factor 1 (visuospatial)	Factor 2 (verbal memory)	Factor 3 (language)	MMSE
β-Amyloid (ng/mL)				
r	0.03	0.43	0.19	0.46
p	0.92	0.03	0.37	0.002
n	17	25	24	43
h-tau (ng/mL)				
r	−0.28	−0.33	−0.26	−0.29
p	0.27	0.11	0.22	0.06
n	17	25	24	44
P-tau (ng/mL)				
r	0.20	0.23	0.26	0.16
p	0.45	0.26	0.22	0.29
n	17	25	24	43
CSF copper (μmol/L)				
r	−0.524	−0.039	−0.324	−0.001
p	0.05	0.869	0.176	0.99
n	14	20	19	42
Copper explained (μmol/L)				
r	0.26	−0.08	0.10	−0.27
p	0.35	0.72	0.68	0.07
n	14	21	20	43
Copper unexplained (μmol/L)				
r	−0.37	−0.45	−0.41	−0.45
p	0.20	0.04	0.07	0.003
n	14	21	20	43

Pearson correlation coefficient (*r*) significant at the 0.05 level.

MMSE = Mini-Mental Status Examination.

Discussion. An excess of serum copper significantly correlates with biologic markers of neurodegeneration in the CSF of patients with AD. In particular, data suggest that the inverse dependence of serum copper on β-amyloid content in the CSF occurred mainly in the control population, whereas the variations of CSF copper amount depending on the nonceruloplasmin-bound fraction were prominent in AD.

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The present in vivo study can provide information on the physiologic and dynamic aspects of copper metabolism, focusing on the move of this metal from serum to brain via a low molecular weight component and from brain to serum via an APP β-amyloid–mediated mechanism.

Copper bound to low molecular weight complexes can penetrate into erythrocytes and hepatocytes and also into brain cells.[31] The move of nonceruloplasmin copper from serum to brain is best exemplified by Wilson's disease in which impairment upon mutation of ATPase7B, which is needed in the liver for the incorporation of copper into ceruloplasmin and for the excretion of copper into the bile, results in the absence of holoceruloplasmin synthesis and liver copper overload. When the capacity for copper hepatic storage is exceeded, cell death ensues, and copper is released into the blood in the form of low molecular weight complexes, which results in deposition of copper in extrahepatic tissues, namely, cornea (with the formation of the diagnostic Kaiser- Fleischer rings) and brain.[31,32] Here it is demonstrated in patients with AD that the amount of serum copper unbound to ceruloplasmin may be associated with the copper contained in the CSF, as revealed by the regression model expressing that, in AD, 1 μM of unexplained copper in the serum accounts for 0.03-μM increase in copper content in the CSF. The idea of a move of copper from brain to serum via an APP β-amyloid–mediated mechanism finds support in a recent biologic model hypothesizing that copper binding to APP in the extracellular milieu could favor the processing of the APP-copper complex in a nonamyloidogenic pathway, resulting in a reduction in β-amyloid in the CSF and copper removal from the neuronal tissue via the bloodstream.[33] In our study, the inverse dependence of serum copper on CSF β-amyloid in the healthy controls fits nicely with the APP molecular model of copper transport, if one considers that the portion of copper from the brain is removed into the blood via APP binding, entering the nonamyloidogenic pathway.

Two opposite fluxes of copper can, therefore, be supposed to influence copper content in the brain: 1) a blood-to-brain inward flux, corresponding to the labile fraction of serum copper, that can diffuse or even be transported across the BBB by copper transporter 1-CTR1,[34] and by ATPase7A (the Menkes' disease copper-transporter)[35]; 2) a brain-to-blood APP-driven outward flux, leading to a reduction in β-amyloid concentrations in the CSF.[33]

Although copper bound to low molecular weight compounds represents only a small fraction of serum copper in AD,[8] excessive levels over time could determine an increase in brain-soluble copper, even considering that the brain has strict conservation mechanisms for this metal.[36,37] Indeed, it is known that the day-to-day and week-to-week variation in serum copper is insignificant.[38-40] Thus, in AD, the inward copper flux, postulated based on the regression model of CSF copper elevation depending on ceruloplasmin unbound copper, could be considered a chronic condition, resulting in a continuous supply of the copper brain reservoir. This small continuous supply would determine an increase in CSF copper content that is not actually measured in the CSF of AD patients (table 2). Indeed, the majority of previous studies have reported no change in the CSF,[41-44] and no change in or even decreased copper levels in the brain of AD patients,[41,44,45] suggesting that an additional process restraining the increase in soluble copper in the AD brain might be operating.[46,47] This could presumably occur via β-amyloid buffering for copper excess, binding and precipitating this metal in amyloid plaques, removing it from the CSF,[46-49] and decreasing β-amyloid itself in the CSF of AD patients. Our data are in line with recent evidence in cholesterol-fed rabbits showing that ingestion of ≈2 μM of copper in drinking water markedly changed the brain β-amyloid burden with no variation of serum ceruloplasmin levels,[50] suggesting that ingested copper supplied the serum exchangeable copper pool.

Copper in AD binds β-amyloid peptide with high affinity and reduces Cu(II) to Cu(I), resulting in catalytic generation of peroxides (H_2O_2) and β-amyloid aggregation.[6] Current results show elevated levels of peroxides, a strong effect of copper on the generation of peroxides (figure, D), and a positive correlation between peroxides and h-tau protein concentrations in the CSF, suggesting a direct effect of oxidative stress on brain tissue damage in AD.[6,51] Furthermore, in line with previous reports,[52] a correlation between serum copper and increased h-tau concentrations in the CSF is found, as a further suggestion for a neurotoxic copper effect because h-tau is released in the extracellular milieu as a cell death byproduct.

Data on plasma β-amyloid content in AD are controversial and require large patient groups.[53-55] Therefore, our results, in line with the evidence that β-amyloid increases with age,[53,55,56] could be only informative about the relationship occurring between β-amyloid content in plasma and in CSF. The correlation found between β-amyloid levels in CSF and in plasma in healthy controls, a correlation that disappears in AD, might suggest impaired transport of β-amyloid through the BBB in AD, as already reported.[57,58] The connection between copper and β-amyloid metabolism is also supported by the observation that, in AD, the exchangeable fraction of copper in serum is positively associated with plasma β-amyloid, further supporting the proposed role of β-amyloid itself as a candidate chaperone of copper.[59] This is also in agreement with the finding that treatment with the copper-binding compound clioquinol in AD patients determines a decrease in plasma β-amyloid levels.[56]

Higher levels of CSF β-amyloid concentrations corresponded to higher MMSE scores, similar to previous reports,[60,61] indicating greater impairment and disease severity in patients with lower CSF β-amyloid levels. In the same patient sample, we

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found that the higher the unexplained copper in the serum was, the lower the MMSE score was, stressing the potential role of this metal as a marker of disease and disease severity. Moreover, higher factor 2 (verbal memory) scores, a neuropsychological function considered most representative of the typical impairment of AD, corresponded to higher β-amyloid concentrations and lower levels of copper unexplained by ceruloplasmin in the serum. Although we are far from demonstrating disease causality, the present results support the idea that an excess of exchangeable serum copper when crossing the BBB and interacting with both β-amyloid and tau proteins might contribute to cognitive decline in AD. Current data replicate, in a new set of patients, our previous findings of greater impairment and disease severity in patients with higher levels of copper,[8,14,15,62] also reinforcing evidence from other groups of a negative correlation between plasma copper and cognitive function in AD.[63] Moreover, the study presented supports the results of copper chelation pilot studies on human subjects, showing positive clinical effects on cognitive loss in AD.[52,56,64] The fact that worse cognitive performance is accompanied by higher levels of serum copper found indirect support also from the evidence that cognitive disturbances in Wilson's disease, seemingly directly related to cerebral copper intoxication, are improved by chelation treatment.[28,65] Our current findings appear to be at variance with those of a recent study of some authors,[40] who reported that higher levels of serum copper correspond to better cognitive performances in a small cohort of patients with AD when excluding patients with AD in the highest plasma copper tertile. However, in contrast to our procedures, the report of those authors[40] did not include data from a healthy control group. Therefore, it is not possible to compare data from the two studies.

Our subject sample, despite its relatively small size, was carefully selected, aiming at the exclusion of vascular dementia, MCI, mixed and other dementias and focusing on a group with likely similar pathogenetic mechanisms. This study has a number of limitations, which include the need for AD selection with possible sampling bias (i.e., mild cases may be underrepresented); the lack of other dementias and neurodegenerative diseases, not permitting similar abnormalities in conditions other than AD to be conclusively ruled out; the insufficient number of neuroradiologic studies in patients and controls to carry out correlations with the biochemical markers under study. All these considerations certainly warrant the extension of the current investigation to other patient populations.

The present study, despite the described limitations, supports the hypothesis of β-amyloid implication in the move of copper from the brain to serum, demonstrates an excess of unbound copper in AD, and reveals a potential toxic interaction of this exchangeable copper component with β-amyloid and tau protein metabolism.

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Longitudinal prognostic value of serum "free" copper in patients with Alzheimer disease

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ABSTRACT

Background: Serum copper not bound to ceruloplasmin ("free") appears slightly elevated in patients with Alzheimer disease (AD). We explored whether a deregulation of the free copper pool can predict AD clinical worsening.

Methods: We assessed levels of copper, iron, zinc, transferrin, ceruloplasmin, peroxides, total antioxidant capacity, free copper, and apolipoprotein E genotype in 81 patients with mild or moderate AD, mean age 74.4, SD = 7.4 years, clinically followed up after 1 year. The association among biologic variables under study and Mini-Mental State Examination (MMSE) (primary outcome), activities of daily living (ADL), and instrumental activities of daily living (IADL) (secondary outcomes) performed at study entry and after 1 year were analyzed by multiple regression.

Results: Free copper predicted the annual change in MMSE, adjusted for the baseline MMSE by means of a linear regression model: it raised the explained variance from 2.4% (with only sex, age, and education) to 8.5% ($p = 0.026$). When the annual change in MMSE was divided into <3 or ≥3 points, free copper was the only predictor of a more severe decline (predicted probability of MMSE worsening 23%: odds ratio = 1.23; 95% confidence interval = 1.03–1.47; $p = 0.022$). Hyperlipidemic patients with higher levels of free copper seemed more prone to worse cognitive impairment. Free copper at baseline correlated with the ADL and IADL clinical scales scores at 1 year.

Conclusions: These results show an association between copper deregulation and unfavorable evolution of cognitive function in Alzheimer disease. Further research is needed to establish whether copper is an independent risk factor for cognitive decline. ***Neurology*® 2009;72:50–55**

GLOSSARY

AD = Alzheimer disease; **ADL** = activities of daily living; **BBB** = blood–brain barrier; **CB** = ceruloplasmin; **CI** = confidence interval; **IADL** = instrumental activities of daily living; **MMSE** = Mini-Mental State Examination; **OR** = odds ratio.

Alzheimer disease (AD) is a form of dementia which progresses at different rates in different patients. Factors influencing or predicting the progression are not well understood.

In a previous article, we reported that the ceruloplasmin-copper relationship, rather than absolute serum copper levels, represented the key to interpreting in vivo copper findings in AD.[1] In particular, we discussed a deregulation of that relationship consisting of an increase in the serum copper portion that did not bind to ceruloplasmin, namely "free" copper,[2] also correlating with the typical deficits and markers of the disease.[1-3] Our observations were recently confirmed by two other groups, one in the Netherlands[4] and one in the United States (Althaus J, personal communication). In particular, data from the latter study indicated that the free copper level in AD sera was nearly twice that in normal sera (AD 2.27 ± 0.16 μmol/L; normal 1.56 ± 0.29 μmol/L). Normally, the majority of human serum copper tightly binds to ceruloplasmin.[5] The remaining copper—i.e., free copper—is distributed and exchanged

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among albumin, amino acids (e.g., histidine), and small molecular weight complexes (0.5–5%; normal value <1.6 μmol/L),[5] which can easily cross the blood–brain barrier (BBB).[6]

Most studies of diagnostic markers focus on the early diagnosis of AD. Fewer studies have aimed at the identification of prognostic markers that influence the variation in rate of decline after patients have received the diagnosis of AD and little is known about the determinants of the variability.

In this study, we explored the hypothesis that a deregulation of the free copper pool in serum could be a determinant of the variability of the progression of cognitive decline in a group of patients with mild or moderate AD.

METHODS A total of 107 patients with probable AD according to National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association,[7] showing mild or moderate cognitive impairment (score ≤2 of the Clinical Dementia Rating)[8] and a Mini-Mental State Examination (MMSE)[9] score of 25 or less (range 16–24), were recruited in two specialized dementia care centers: the Department of Neuroscience, Fatebenefratelli Hospital, Isola Tiberina, Rome, Italy, and the IRCCS, Fatebenefratelli Hospital of Brescia, Italy.

Criteria for exclusion were conditions known to affect copper metabolism and biologic variables of oxidative stress on the basis of the past medical history and screening laboratory tests, reported in detail elsewere.[1] We also excluded patients with a history of stroke, presence of focal neurologic signs, severe subcortical leukoencephalopathy, presence of hemodynamically significant neck and intracranial arteries stenosis or occlusion, or cardiopathy.

When admitted to the study, patients underwent a structured clinical interview, a neurologic examination, brain MRI, an extensive neuropsychological assessment, biochemical measurements, and apolipoprotein E (APOE) genotyping. Blood was drawn on the same day of the clinical interview. Follow-up was performed 12 months after inclusion in the study. During this period, patients received the acetylcholinesterase inhibitor donepezil (5 mg daily for 3 months and then 10 mg daily) and compliance to the therapy was checked periodically by means of telephone contacts and visits performed at 3-month intervals.

Progression of cognitive decline was evaluated by means of the MMSE (primary outcome) and activities of daily living (ADL) and instrumental activities of daily living (IADL) (secondary outcomes) performed at study entry and at the end of the follow-up period.

The study was approved by the local ethical committee. All patients or caregivers gave written consent to be included in the study.

Biochemical and molecular 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 method of Abe et al. (Randox Laboratories, Crumlin, UK)[10] and by an A Analyst 300 Perkin Elmer atomic absorption spectrophotometer equipped with a graphite furnace with platform HGA 800 (Foster City, CA).[11] Ceruloplasmin was analyzed by immunoturbidimetry assay (Horiba ABX, Montpellier, France).[12] For each serum copper and ceruloplasmin pair we computed the amount of copper bound to ceruloplasmin (CB) and the amount of copper not bound to ceruloplasmin (free copper) following standard procedures (appendix 1 of reference 5: "Calculation of 'free copper' concentration")[5]; briefly: CB = ceruloplasmin (mg/dL) × 10 × n; n = 0.0472 (μmol/mg); free copper = absolute serum copper − CB.[5] This calculation expresses free copper in μmol/L and is based on the notion that ceruloplasmin contains 0.3% copper.[5] Thus, for a patient with a serum absolute (or total) copper concentration of 17.3 μmol/L and a serum ceruloplasmin concentration of 33 mg/dL, the bound copper concentration = 33 × 10 × 0.0472 = 15.6 μmol/L, and the free copper concentration = 17.3–15.6 = 1.7 μmol/L.

Moreover, we have recently set up an automated procedure to measure ceruloplasmin oxidase activity which utilizes o-diansidine dihydrochloride as a substrate, according to previous methods.[13,14] In fact, it is well known that values of ceruloplasmin obtained immunologically, as we showed them in this study, result in higher values than those obtained enzymatically, i.e., monitoring the protein's oxidase activity.[5,13,14] This is because the apo-form of ceruloplasmin is biologically inactive.[5] In normal controls, the two detection methods showed a significant correlation (data not shown), as also reported by other authors.[5,13,14] However, quantification of ceruloplasmin by the enzymatic method based on standard ceruloplasmin solutions has not been considered by the majority of previous authors,[13] because of its cost, the variable purity of commercially available ceruloplasmin, and the general recommendation to report serum enzymes in International Units.[13] We have tried to quantify the amount of ceruloplasmin starting from the protein's oxidase activity with a commercial standard (Human Serum Ceruloplasmin, Sigma-Aldrich), but the spectroscopic inspection of the latter revealed a decay in the protein peak of absorbance in day-to-day assays (data not shown), decreasing our confidence in using the enzymatic detection to quantify the protein amount, necessary to estimate the free copper value.

Measurements of the other biologic variables of oxidative stress are described in detail elsewhere.[15] Briefly, hydro-peroxide content was assessed by d-ROMs test (Diacron, Italy) and expressed in arbitrary units (U.CARR), 1 U.CARR corresponding to 0.08 mg/100 mL of hydrogen peroxide. Normal range was between 230 and 310 U.CARR.[16] Total radical trapping antioxidant capacity, or TRAP, was assayed by the TAS kit (Randox Laboratories, Crumlin, UK), based on published methods.[17] The serum reference range is 1.30–1.77 mmol/L.[17] Transferrin level was analyzed by immunoturbidimetry assay,[18] and iron using Ferene.[19] All these reagents were ABX Pentra from Horiba ABX (Montpellier, France). Zinc levels were measured using the specific complexant 5-Br-PAPS [(2-5-bromo-2-pyridylazo)-5-(N-propyl-N-sulfo-propylamino) phenol][20] according to manufacturer's instructions (Zinc, Sentinel Diagnostic, Milan, Italy).

All biochemical measures were automated on a Cobas Mira Plus (Horiba abx, Montpellier, France) and performed in duplicate.

APOE genotyping was performed according to standard methods.[21]

MRI evaluation. Brain MRI was performed using a 1.5 Tesla superconductor magnet. The imaging protocol consisted of axial T2 W double spin echo sequences and T1 W spin echo images in axial, coronal, and sagittal planes, with 5 mm slice thickness and intersection gap = 0.5 mm. An extensive MRI evaluation was performed (data not shown). Briefly, the spin echo technique and T1 W, T2 W, and fluid-attenuated inversion-recovery sequences detected pos-

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Table 1 Baseline characteristics, Mini-Mental State Examination (MMSE) scores, and circulating copper metabolism evaluation (baseline and at 1 year) of the 81 patients with Alzheimer disease

Characteristics	Values
Age, y, mean (SD)	74.4 (7.4)
Sex, % men	17
MMSE score, mean (SD)	20.5 (3.5)
Risk factors, %	
Hypertension	36
Hyperlipidemia	13
Smoking	33
Cardiopathy	9
MRI findings, %	
Subcortical infarcts (grade I)	44.5
Medial temporal lobe atrophy	71
Drugs, %	
Antihyperlipidemia agents	23
Statins	24
Oral hypoglycemic agents	11
Antiplatelet aggregation agents	43
Antihypertensive drugs	39
β-Blockers	11
Calcium antagonists	17
Diuretics	14
APOE ε4 frequency (%)	24.5
Biological variables of copper in serum, mean (SD)	
Basal copper (μmol/L)	15.6 (3.5)
Basal ceruloplasmin (mg/dL)	27.7 (6.3)
Basal free copper (μmol/L)	2.5 (2.9)
Cognitive evaluation, mean (SD)	
Basal MMSE scores	20.5 (3.5)
One-year MMSE scores	17.3 (4.7)

sible white matter lesions. These were graded according to published protocols.[22] Only patients without vascular lesions (grade 0) or with small subcortical focal lesions defined as high signal intensity areas on T2-weighted images, but isointense with normal brain parenchyma on T1-weighted images and classified as grade I (44.5%; table 1), were included. Volumetric analysis of the hippocampus was visually performed by scoring every patient according to the width of the choroid fissure and the temporal horn, following the criteria of a rating scale of temporal lobe atrophy previously reported (five point rating scale of medial temporal lobe atrophy [MTA]).[1,22] MRI revealed that atrophy of the medial temporal lobes with a reduction in hippocampal volume (score 2–4) was prominent (71%; table 1) in the patients.

Statistical analyses. Formal sample size calculation was not performed because this was an exploratory study of possible correlation between metal and oxidative biologic variables and impairment and severity of progressive cognitive decline. However, we considered as relevant a bivariate correlation of 0.40 (corresponding to 16% of accounted variance) and calculated that a sample size of 50 patients would result in a power of 86% (with bilateral α at 0.05).

The aim of the study was to see whether and how severe the progression of cognitive decline was in relation to metal and oxidative biologic variables alterations. Attaining this goal was best served by a regression model. A multiple regression analysis was applied to test the potential confounding role of demographic characteristics (sex, age, and education) and vascular risk factors (hypertension, smoking habits, and hyperlipidemia), then the linearity of the relationship between metal and oxidative biologic variables alterations and severity of progression of cognitive impairment was tested using a polynomial regression and the best-fitting model (maximum R^2 and significant R^2 change vs the previous model) was chosen.

To provide additional information potentially useful for clinicians, outcome cognitive variables were dichotomized in two groups: above or equal to 3 points MMSE decrease (severe cognitive decline) and below 3 (milder cognitive decline). Impairment was evaluated using a logistic regression model. Odds ratios (ORs) and corresponding 95% confidence intervals (CIs) were calculated.

RESULTS

Patients. Eighty-one of the 107 subjects completed the follow-up after 1 year. The main baseline demographic and medical characteristics of these patients are reported in table 1, along with evaluations of free copper and MMSE at baseline and at the 1 year follow-up. Patients were affected mainly by hypertension (36%), cardiopathy (9%), hyperlipidemia (13%), diabetes (11%), and smoking (33%). The most frequent drugs taken by patients were antiplatelet aggregation agents (43%), statins (24%), and antihypertensive drugs (39%), mostly represented by calcium antagonist (17%). During the study, all patients received acetylcholinesterase inhibitors. Allele APOE ε4 frequency was 24.5%. The APOE ε4 allele was more prevalent in patients born in northern Italy (35%) than in central/southern Italy (17%; $p = 0.013$). Patients at baseline had mild to moderate dementia (MMSE: mean = 20.5, SD = 3.5).

Sixty-five percent of patients had a higher than normal free copper (<1.6 μmol/L).[5] Patients who were carriers of the APOE ε4 allele had higher levels of free copper than noncarriers (data not shown).[11]

After 1 year, MMSE decreased by 3.2 points (SD = 3.9; $p < 0.001$; table 1).

Prediction of cognitive decline. Baseline cognitive status could be the first predictor of cognitive deterioration. In consideration of this, the annual change in MMSE was adjusted for the baseline MMSE by means of a linear regression model, even though only 4% of change variance was accounted for by baseline measurement. We then analyzed the correlations between cognitive decline at 1 year and the biologic variables under study. The analysis revealed that the higher the annual change in MMSE, the higher both

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Table 2 Correlations between annual change in Mini-Mental State Examination and biological variables of metals and oxidative stress in serum

Serum biological variable of metals and oxidative stress at baseline	Annual change in MMSE
Copper	
Pearson correlation	0.219*
Significance (two-tailed)	0.050
No.	81
Zinc	
Pearson correlation	−0.218
Significance (two-tailed)	0.103
No.	57
Iron	
Pearson correlation	0.058
Significance (two-tailed)	0.640
No.	68
Transferrin	
Pearson correlation	−0.111
Significance (two-tailed)	0.347
No.	74
Ceruloplasmin	
Pearson correlation	−0.001
Significance (two-tailed)	0.990
No.	81
Free copper	
Pearson correlation	0.256*
Significance (two-tailed)	0.021
No.	81
Peroxides	
Pearson correlation	−0.051
Significance (two-tailed)	0.649
No.	81
Total radical trapping antioxidant capacity	
Pearson correlation	0.169
Significance (two-tailed)	0.137
No.	79

*Correlation is significant at the 0.05 level (two-tailed).

the absolute copper and free copper (table 2), while the MMSE at baseline did not correlate with copper biologic variables (all p values >0.17). The relationship between copper variables and cognitive decline was studied further: when the annual change in MMSE was entered as a dependent variable in a linear regression model, free copper proved to be a significant covariate, since it raised the explained variance from 2.4% (with only sex, age, and education) to 8.5%. In particular, the slope of the regression was b = 0.316 (SE = 0.140; $p = 0.026$) indicating that about 3 μmol/L units of free serum copper explained a one-point loss on the annual change in the MMSE. To better quantify our model the analysis of influential statistics was applied (based on changes in regression coefficients and on leverage measures): this analysis indicated that the exclusion of outlier cases with a potentially detrimental effect on the regression model did not change the relationship between free copper and MMSE changes (data not shown).

We also observed an effect of hyperlipidemia on cognitive decline. Hyperlipidemia was associated with a 2.6 point loss on the annual change in MMSE. Hyperlipidemia accounted for 4.8% of residual variance ($p = 0.058$). Hyperlipidemic patients with higher levels of free copper seemed more prone to higher cognitive impairment.

When the annual change in MMSE scores were divided into <3 or ≥ 3 points, the multiple logistic regression indicated that free copper was the only predictor of more severe decline; for each additional μmol/L unit of free copper, the odds of MMSE worsening increased by about 23% (OR = 1.23; 95% CI = 1.03–1.47; $p = 0.022$). Those patients from the current study panel who had a value of free copper higher than 2.1 μmol/L had an increased probability of worsening than those patients who had their free copper values below such levels (figure).

Diabetes had no effect in explaining cognitive decline even after adjusting for free copper values ($p > 0.2$).

Drug treatment effects were taken into account in the analysis. Concentrations of free serum copper [$t(79) = 3.746$; $p < 0.001$] and cognitive decline [$t(79) = 3.716$; $p = 0.005$] were lower in those patients who were administered with calcium antagonists. When this effect was included in the multiple regression analysis, free copper could not enter the model for prognosis evaluation.

Free copper correlated with the ADL and IADL clinical scales which evaluate the disabilities in daily living of the patients. At baseline, higher levels of free copper corresponded to lower ADL scores at the 1-year evaluation (rho = -0.299, $p = 0.018$). The higher were free copper levels, the lower were IADL scores either at baseline (rho = -0.0349, $p = 0.001$) or at 1 year (rho = -0.359, $p = 0.004$).

DISCUSSION The most important result of this study suggests that a deregulation of free copper could determine the variability of the progression of cognitive decline in AD. However, the implication of free copper in this disease was based on correlative studies. Therefore, the present study does not allow a conclusive interpretation about a causal pathophysio-

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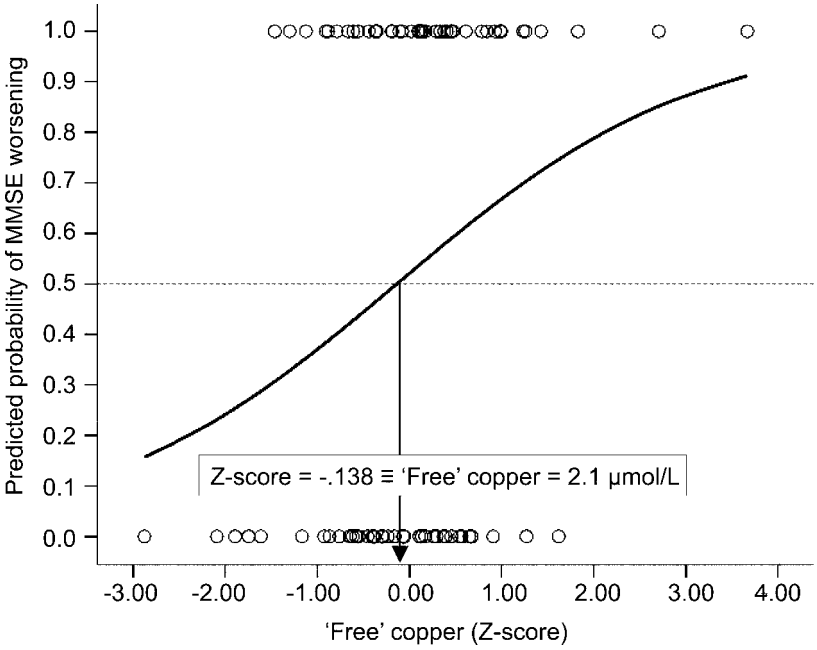
Figure Model to predict the probability of Mini-Mental State Examination (MMSE) worsening in patients affected by Alzheimer disease (AD) according to "free" serum copper levels



Circles represent the value of free serum copper of the patients. The line represents the model of the predicted probability of MMSE worsening. Free copper levels in individual patients with AD are expressed in *z*-score, i.e., in terms of standard deviations from their mean value. Those patients from the current study panel who had a *z*-score higher than −0.138, corresponding to a free copper value of 2.1 μmol/L, had an increased probability to worsen than those patients who had their free copper values below such levels.

logic effect of serum copper abnormalities on cognitive decline. Our current findings show that higher levels of free copper could explain a decline in MMSE scores.

In spite of the relatively low percentage of explained variance of free copper, the most interesting aspect of our study is the possibility of obtaining useful information for identification of patients with AD at higher risk for a rapid and pronounced evolution of cognitive impairment by means of a simple blood test. The majority of studies report that there is heterogeneity in the progression of AD. The severity of cognitive impairment at baseline,[23] as well as hippocampal atrophy,[24] are suggested to be important predictors of progression. With regard to those patients with a free copper dysfunction, estimated to be 65% of our patients, a free copper value higher than normal seems to represent a determinant of progression variability. In particular, a value of free copper higher than 2.1 μmol/L increased the probability of loss of 3 points in a year on the MMSE. These results are consistent with the hypothesis that even a small increase of the serum low molecular weight copper, i.e., free copper, can be of significance, particularly over a long period of time. In fact, free copper is potentially toxic as it can promote oxidative stress,[25] because this low molecular weight copper can easily cross the BBB and supply the brain with a continuous flux of noxious redox copper.

This interpretation is supported by results of an experiment with mice to test the effects of radiocopper [67Cu(II)] brain uptake during a single passage through the telencephalon-diencephalon microcirculation which demonstrates that net brain copper uptake parallels the increase of the free copper level in the injectate, starting from a concentration of Cu (II) of 3.2 ng/mL, corresponding to 0.05 μmol/L.[26]

In the present study, changes in ADL and IADL—which predict future functional decline, institutionalization, and death—were evaluated in relation to copper status. Patients with higher levels of free copper at baseline seemed to have a faster and more pronounced evolution of disabilities, in parallel with the progression of cognitive decline.

Our data indicate that hyperlipidemia was the only risk factor associated with MMSE worsening. Even though it is relevant in a small number of patients, we demonstrated an interaction between free copper and hyperlipidemia on cognitive decline in agreement with other authors who used different study design approaches.[27,28]

The impact of serum copper on cognitive decline in this study was not affected by age, sex, or education.

We have tried to control confounders by selecting patients with no evidence of additional pathologic conditions, and by taking into account cardiovascular risk and drug treatment. In this patient sample we noted that subjects who were taking a calcium antagonist therapy had lower levels of circulating free copper. A possible explanation is a diminished rate of copper absorption at the intestinal level.

This study has a number of limitations, which include the shortage duration of the clinical follow-up, the need for patient selection that possibly resulted in sampling bias, and the refusal to perform follow-up evaluation by some patients that reduced the number of subjects initially involved in the study. Another interesting result of this study concerns the possibility that a therapeutic intervention aimed at metal levels could have favorable practical implications for the management of AD.[29] Therapeutic strategies available for copper's diseases, i.e., Wilson disease, at least in a subgroup of patients with AD with impaired copper metabolism, deserve further study.

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Measurements of serum non-ceruloplasmin copper by a direct fluorescent method specific to Cu(II)

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Abstract

Background: Meta-analyses indicated the breakdown of copper homeostasis in the sporadic form of Alzheimer's disease (AD), comprising copper decreases within the brain and copper increases in the blood and the pool not bound to ceruloplasmin (non-Cp Cu, also known in the literature as "free" copper). The calculated non-Cp Cu (Walshe's) index has many limitations.
Methods: A direct fluorescent method for non-Cp Cu detection has been developed and data are presented herein. The study included samples from 147 healthy subjects, 36 stable mild cognitive impairment (MCI) and 89 AD patients, who were tested for non-Cp Cu through the direct method, total serum copper, ceruloplasmin concentration and *o*-dianisidine ceruloplasmin activity. The indirect non-Cp Cu Walshe's index was also calculated.
Results: The direct method was linear (0.9–5.9 μM), precise (within-laboratory coefficient variation of 9.7% for low and 7.1% for high measurements), and had a good recovery. A reference interval (0–1.9 μM) was determined parametrically in 147 healthy controls (27–84 years old). The variation of non-Cp Cu was evaluated according to age and sex. Non-Cp Cu was 1.5 times higher in AD patients (regarding the upper value of the reference interval) than in healthy controls. Healthy, MCI and AD subjects were differentiated through the direct non-Cp Cu method [areas under the curve (AUC) = 0.755]. Considering a 95% specificity and a 1.91 μmol/L cut-off, the sensitivity was 48.3% (confidence interval 95%: 38%–58%). The likelihood ratio (LR) was 9.94 for positive test results (LR+) and 0.54 for negative test result (LR−).
Conclusions: The direct fluorescent test reliably and accurately measures non-Cp Cu, thereby determining the probability of having AD.

Keywords: Alzheimer's disease; ceruloplasmin; copper; metal; mild cognitive impairment; non-ceruloplasmin copper.

Introduction

Copper is an essential metal and a co-factor in numerous physiological proteins and enzymes that grant cell functioning and central nervous system development. It serves as a catalyst for reduction-oxidation (redox) reactions in ubiquitous or tissue-specific enzymes for a number of molecular pathways, including energy generation, hematopoiesis, and iron homeostasis. In the liver, the protein ATP7B loads copper into nascent ceruloplasmin, a ferroxidase which structurally binds the 85%–95% serum copper [1]. Residual serum copper is bound to low molecular weight molecules (e.g. amino acids like histidine, or peptides) or to albumin. This fraction represents an exchangeable pool of circulating copper, referred to as copper non-bound to ceruloplasmin (non-Cp Cu) also named "free copper" in the literature. It is bioavailable for tissues and organs, including the brain, as it easily crosses the blood-brain barrier. The copper pump ATP7B is also physiologically involved in redirecting copper from the brain to the blood [2, 3]. Changes in the metal oxidation state can affect the redox status of the entire cell or matrix and can trigger radical-mediated toxicity. The paradigm of non-Cp Cu overload is Wilson's disease (WD), a rare monogenic recessive disorder caused by more than 500 mutations in the *ATP7B* gene. Mutations in the gene generate a defective ATP7B, which cannot supply copper to the Golgi apparatus [4]. The metal is either not properly loaded into ceruloplasmin or excreted through the bile [5].

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Copper and ceruloplasmin dysregulation affect the pathogenesis of both Alzheimer's disease (AD) [6, 7] and Parkinson's disease [8].

The value of the systemic non-Cp Cu index is routinely calculated by measuring the copper and ceruloplasmin pair in a serum sample. The amount of copper bound to ceruloplasmin (CB) and the amount of non-Cp Cu (historically named "free copper") can be computed following standard procedures [9]. This index (known as Walshe's index [9]) has some limitations, including paradoxical results of negative non-Cp Cu values, and is not as reliable as a direct assay. We addressed these issues in a previous study [7] (information available at http://www.j-alz.com/node/182). For this reason, a new patented method for direct non-Cp Cu detection in serum through a fluorescent probe has been set-up [10].

Validation data for the CE mark in Europe (C4D test, 2012 CE certified test code no. 1211662), the normal reference range interval, and detection in AD and mild cognitive impairment (MCI) subjects are reported herein.

Materials and methods

Subjects

Serum samples from 147 healthy subjects were obtained by the Blood Donors Unit of Fatebenefratelli Hospital (Isola Tiberina, Rome, Italy). The healthy volunteers were screened for neurological, psychiatric, and cardio-cerebro-vascular diseases. A minimental state examination (MMSE) test [11] was conducted. An additional 48 healthy subjects (26 F), of a mean [standard deviation (SD)] age of 61.9 years (10.5) ranging from 28 to 76 years were recruited from the IGEA Research Corporation (Miami, FL, USA) between September 2015 and May 2016 and were separately evaluated for non-Cp Cu values measured with the direct fluorescent method in IGEA Research Corporation Laboratories, confirming values of non-Cp Cu in another healthy control group.

Mild cognitive impairment and AD subjects (36 and 89, respectively) were recruited from the Department of Neuroscience of Fatebenefratelli Hospital. Recruitment from the Italian centers was carried out between February 2013 and April 2014. Details about the clinical protocols for MCI and AD diagnosis were reported elsewhere [6, 12]. Patients and controls with diabetes, abnormal values of thyroid, liver, kidney and cardiac functions were excluded from the study, as were patients with a history of stroke, focal neurological signs, severe subcortical leukoencephalopathy, hemodynamically significant neck and intracranial artery stenosis or occlusion, or cardiopathy.

Blood was drawn the morning after an overnight fast, collected in a test tube (Vacutainer® BD Thrombine) containing an activator of coagulation, and quickly stored at – 80 °C.

All procedures involving experiments on human subjects complied with the ethical standards of the Committee on Human Experimentation of our institution and the Helsinki Declaration of 1975 (revised 2008). Blind procedures for data collection and analysis were applied. Serum were collected with tubes certified to be free of trace elements (VWR, Metal-Free Centrifuge Tubes). A 500 µL serum aliquot of the subjects recruited from the Fatebenefratelli Hospital, was shipped to the Spin-off Biofordrug Laboratory University "Aldo Moro" of Bari, Italy, for direct fluorescent non-Cp Cu measurements. Measurements of serum copper, ceruloplasmin concentration and activity were performed in the Laboratory of Biology, Fatebenefratelli Foundation, Fatebenefratelli Hospital.

Assay

The direct fluorescent non-Cp Cu test is an in vitro method with two steps [10]: step (i) a size exclusion solid-phase extraction (SPE) flow system separates the portion of low molecular weight non-Cp Cu present from the protein-bound copper; step (ii) a coumarin fluorescent probe specific to Cu(II) is added to the eluate from the previous step, and a direct fluorescent assay is performed on a micro-plate device. When the probe captures the labile non-Cp Cu – as a Cu(II) – from small peptides and amino acids, there is a decay in its fluorescence emission, proportional to non-Cp Cu concentration.

During the step (i) serum is loaded to a SPE chromatography (ultra-high molecular weight polyethylene; micro-column with a resevoir of 3 mL capacity). The stationary phase is compressed with 6 mL physiological solution (0.9% NaCl) using a peristaltic pump (ISMATEC IPC, Cole-Parmer GmbH, Germany) at a flow rate of 500 µL/min. Serum (200 µL) was charged on the column and eluted at a flow rate of 200 µL/min. The fraction eluted, containing non-Cp Cu, is collected in polypropylene tubes. During step (ii) 50 µL of the fraction eluted (in duplicate) is co-incubated with the fluorescent probe at a final concentration of 10^{-5} M into the well of a 96-well microplate reader, and the complex is read in a Victor3 fluorimeter (Perkin Elmer), at excitation and emission wavelengths of 380 and 495 nm, respectively. The fluorescent probe interacting with Cu(II) present in the eluate of step (i) switches off the fluorescent probe. The intensity of the decay of the fluorescent emission is proportional to the quantity of non-Cp Cu present in the original specimen. Studies were performed to evaluate potential copper contamination originating from disposable material and interference [10]. Because hemoglobin has an intrinsic fluorescence, collecting serum samples correctly is fundamental to avoiding hemolysis. The fluorescent chelator is inactive towards other bivalent ions like Zn(II), Fe(II), Mg(II), and Ca(II). Indeed, the formation constant (Kf) is 10 times higher for Cu(II). Therefore, the probe is unable to bind other ions in biological fluid.

The Spin-off Biofordrug Laboratory University "Aldo Moro" of Bari, Italy, and the IGEA Research Corporation Laboratories share the same Standard Operation Procedures.

Biochemical and molecular investigations: standard copper studies

Total serum copper levels were analyzed in a graphite furnace atomic absorption spectrophotometer (Perkin Elmer A Analyst 600). The samples and calibration standards for copper measurement were 1 : 20 dilutions with 0.2% HNO_3 in 0.1% Triton. Commercial copper calibrators were used as standards (1.000 mg/L) through serial dilution, and samples were evaluated according to a standard

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curve [13]. A seronorm™ (SERO AS, Norway) was employed as Quality Control.

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 [5]. Both the ceruloplasmin concentration and ceruloplasmin activity measures were automated on a Pentra 400 (Horiba ABX, Montpellier, France) and performed in duplicate.

For the non-Cp Cu index calculation we used values of ceruloplasmin obtained immunologically [9]. For each serum copper and ceruloplasmin pair, we computed the amount of copper bound to ceruloplasmin (CB) and non-Cp Cu, following standard procedures (Appendix 1: "Calculation of 'free copper' concentration") [9]: CB (μmol/L) = ceruloplasmin (mg/dL) * 10 * n (μmol/mg); where n = 0.0472 μmol/mg is obtained from the molecular weight of copper, 63.546 (g/mol) divided by the molecular weight of Cp, 132 kDa. Finally: non-Cp Cu (μmol/L) = absolute serum copper (μmol/L) – CB (μmol/L).

The normal reference range with this index has been set lower than 1.6 μmol/L in previous studies [9, 14]. This calculation expresses non-Cp Cu in μmol/L because ceruloplasmin binds 6(-8) atoms of structural copper, which are equivalent to ceruloplasmin containing 0.3% copper (weight percentage of copper in Cp); more details are available at http://www.jalz.com/letterseditor/index.html#March2013.

Copper : Ceruloplasmin (Cu : Cp) is an index (dimensionless value) that effectively determines the ceruloplasmin calibration, providing information about the actual stoichiometry between copper and ceruloplasmin in the specimens. The Cu : Cp ratio was calculated as reported by Twomey et al. [15], whose equation is as follows: [copper μM]* [132000 g/mol]/[Cp mg/dL*10(4)].

Twomey et al. [15] proposed that 6.6 is the theoretical optimal mean value of this ratio in healthy subjects.

Testing procedures

Procedures from the Clinical and Laboratory Standards Institute (CLSI) were employed, including the EP05-A [16], the EP06-A method evaluation [17], EP17-A [18], and EP15-A2 [19]. StatisPro Method evaluation software (from CLSI version 3.02.2) was used for these analyses.

Test precision was evaluated on 20 observations (10 runs, 5 days, two replicates per run), separately for low (mean 1.25) and high (mean 3.18) levels, with a 95% confidence level and an acceptance criterium of 10% max imprecision. Replication studies were performed at the IGEA Research Corporation. Linearity was tested on seven levels of $CuCl_2$ (0.9, 1.1, 2.0, 2.5, 4.5, 5.4, and 5.9 μmol/L) with two replicates and an acceptance criterium of 5% non linearity. The parametric limit of blank (LoB = mean blank + 1.645 SD blank) and non-parametric limit of detection (LoD) were calculated from software on five samples (12 replicates), considering alpha and beta errors at 5%.

Recovery was determined by adding five different concentrations (0.5, 1, 2, 3, and 5 μmol/L) of $CuCl_2$ as reference material to a standard seronorm (SERO AS) after SPE [step (ii) of the procedure]. Nine replicates of the resulting mixtures at each concentration were analyzed. The mean percentage of recovery was calculated.

Reference intervals for the direct fluorescent non-Cp Cu values in the larger Italian sample of healthy controls were calculated through non-parametric CLSI procedures [20].

Statistical analysis

Linear and quadratic regression statistical models were implemented to test comparability between fluorescent direct non-Cp Cu and the calculated Walshe's index non-Cp Cu values, and R^2 change was used to evaluate fitness. Piecewise regression was used to evaluate breakpoints. Intraclass correlation and a 95% confidence interval (95% CI) were calculated to test consistency. Analysis of variance (ANOVA) was used to evaluate the influence of sex and age on fluorescent direct non-Cp Cu, and the age-adjusted value was calculated as: Age-adjusted non-Cp Cu = non-Cp Cu – [0.009 * (age-49.05)] μmol/L.

Correlation analyses were performed (Pearson's r). ANOVA was used to evaluate discriminant validity among controls, MCI, and AD patients. Receiver operating characteristic (ROC) curves were used to evaluate the diagnostic accuracy of both fluorescent direct non-Cp Cu and calculated Walshe's index values in discriminating subjects grouped on the basis of the diagnosis: their areas under the curve (AUC) were compared. Diagnostic sensibility (SE) for the discrimination of AD patients vs. healthy controls and MCI subjects (and its 95% CI) were calculated considering a specificity (SP) of 95% and the upper reference limit value. Positive and negative likelihood ratios (LR) were calculated, using SPSS v 16 statistical software (IBM Corporation).

A p-value < 0.05 was considered statistically significant.

Results

One hundred and forty-seven healthy controls (77 women, 52.7%), mean (±SD) age 49 ± 12.7 years old (25–84) with MMSE score 29 ± 1, 36 MCI subjects (26 women, 70.3%), mean age 73 ± 8.3 years old (35–85), MMSE 24 ± 2.5 and 89 AD patients (52 women, 67.5%), mean age 73 ± 8.5 (50–86), MMSE 19 ± 4.7 were recruited. In healthy controls, the correlation between: ceruloplasmin concentrations and serum copper levels was 0.939 ($p < 0.001$), between activity values and serum copper levels was 0.971 ($p < 0.001$), and between ceruloplasmin concentrations and ceruloplasmin activity values was 0.930 ($p < 0.001$); (Figure 1A–C, respectively). The Cu : Cp mean ratio in healthy controls was 6.8 (SD = 0.95), demonstrating a good accordance between copper and ceruloplasmin allowing the application of the Walshe's formula.

The direct fluorescent non-Cp Cu test showed an appropriate precision with a within-laboratory coefficient of variation (CV) of 9.7% for low measurements and 7.1% for high measurements (Table 1). We replicated the calculation of the within-laboratory CVs at the IGEA Research Corporation Laboratory and obtained similar results (a low CV of 9% and a high CV of 5%).

The linearity of the method is shown in Figure 2.

The LoB is 0.0492 μmol/L and the delta percentile LoD is 0.349 μmol/L.

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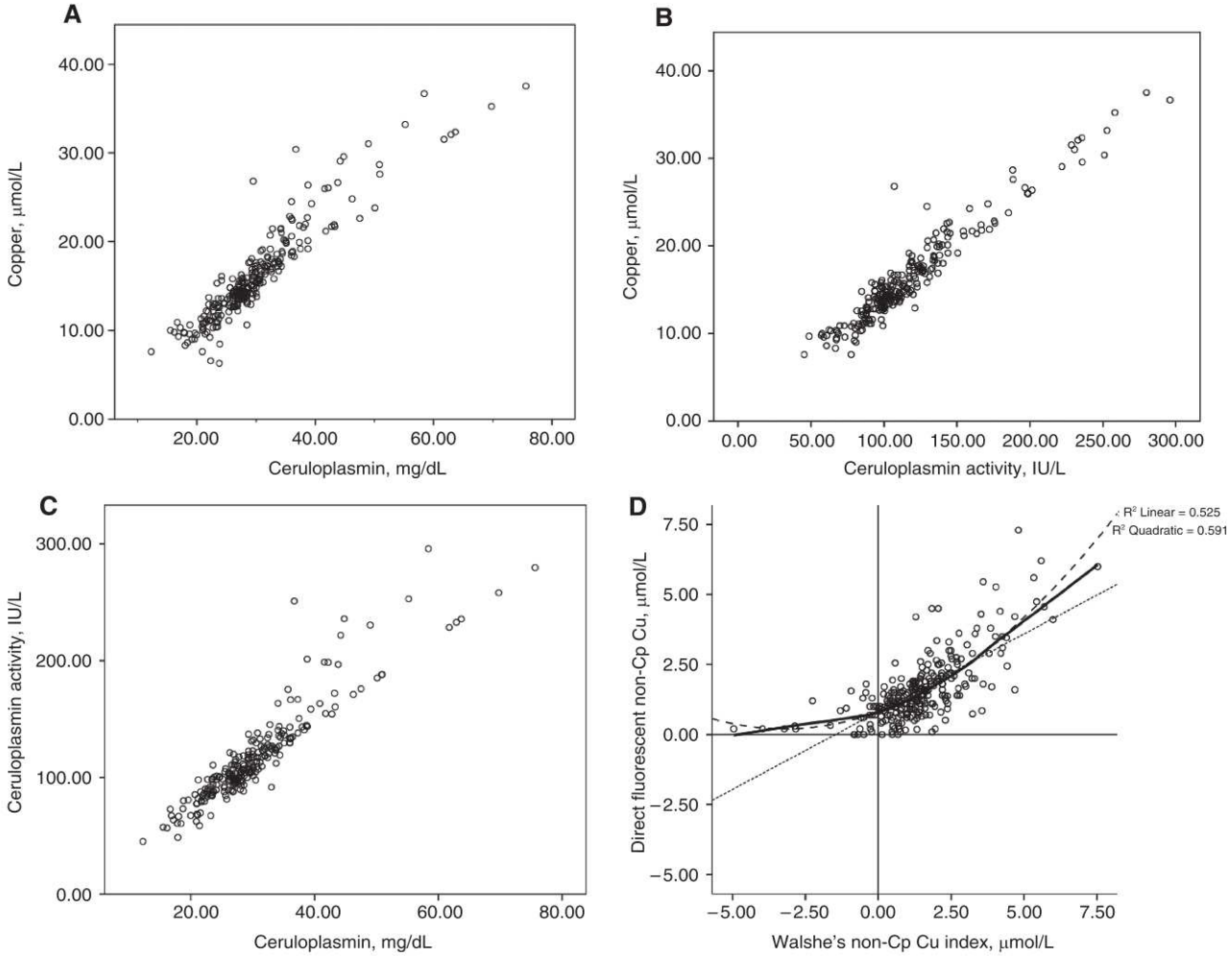

Figure 1: Scatter plots of Cu and Cp concentration (A), Cu and Cp activity (B), Cp concentrations and Cp activity (C), Walshe's index and direct non-Cp Cu (D).

Table 1: Non-Cp Cu direct test precision of measured method on 20 observations (10 runs, 5 days, two replicates per run).

	SD	95% CI	CV	Allowable total SD
Level high				
Repeatability	0.18	0.13–0.32	5.7%	–
Between-run	0.13		4.2%	–
Between-day	0.00		0.0%	–
Within-laboratory	0.23	0.17–0.36	7.1%	0.32
Level low				
Repeatability	0.09	0.06–0.15	7.0%	–
Between-run	0.07		5.7%	–
Between-day	0.00		0.0%	–
Within-laboratory	0.11	0.08–0.18	9.0%	0.12

The mean recovery for the added concentration of $CuCl_2$ equaling 0.5, 1.0, 2.0, 3.0, and 5.0 µmol/L was 112.5%.

A better R^2-value was found using quadratic, rather than linear, regression (from 0.525 to 0.591, $p < 0.001$), analyzing comparability between the non-Cp Cu direct fluorescent assay and the data calculated with Walshe's index (Figure 1D). Piecewise regression identified zero as a breakpoint. Negative values (information available at http://www.j-alz.com/node/182) found in non-Cp Cu (calculated with Walshe's formula) were not considered in the regression and correlation analyses. The Intraclass correlation was 0.75 (CI 95%, 0.69–0.80). The mean (SD) difference between the two methods was 0.003 (0.86), and Table 2 and Figure 1D show a good linearity fit.

The healthy subject sample was analyzed to evaluate the reference interval on the test. Non-Cp Cu values were slightly influenced by age ($p = 0.035$, $\eta^2 = 0.035$), but not by sex ($p = 0.359$). The age effect did not differ between men and women ($p = 0.428$). A mean increment of 0.09 µmol/L every 10 years was estimated. The upper reference limit (95%) was 1.91 µmol/L (CI 90%, 1.78–2.06).

The mean (SD) value of non-Cp Cu measured with the direct fluorescent method in the 48 healthy US subjects

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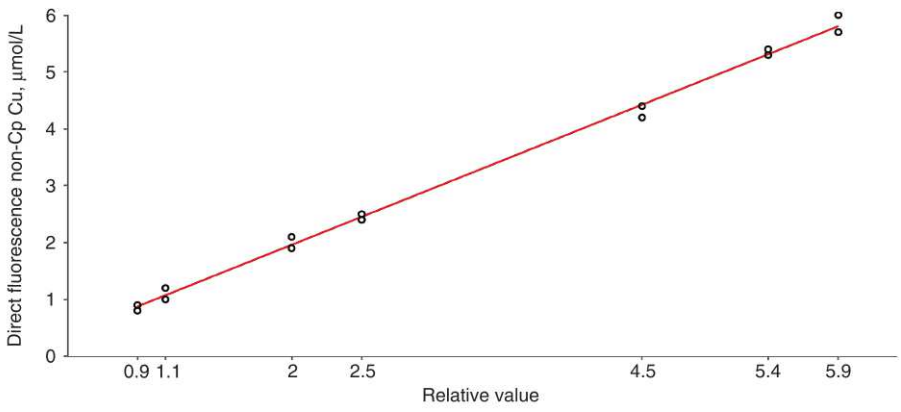

Figure 2: Linearity of new direct fluorescent method measuring non-Cp Cu.

Table 2: Linearity of measured method.

Level	Mean	Linear fit	Non linear fit	Non linearity	Allowable nonlinearity
1	0.85	0.88	–	–	0.04
2	1.10	1.07	–	–	0.05
3	2.00	1.96	–	–	0.10
4	2.45	2.45	–	–	0.12
5	4.30	4.42	–	–	0.22
6	5.35	5.31	–	–	0.27
7	5.85	5.80	–	–	0.29

was 1.7 (0.2) µmol/L (range 0.07–2.19 µmol/L). In this confirmatory healthy subjects sample, only two individuals had non-Cp Cu higher than 1.91 µmol/L, ratifying the consistency of 1.91 µmol/L individuated as the upper reference limit for healthy subjects.

Both, the values of the Walshe's index and the direct fluorescent method for non-Cp Cu detection from the Italian samples were analyzed for their reliability in discriminating subject groups on the basis of the diagnosis. As shown in Figure 3A, healthy subjects, MCI, and AD patients were well discriminated by the Walshe's index of

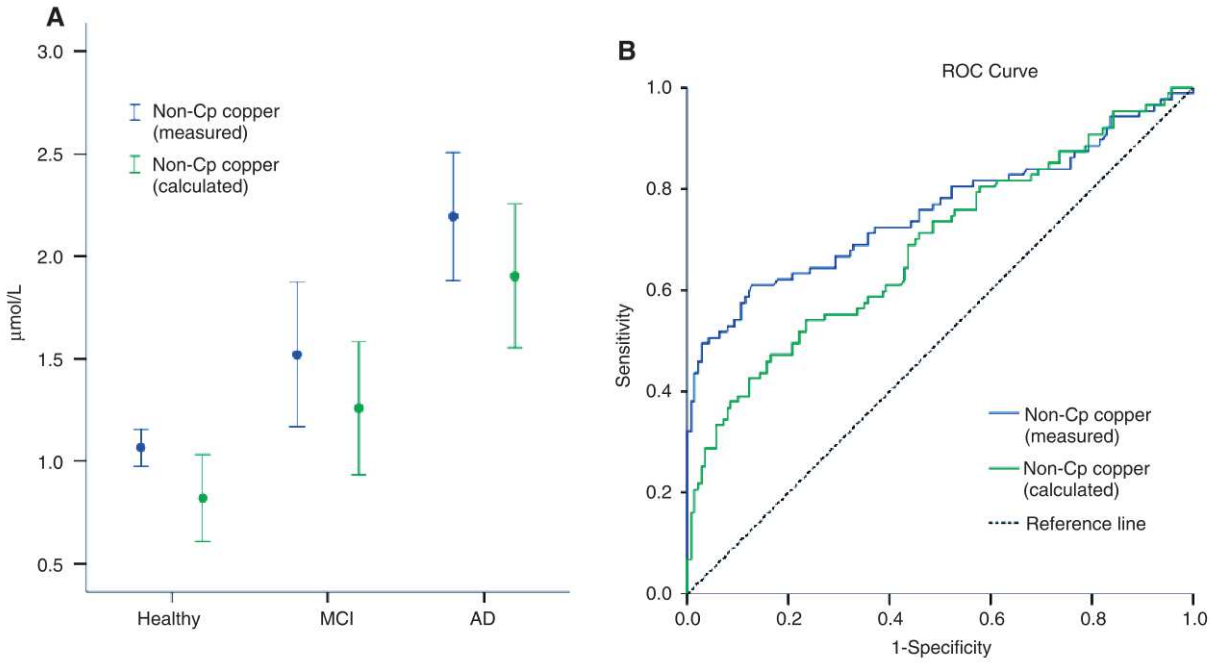

Figure 3: Walshe's and direct Non-Cp Cu mean value in healthy, MCI and AD (A), ROC curves of direct (AUC = 0.755) and Walshe's non-Cp Cu (AUC = 0.687) discriminating healthy vs. AD (B).

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non-Cp Cu [$F(2,265) = 17.702$, $p < 0.001$, $\eta^2 = 0.119$] and by the direct fluorescent non-Cp Cu method [$F(2,265) = 32.536$, $p < 0.001$, $\eta^2 = 0.196$]. Comparing healthy subjects and AD patients, the direct fluorescent method showed a better AUC than the Walshe's index (0.755 vs. 0.687, Figure 3B). Considering a 95% SP and 1.91 μmol/L cut-off, the SE is 48.3% (CI 95%, 38%–58%). The LR for positive test results (LR+) was 9.94, and the LR for negative test results (LR−) was 0.54. Figure 4 shows a distribution of direct fluorescent method values for healthy control subjects, MCI and AD patients.

Discussion

Non-Cp Cu concentration in serum represents the active fraction of copper in circulation that could eventually lead to the accumulation of labile copper in tissues and organs. The most dramatic clinical example of this copper overload is WD. The evidence demonstrated that copper is also involved in AD pathology. Meta-analyses indicated the breakdown of copper homeostasis in AD, consisting of copper decreases in the brain [21], copper increases in the blood [22], and copper increases in the serum fraction of non-Cp Cu [7]. Increases in the non-Cp Cu pool when brain copper decreases in AD is biologically plausible on the basis of the Long Evans Cinnamon rat model of WD. These rats exhibit an inbred *ATP7B* mutation, and, as a consequence, they have increased non-Cp Cu in serum, decreased total copper in the brain, and increased redox active labile copper in the brain [23]. James and colleagues [24] demonstrated decreased levels of total copper in the AD brain in those areas where the redox active labile copper fraction was increased. The parallel between the two neurological disorders strongly suggests a copper failure occurring also in AD. As a multifactorial or complex disease, AD includes a broad spectrum of phenotypes [25], among which the "copper AD" phenotype is credited [26–30]. This finding is further substantiated by current data, demonstrating via a direct measurement method the existence of non-Cp Cu abnormalities in AD and MCI subjects.

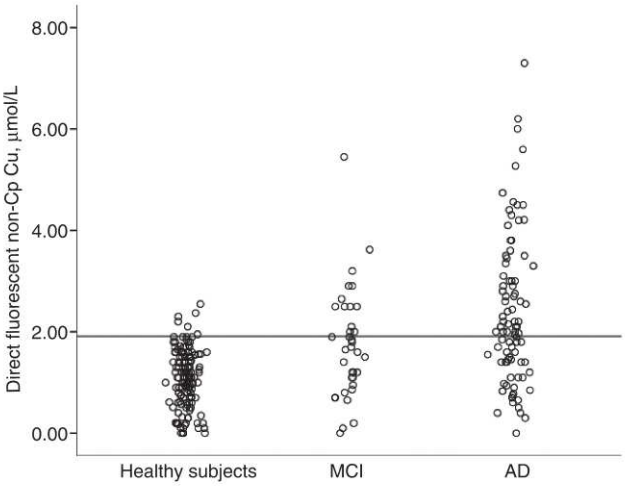

Figure 4: Distribution of actual measured non-Cp Cu values in AD, MCI and healthy controls.

Excesses of non-Cp Cu in serum have been associated with a decline in cognitive function, with levels of β-amyloid [31] and Tau proteins in cerebrospinal fluid, and with synaptic loss in AD, as revealed by electroencephalography studies [32], with cognitive worsening in longitudinal studies [6, 12]. The Walshe's formula was very useful in these studies; however, it has some limitations [7] briefly reported upon as follows: (1) it assumes that ceruloplasmin is saturated, that ceruloplasmin and copper measurements are in the correct accordance (Cu : Cp) [15], and that the amount of copper bound to proteins other than ceruloplasmin is negligible. (2) Ceruloplasmin analytic methods are known to produce variable results in laboratories. (3) Ceruloplasmin is overestimated by immunologic assay, which generates negative values of non-Cp Cu if the Cu : Cp ratio is not in the correct range [9, 15]. (4) The ceruloplasmin activity assay measures the holo-form of ceruloplasmin; even more reliable than the immunologic assays, it can not be used in the non-Cp Cu calculation, because commercial ceruloplasmin standards for conversion of activity into concentration are not recommended as a result of their variable purity [5, 6]. The use of Cu : Cp has been proposed to overcome such problems, providing an internal control for the accordance between copper and ceruloplasmin necessary to correctly apply Walshe's formula [15, 33]. However, Cu : Cp may behave differently, depending on the ceruloplasmin assay, as the measurement of activity of this protein is not standardized [33].

Previously published methods for direct detection of non-Cp Cu [14, 34] have demonstrated a certain feasibility. In line with the test named "free copper" by McMillin and colleagues [14], we have demonstrated the feasibility of separating the fraction of labile non-Cp Cu from total copper in serum. The R^2-coefficient of regression of 0.5 corresponds to the fact that the direct technique measures the Cu(II) specifically, while the Walshe's calculated method combines Cu(II) and Cu(I). With respect to the McMillin's "free copper" test [14], the fluorescent method presented is specific to Cu(II); it is also faster and cheaper and could be employed easily for diagnostics. The fluorescence signal is detected on 96-well plates quickly with a fluorimeter, an instrument that is easy to use and set in diagnostic laboratories.

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Current data demonstrate that the direct method for non-Cp Cu detection is more accurate than Walshe's index in differentiating AD patients from healthy controls. More precisely, setting the false positive proportion at 5% (SP = 95%), the SE of the direct measurement method reaches almost 50% vs. 29% of the calculated non-Cp Cu. MCI subjects had values of non-Cp Cu between healthy controls and AD patients, as per previous reports [35, 36].

Overall, the direct fluorescent method reveals an indisputable superiority over non-Cp Cu index assessments.

Diagnostic accuracy related to the ability of non-Cp Cu to discriminate between AD patients and healthy controls demonstrated that it is not sensitive for AD diagnosis, but that the test has a validity in the definition of a specific subset of AD patients. In accordance with previous literature, the copper imbalance appears to be related to a percentage of AD patients and MCI subjects ranging from 50% to 65% [29, 30, 37]. In the current investigation, upon setting a cut-off of 1.91 μmol/L, the accuracy of the test was evaluated at 48% SE and 95% SP, in line with previous literature [12, 29, 30, 37].

The LR + estimated in the current subject sample was close to 10, indicating a significant contribution to the diagnosis of the test, while the LR – was 0.54, reflecting the test's ability to discriminate only a subset of AD patients.

The data presented show that the direct fluorescent test reliably detects non-Cp Cu variation and offers great contributions in diagnosing copper failure disorders. It could be used to identify subjects at a higher risk for cognitive decline regarding the disturbed copper metabolism, in line with previous reports [12, 38]. The test could be also very useful as diagnostic tool for WD, in the scoring for the diagnosis of this rare disease [39]. Studies investigating the accuracy of this test in discriminating copper AD [29] in other clinical settings, along with studies determining how well the test detects WD copper failure, are in progress.

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Meta-Analysis of Serum Non-Ceruloplasmin Copper in Alzheimer's Disease

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Abstract. The fraction of copper not bound to ceruloplasmin seems altered in Alzheimer's disease (AD). We have addressed this notion evaluating all the studies carried out from 1996 until March 2013 by means of meta-analysis. We performed our analysis on diverse indices evaluating the relationship between copper and ceruloplasmin in general circulation, namely 'Non-Cp copper', '% Non-Cp copper', and 'Adjusted copper'. For Non-Cp copper and % Non-Cp copper, the correct stoichiometry between copper and ceruloplasmin (6–8 atoms of copper for each ceruloplasmin molecule) in healthy controls has been adopted as criterion for the study to be included in the meta-analysis evaluating data with the canonic Walshe's formula for Non-Cp copper. Copper to ceruloplasmin ratio (Cu:Cp), which is an internal quality control check for ceruloplasmin calibration, was used as an index of the actual stoichiometry in the specimens. Adjusted (Adj-Cp) copper, even though less reliable, was calculated, allowing the evaluation of all the studies selected. An additional meta-analysis of systemic total copper was re-calculated accounting for all the studies carried out from 1983 to March 2013. Ten studies were analyzed in the meta-analysis for Non-Cp copper and % Non-Cp copper reaching a pooled total of 599 AD subjects and 867 controls. For Adj-Cp copper, 14 studies were analyzed with a pooled total of 879 AD and 1,712 controls. 27 studies were considered for systemic total copper meta-analysis, with a pooled total of 1,393 AD and 2,159 controls. All the copper indices analyzed were significantly higher in AD subjects compared to healthy controls.

Keywords: Alzheimer's disease, ceruloplasmin, copper, metal, plasma, serum

INTRODUCTION

The *Journal of Alzheimer's Disease* has recently welcomed a debate on the robustness of copper as a biomarker of Alzheimer's disease (AD) (available at http://www.j-alz.com/node/182). The debate focused on the portion of copper not bound to ceruloplasmin, also known as 'free' copper or labile copper. Besides the academic or theoretical nature of the debate, it is necessary to reach an interdisciplinary consensus regarding the role of copper not bound to ceruloplasmin as a risk factor for AD. Published studies addressing the hypothesis of copper not bound to ceruloplasmin dysfunction in AD have been based on heterogeneous samples, with diverse ethnic and geographical populations, as well as different methodological approaches, which are sensitive to ceruloplasmin calibration. These limitations influence statistical power which impact on the validity and utility of these measures across different research

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centers. Meta-analysis can help bridge these caveats by comparing all the covariates.

Copper is essential for life, but when inappropriately compartmentalized can catalyze oxidative stress, via Fenton and Haber-Weiss chemistry. Text books [1, 2] report that 85–95% of systemic copper is bound to ceruloplasmin (i.e., it is a ceruloplasmin-bound copper) and that the remaining copper, corresponding to 0–1.6 μmol/L is loosely bound to and exchangeable among albumin, transcuprin, various peptides, and amino acids and consequently, represents a pool of bioavailable copper for cells and tissues. These values refer to copper not bound to ceruloplasmin measured after an overnight fast, since copper from foods, after digestion through gastrointestinal secretions in the upper gastrointestinal tract reaches the liver via the portal circulation in the form of low-molecular-weight-soluble complexes of copper, mainly bound to amino acids, small peptides, micronutrients, and albumin. Copper not bound to ceruloplasmin has been proposed to be a superior marker of serum copper status [3]. It is effective in distinguishing patients with copper metabolic disorders, such as Wilson's disease, a rare but treatable autosomal recessive disease caused by mutations in the *ATP7B* gene.

Ceruloplasmin binds 6–8 atoms of copper, six structurally mounted in the holo-form of the protein, and an additional two easily exchangeable [4]: from this derives the correct stoichiometry for copper and ceruloplasmin. This evidence has been confirmed using size exclusion inductively coupled mass spectrometry (SE-ICPMS) and from crystal structure analysis [4–6]. Walshe [7] provides an equation for Non-Cp copper calculation (appendix of [7]) based on the measures of copper and ceruloplasmin in serum. Walshe's formula assumes that the amount of copper in ceruloplasmin is approximately 0.3% g/g of ceruloplasmin, corresponding exactly to 6.23 atoms of copper bound to ceruloplasmin. Through the text we use the term 'Non-Cp copper' to referring to the fraction of copper not bound to ceruloplasmin when it is calculated with the canonical Walshe's formula [7].

Copper:Ceruloplasmin (Cu:Cp) is an additional copper index which serves as a useful internal quality control verification of ceruloplasmin calibration, and provides information about the actual stoichiometry between copper and ceruloplasmin in the specimens. A Cu:Cp value of 6.6 has been advocated as the theoretical optimal ratio for healthy subjects [8]. From the knowledge that the correct stoichiometry for copper and ceruloplasmin is 6–8 atoms of copper per ceruloplasmin molecule [4–6], the plausible theoretical values of Cu:Cp, which can be effectively measured in specimens of healthy controls, should range between 6–8, even though this ratio can yield diverse values. On this basis, we decided to apply the criterion of having a correct stoichiometry for copper and ceruloplasmin within the 6–8 range for healthy controls, for a study to be included in the meta-analyses of Non-Cp copper [7] and % Non-Cp copper [8, 9] (data calculated with the Washe's formula). We used the mean value of the Cu:Cp obtained in healthy controls as an index of the actual stoichiometry for copper and ceruloplasmin in those specimens. In other words, we used the Cu:Cp ratio as an index to verify how close the actual stoichiometry measured in the healthy control specimens was to the theoretical correct one. In fact, not fulfilling the criterion of 6–8 copper/ceruloplasmin stoichiometry results in Non-Cp copper in the negative range (for a stoichiometry below 6) and in an overestimation of Non-Cp copper (for a stoichiometry above 8). Caution should be also taken when applying the Walshe's formula [7] to copper and ceruloplasmin pairs with a stoichiometry below 6.23, since it also yields results in the negative range. Conversely, when estimating copper non-bound to ceruloplasmin with diverse indices, we do not adopt any internal criterion. In fact, several different mathematical approaches using serum copper and ceruloplasmin levels have been proposed as markers of copper status, for example, the Cu:Cp ratio itself [8]. In addition, the '% of Non-Cp copper' and the 'adjusted copper' (Adj-Cp copper) [10] formulae have been proposed. Even though they are similar, these indices reflect the diverse biochemical relationship linking copper and ceruloplasmin. Specifically, the Cu:Cp is a valid cross-check of the reliability of the measures of copper and ceruloplasmin obtained in the specimen as detailed above [8]. The Adj-Cp copper is an index of serum copper status clinically equivalent to Non-Cp copper [10]: it adjusts the value of copper concentration to that of ceruloplasmin. This strategy overcomes the issues associated with estimating how many copper atoms are bound to each ceruloplasmin molecule, and consequently, even though less robust, indicates copper not bound to ceruloplasmin status, overcoming the negative values for Non-Cp copper that can be found, as well as age and gender variations in ceruloplasmin concentration.

Despite the proposed utility of Cu:Cp, Non-Cp copper, and % Non-Cp copper, as well as Adj-Cp copper for clinical interpretation of a copper dysfunction, these measures suffer largely from the methodological caveats related to the immunological methods used to quantify ceruloplasmin concentration, since

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antibodies against the holo-form of ceruloplasmin (containing 6–8 atoms of copper) cross-react with fragments of the apo-form of the protein, a copper depleted form of ceruloplasmin, which is rapidly cleaved and removed from general circulation. The cross-reaction determines an overestimation of ceruloplasmin concentration [3, 7, 8].

We have previously published meta-analyses, a statistical method that combines the results of different studies, to address the question if total serum copper is changed between AD subjects compared to healthy controls [11, 12]. Here we present meta-analyses of systemic Non-Cp copper, % of Non-Cp copper, and Adj-Cp copper data, clinically equivalent indices of copper not bound to ceruloplasmin, in AD and healthy controls, considering all the studies, evaluating both copper and ceruloplasmin in general circulation, published from 1996 until March 2013, also accounting for the confounding factors described above. Moreover, we have re-run a meta-analysis of systemic total copper in AD [11, 12] adding any newly published studies, and discussed all these results in the context of recent meta-analytic data published on copper concentrations in the AD brain. A critical evaluation based on meta-analytic data about copper and copper not bound to ceruloplasmin in AD, and their tentative elaboration in a holistic perspective is reported herein.

METHODS

To identify eligible studies for meta-analysis, we entered the keywords "Alzheimer's disease", "copper", "ceruloplasmin", "free copper", "Non-Cp copper", "serum", metals" and their combinations into PubMed (ncbi.nlm.nih.gov/pubmed/) and selected studies from 1996 to 2013. We also identified other studies using the "Scopus" and "ISI Web of Knowledge" databases. After reviewing the contents of this first selection, we also reviewed their reference lists to search for additional studies via "Google Scholar".

We only considered papers showing comparative analyses of AD and healthy populations, presenting original results, and published in peer-reviewed journals. In all included studies, the severity of the AD subjects' cognitive decline had been assessed by Mini-Mental State Examination (MMSE, [13]). The primary aim of our meta-analysis was the comparison of the Non-Cp copper values between AD subjects and controls.

Our first selection consisted of 18 studies [14–30]. At this point, we further required that articles explicitly report the means of Non-Cp copper values or of copper and ceruloplasmin, together with standard deviations for both patient and control groups. When not provided by the authors, the variance was calculated with standard procedures. Specifically, the variance was calculated with the formula for the calculation of the variance of the linear combination of two variables, which are not independent of each other. From the first selection, four studies from our laboratory were excluded since their patient samples partially overlapped [26, 28–30].

For the studies analyzed with the Walshe's formula in the Non-Cp copper and % Non-Cp copper meta-analyses, the additional criterion of the correct stoichiometry for copper and ceruloplasmin, i.e., hav-

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Table 1
Demographic data of the eligible studies for meta-analyses

References	AD				Healthy controls				p value
	N°	Gender (%F)	Mean age (y)	MMSE	N°	Gender (%F)	Mean age (y)	MMSE	
Molaschi et al. [18]	31	100%	77.2 ± 2.4		421	100%	77.6 ± 2.3		nd
Snaedel et al. [21]	44	72.7%	74.3 ± 10.7	15.9 (6–28)	44	72.7%	74.3 ± 11		nd
Squitti et al. [24]	47	74.5%	75.6 ± 7.7	18.6 ± 4.7	44	45.5%	71.1 ± 11	28.1 ± 1.3	0.001
Squitti et al. [22]	28	71.4%	71.4 ± 8.6	15.5 ± 6.2	25	68%	70 ± 9.6	28.4 ± 1.2	<0.001
Sedighi et al. [20]	50	48%	76.4	14.3 ± 4.6	50	50%	67.8	25.8 ± 1.5	nd
Squitti et al. [26]	51	78.4%	73 ± 8	19.2 ± 4.2	53	66%	70 ± 10	28.5 ± 1.2	0.002
Agarwal et al. [14]	50	38%	59.9 ± 11.6	14.1 ± 7.6	50	34%	55.32 ± 11		nd
Zappasodi et al. [27]	54	81.5%	73.7 ± 8.7	19.5 ± 3.8	20	65%	71.5 ± 9.2	28 ± 1.7	0.013
Arnal et al. [15]	110	58.2%	74.7 ± 4.1	16.6 ± 2.3	79	48%	77.8 ± 3.7	28.6 ± 0.9	<0.05
Brewer et al. [16]	28	46%	76.2 ± 11.6	24 ± 3.91	29	69%	68.6 ± 6.3	29.8 ± 0.7	0.24*
Squitti et al. [23]	105	78%	74 ± 8	19.6 ± 4.6	100	57%	69 ± 9.7	25.5 ± 1.3	<0.001
Squitti et al. [25]	93	77%	75.14 ± 8.7		48	48%	70.29 ± 9		<0.001
Lopez et al. [17]	36	55.5%	77.7 ± 5.3	20.7 ± 4.4	33	63.6%	74 ± 5.0	28.9 ± 1.3	nd
Rembach et al. [19]	152	59.9%	77 ± 7.9	19.3 ± 5.2	716	57.5%	69 ± 6.8	28.9 ± 1.2	$p > 0.05$

MMSE, Mini Mental State Examination; p value, the significance reported by the authors in their studies relatively to the comparison for Non-Cp copper between AD patients and healthy controls; nd, not determined in the original study by the authors. *% of Non-Cp copper between AD and healthy controls reported by the authors is $p = 0.01$.

ing the internal quality control check of ceruloplasmin calibration ratio in healthy controls within the 6–8 range, had to be fulfilled [8]. This criterion was based on the evidence that each ceruloplasmin molecule has 6 to 8 sites for copper atom binding and was verified considering the actual Cu:Cp in healthy subjects specimens. Four studies were not included for Non-Cp and % Non-Cp copper meta-analyses since they did not fulfill this internal quality control check of ceruloplasmin calibration [14, 16, 19, 20].

Cu:CP ratio was calculated as reported in Twomey et al. [8]. These authors provided the equation as follows: [copper μM]* [132000 g/mol] / [CP mg/dL*10^4].

In the studies beyond ours in which the authors did not report the value of Non-Cp copper, we computed the amount of copper bound to ceruloplasmin and the amount of Non-Cp copper following standard procedures (Appendix 1 of [7]: "Calculation of 'free copper' concentration"); briefly: CB (copper bound to ceruloplasmin) = ceruloplasmin (mg/dL) * 10* n; $n = 0.0472$ (μmol/mg); Non-Cp copper = total serum copper – CB [7].

Additionally, we compared Adj-Cp copper in the meta-analysis incorporating data from14 studies (Tables 1 and 2), and total copper in a meta-analysis including data from 27 studies [14–20, 22–27, 31–44] (Table 3).

Adj-Cp copper was calculated as reported by Twomey et al. [10], who provided the formula which follows: [Adj-Cp copper](μmol/L) = [total copper] (μmol/L) – 0.052 x [ceruloplasmin (mg/L)] + 17.5 (μmol/L).

In order to evaluate the influence of differences in percentages of females or the mean age in each cohort on Non-Cp copper concentrations, we carried out a meta-regression, assuming the unified estimation of Non-Cp copper as a variable of response and the different sample composition as an explicative variable.

Statistical analysis

Data was entered into STATA 10.1. Meta-analysis was performed, where appropriate, using a random-effects model to obtain summary statistics for the overall difference in copper. We computed the Standardized Mean Difference (SMD), which standardizes the outcome for each individual study to the effect size found in terms of the standard deviation observed in the study. SMD is generally used as a method for pooling data from different scales. We evaluated heterogeneity via the Cochran's Q test and quantified through the I^2. The I^2 describes the rate of variation across studies due to heterogeneity rather than chance, ranging from 0 (no heterogeneity) to 100 (maximal heterogeneity). To evaluate the influence of the subjects' mean age and gender on study effect size, we used meta regression. All results are reported with 95% confidence intervals (CI) and all *p*-values are two-tailed. Publication bias was evaluated with funnel plot and the "Trim and Fill" procedure that tests how the effect size will shift, when the apparent bias was removed. More precisely, funnel plot is a simple scatterplot of the differences of the estimates (Non-Cp copper, % Non-Cp, or Adj-Cp copper) between AD patients and healthy controls estimated from individual studies (horizontal axis) against the standard error of the estimates (vertical axis) [45].

Trim-and-Fill is a funnel-plot-based method to test and adjust for publication bias in meta-analysis. The procedure estimates the number of unpublished hidden studies and then corrects the meta-analysis by imputing the presence of these missing studies to yield an unbiased pooled estimate [46].

RESULTS

Demographic data from the 14 studies eligible in our meta-analyses are listed in Table 1. Biochemical indices of copper status of the same studies are reported in Table 2.

Meta-analyses of Non-Cp copper and % Non-Cp copper

Among the 14 studies reported in Table 2, only 10 fulfilled the Cu:Cp criterion (Fig. 1, Table 2). The subject samples obtained from these 10 studies reached a pooled total of 599 subjects and 867 healthy controls. The patient sample size ranged from 31 [18] to 105 [23] and control sample size from 20 [27] to 421 [18]. The mean age of the patient groups was > 70. The percentage of female AD subjects ranged from 56% [17] to 100% [18]. There was a moderate heterogeneity among the included studies ($I^2 = 64.6\%$), therefore it was decided to perform a model for casual effects. Results, shown in Fig. 1, indicate that AD subjects had higher levels of Non-Cp copper than healthy controls (SMD = 0.63, 95% CI 0.419–0.842, $p < 0.0001$).

In order to check for a possible publication bias, we performed a funnel plot (Fig. 2) that suggested an asymmetry confirmed by the Trim-and-Fill procedure. This method indicated that two hypothetical studies were missing. The statistical significance of

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Table 2
Indices of serum copper status in all the 14 studies analyzed

References	AD										Healthy Controls										
	N	Copper (mean) (μmol/l)	Copper (SD)	Cp (mean) (mg/dl)	Cp (SD)	Non-Cp copper (mean) (μmol/l)	Non-Cp copper (SD)	Adj-Cp copper (mean) (μmol/l)	Adj-Cp copper (SD)	% Non-cp copper	N	Copper (mean) (μmol/l)	Copper (SD)	Cp (mean) (mg/d)	Cp (SD)	Non-Cp copper (mean) (μmol/l)	Non-Cp copper (SD)	Cu:Cp ratio	Adj-Cp copper (mean) (μmol/l)	Adj-Cp copper (SD)	% Non-Cp copper
Molaschi et al [18] (1996)	31	18.9	3.4	38.8	7.1	0.5	2.7	16.2	2.8	2.9	421	19.3	3.8	40.6	9.1	0.1	3.6	6.27	15.7	3.9	0.7
Snaedal et al [21] (1998)	44	19.1	3.9	38.2	6.8	1.1	2.9	16.7	2.9	5.6	44	19.4	2.2	38.3	8.1	1.3	3.0	6.69	17.0	3.4	6.8
Squitti et al [24] (2005)	47	17.2	5.9	30.1	5.9	3.0	4.5	19.1	4.4	17.4	44	12.6	2.5	26.5	4.7	0.1	2.1	6.28	16.3	2.2	0.7
Squitti et al [22] (2006)	28	16.2	3.2	27.9	5.3	3.0	2.4	19.2	2.4	18.7	25	12.8	2.3	24.6	2.9	1.2	1.8	6.87	17.5	1.8	9.3
Sedighi et al [20] (2006)*	50	21.7	3.1	27.7	9.6	8.6	3.3	24.8	3.7	39.7	50	20.8	2.5	31.1	5.4	6.2	2.2	8.84	22.2	2.4	29.5
Squitti et al [26] (2007)	51	16.1	5.3	28.4	4.7	2.7	4.1	18.8	4.0	16.7	53	13.0	2.8	26.8	5.3	0.4	2.4	6.40	16.6	2.5	2.7
Agarwal et al [14] (2008)*	50	24.6	4.8	41.6	9.6	4.9	3.7	20.4	3.9	20.1	50	21.1	5.0	28.8	8.0	7.5	4.1	9.68	23.7	4.1	35.7
Zappasodi et al [27] (2008)	54	15.1	3.4	27.6	5.9	2.1	2.5	18.3	2.6	13.7	20	12.9	3.0	27.0	5.3	0.2	2.5	6.31	16.4	2.6	1.2
Brewer et al [16] (2010)*	28	17.0	2.4	24.4	3.0	5.5	1.8	21.8	1.8	32.2	29	18.4	3.1	25.7	3.7	6.3	2.5	9.45	22.5	2.5	34.1
Arnal et al [15] (2010)	110	15.7	0.8	25.2	10.7	3.8	4.5	20.1	5.1	24.3	79	12.7	0.9	26.5	7.0	0.2	2.8	6.34	16.5	3.2	1.8
Squitti et al [23] (2011)	105	16.0	3.7	28.0	5.4	2.8	2.7	18.9	2.7	17.4	100	14.0	2.7	28.0	5.6	0.8	2.4	6.60	16.9	2.5	5.6
Lopez et al [17] (2013)	36	15.8	2.9	25.9	4.1	3.6	2.1	19.9	2.1	22.8	33	13.8	3.7	25.8	4.3	1.6	3.0	7.06	17.9	3.0	11.8
Rembach et al [19] (2013)*	152	13.9	2.7	31.6	7.9	−1.0	2.7	15.0	3.0	−7.1	716	14.3	2.9	31.7	7.9	−0.6	3.1	5.97	15.4	3.3	−4.4
Squitti et al [25] (2013)	93	15.8	5.1	27.8	5.7	2 7	3.8	18.9	3.8	17.2	48	12.8	2.1	26.2	5.3	0.2	2.1	6.42	16.6	2.3	1.9
AFAR Fatebene-fratelli	378	16.0	4.5	28.2	5.5	2.7	3.3	18.8	3.3	16.9	290	13.2	2.6	26.9	5.1	0.5	2.2	6.31	16.7	2.3	3.8

*indicates the four studies not fulfilling the Cu:Cp criterion (values below 6 and higher than 8 in healthy controls), which determined their exclusion from the Non-Cp copper and % Non-Cp copper metanalyses. They were included in the Adj-Cp copper meta-analysis; AFAR Fatebenefratelli represents all the studies from AFaR Fatebenefratelli collapsed in a sole study with a weighted mean value.

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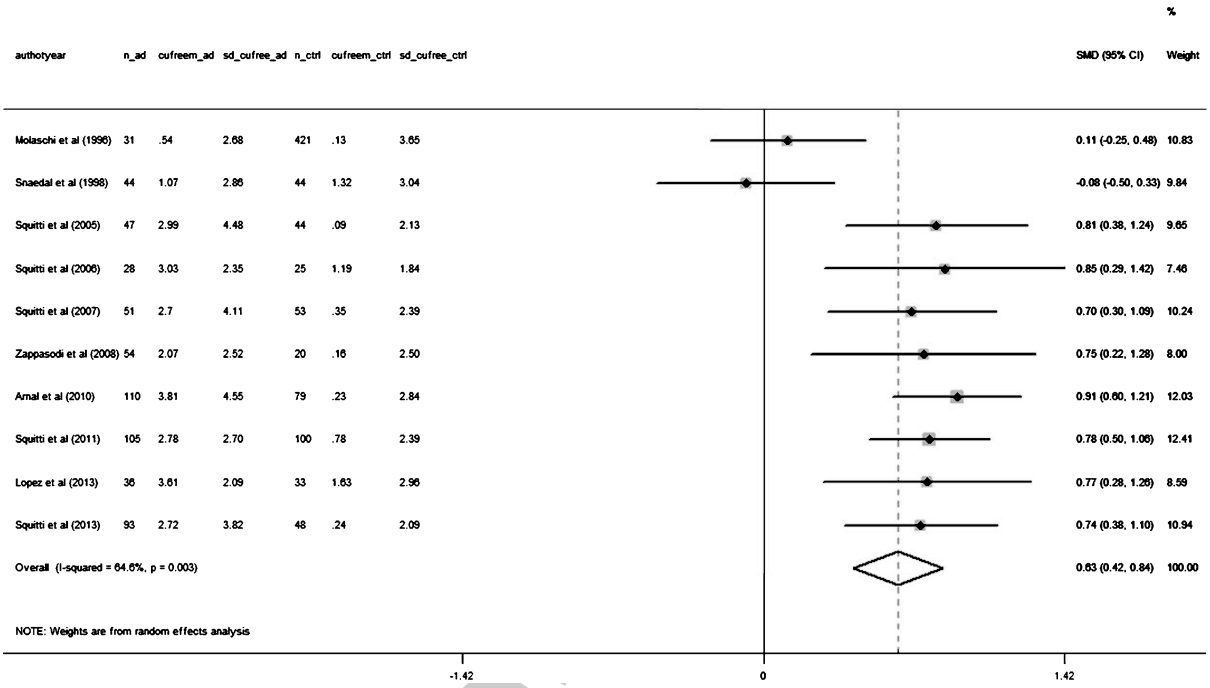

Fig. 1. Standardized mean difference (SMD) computed from the studies of Non-Cp copper (μmol/L) using random-effect meta-analysis. SMDs between AD subjects and controls are represented by squares, whose sizes are proportional to the sample size of the relative study. The whiskers represent the 95% confidence interval (CI). The diamond represents the pooled estimate based on the random effects model, with the center representing the point estimate and the width the associated 95% CI. Heterogeneity Chi-squared = 25.41 (d.f. = 9), $p = 0.003$. I^2 (variation in SMD attributable to heterogeneity) = 64.6%. Estimate of between-study variance Tau-squared = 0. 0724; Test of SMD = 0: z = 5.85, $p = 0.000$. Dashed line represents the weighted mean value of all the studies analyzed. The solid line represents the zero of the difference (Δ) between AD and healthy controls. Studies overlapping the zero line are not different between AD and controls in terms of Non-Cp copper. Studies on the right of the solid line show higher Non-Cp copper in AD compared to healthy controls. Studies on the left of the solid line show lower Non-Cp copper in AD compared to healthy controls. No studies showed lower levels of Non-Cp copper in AD compared to healthy controls.

these two studies fell into the low statistical significant area ($p > 0.10$), suggesting studies showing a non-significantly difference of Non-Cp levels between AD and healthy controls were missing. Therefore, the observed asymmetry in the funnel plot was plausible due to the publication bias. Adding these hypothetical studies in the analysis, we reached a SMD = 0.571 (95% CI 0.376–0.767) which remains significant ($p = 0.0001$). We ran an additional analysis collapsing all the studies from AFaR Fatebenefratelli in a sole study with a weighted mean value (Fig. 3). This meta-analysis confirmed higher levels of Non-Cp copper in AD subjects compared to healthy controls (SMD = 0.51, 95% CI 0.154–0.866, $p = 0.005$). Moreover, when considering only one study from Squitti and colleagues [23], Non-Cp copper was still higher in AD subjects compared to healthy controls (SMD = 0.51, $p = 0.01$; I-squared = 82.5%).

When the % Non-Cp copper was considered, AD subjects were different from healthy controls ($p < 0.0001$), with an increase of 11% Non-Cp copper when considering the 10 studies, and 9.5% Non-Cp copper when considering the analysis with the weighted study of AFaR Fatebenefratelli ($p = 0.021$).

Meta-analysis of Adj-Cp copper

Although Adj-Cp copper is not a robust measure, as it does not take into account the correct stoichiometry, it still has the advantage of being calculated on the basis of copper and ceruloplasmin concentrations in the same specimen (see Methods), independently of the verification of ceruloplasmin calibration [10]. This analysis, comprehensive of all 14 studies (Tables 1 and 2), reached a pooled total of 879 AD subjects and 1,712 healthy controls. This meta-analysis indicated higher levels of Adj-Cp copper in AD compared to healthy controls ($p = 0.004$; Fig. 4). However, when considering the analysis with the weighted study of AFaR Fatebenefratelli, or considering a single study from Squitti and colleagues [23], there was no difference in Adj-Cp copper ($p = 0.230$).

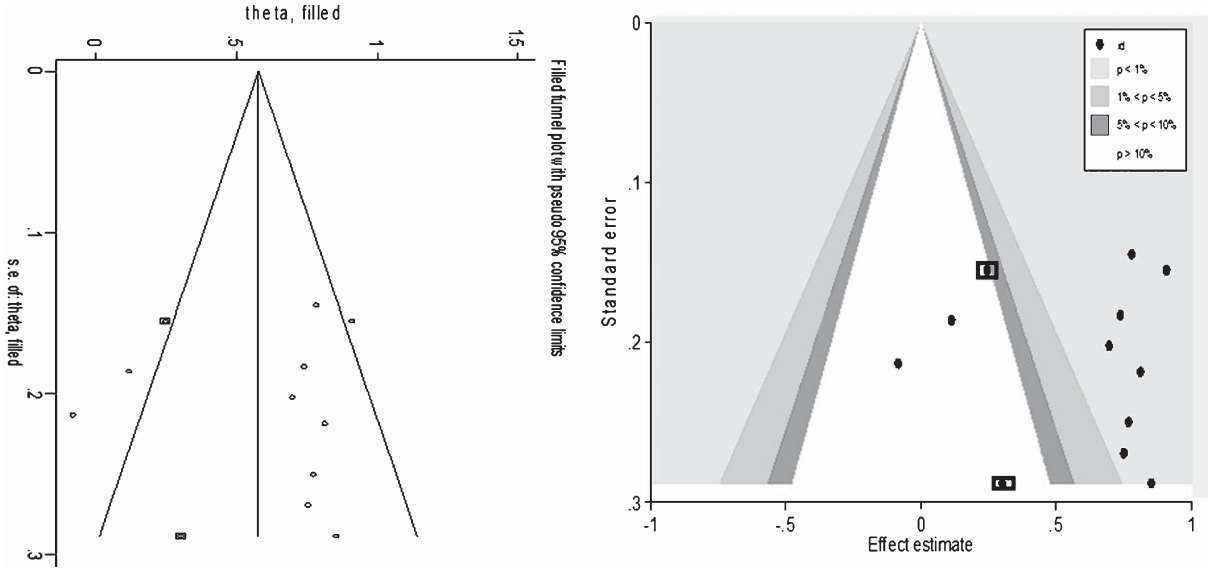

Fig. 2. Funnel plot suggested the presence of publication bias. This result was confirmed by the Trim-and-Fill procedure. Two hypothetic studies should be expected to resolve asymmetry, reaching an SMD = 0.571 (95%CI 0.376–0.767), which remains significant ($p = 0.0001$) (a). These hypothetical studies would fall in the low significant area and the obtained results do not provide sufficient evidence to doubt the validity of the findings (b). The solid vertical line represents the standardized mean difference (SMD), the diagonal lines represent the 95% confidence limits, and the points in the square are the studies filled by trim and fill.

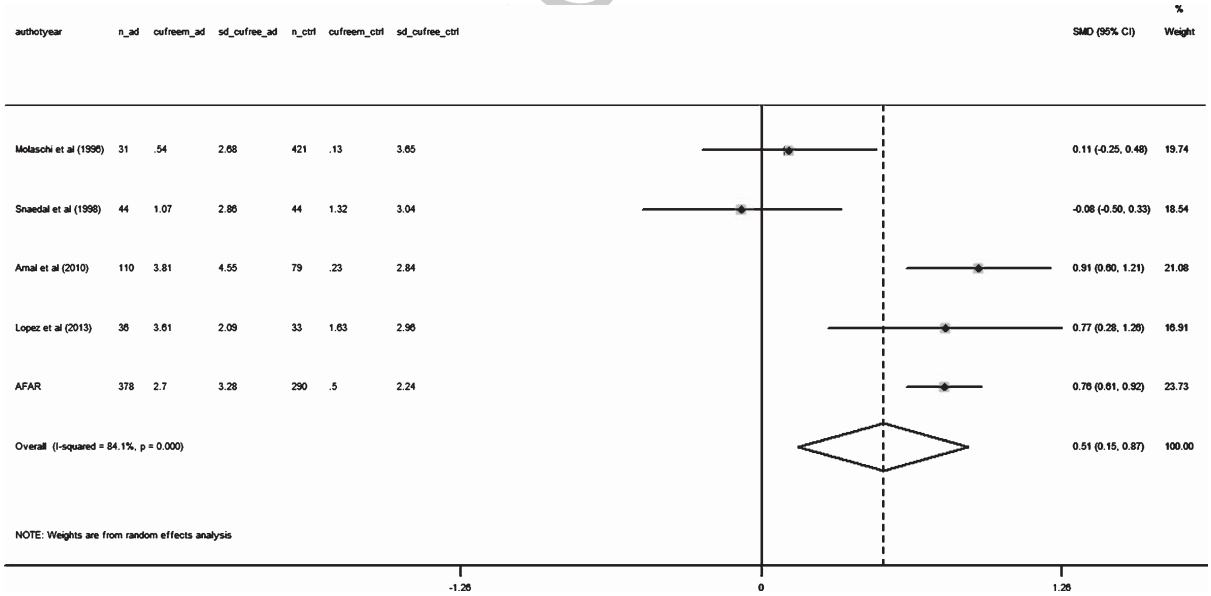

Fig. 3. Standardized mean difference (SMD) computed from the studies on Non-Cp copper (μmol/L). SMDs between AD subjects and controls are represented by squares, whose sizes are proportional to the sample size of the relative study. The whiskers represent the 95% confidence interval (CI). The diamond represents the pooled estimate based on the random effects model, with the center representing the point estimate and the width the associated 95% CI. FBF: represents all the studies from AFaR Fatebenefratelli collapsed in a sole study with a weighted mean value. Heterogeneity Chi-squared = 25.14 (d.f. = 4), $p = 0.000$. I^2 (variation in SMD attributable to heterogeneity) = 84.1%. Estimate of between-study variance Tau-squared = 0.13; Test of SMD = 0: z = 2.81, $p = 0.005$.

We ran additional checks for Non-Cp copper or Adj-Cp copper to evaluate the effect of gender, age, and ethnicity. We analyzed the contribution of gender and age by meta-regression separately and observed that both these factors do not account for the results. For Non-Cp copper, we observed a non-significant effect

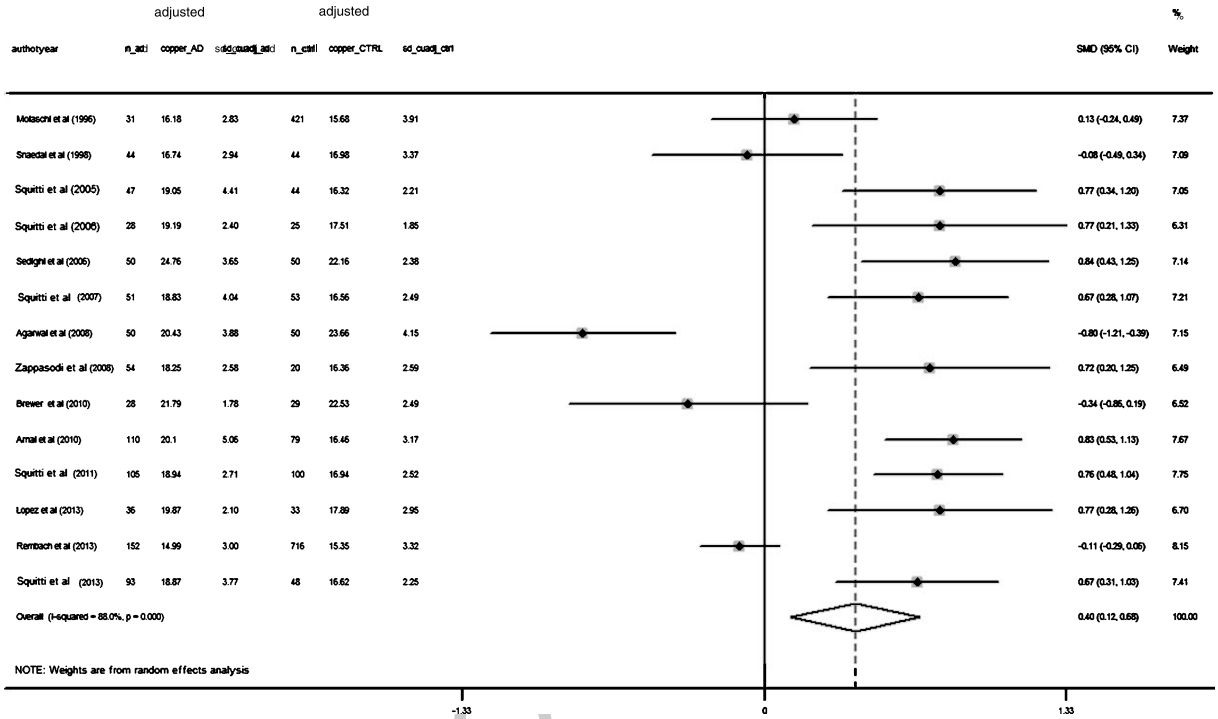

Fig. 4. Standardized mean difference (SMD) computed from the studies on ADj-Cp copper (μmol/L). SMDs between AD subjects and controls are represented by squares, whose sizes are proportional to the sample size of the relative study. The whiskers represent the 95% confidence interval (CI). The diamond represents the pooled estimate based on the random effects model, with the center representing the point estimate and the width the associated 95% CI. Heterogeneity Chi-squared = 112.72 (d.f. = 13), $p = 0.0001$. I^2 (variation in SMD attributable to heterogeneity) = 88.5%. Estimate of between-study variance Tau-squared = 0.26; Test of SMD = 0: z = 2.92, $p = 0.004$.

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for gender ($p = 0.167$) or age ($p = 0.447$) on the pooled estimates. We obtained the same results considering Adj-Cp copper ($p = 0.098$ and $p = 0.480$) for gender and age, respectively.

Controlling for geographic differences, we ran meta-analyses stratifying studies on the basis of the ethnicity of the populations analyzed. Concerning Non-Cp copper, all the studies entered in the original meta-analysis (Fig. 1) have been carried out in European populations, thereby limiting the geographic effect. Concerning Adj-Cp copper there was only one study carried out in a Median-Asian ethnic population [20] and one in a central Asian ethnic group [14], while the remaining 12 studies were all from a European ethnicity. Considering meta-analysis in this European restricted group of studies, Adj-Cp copper was higher in AD subjects than in controls (SMD = 0.46, $p = 0.001$; $I^2 = 84.8\%$), however, the meta-analysis of Adj-Cp copper considering the weighed AFaR study resulted in no significant differences (SMD = 0.228; $p = 0.136$; $I^2 = 91.8\%$). Assuming that ethnic heterogeneity of the Australian population [47] analyzed by Rembach and colleagues [19] prevented the inclusion of this data among the European cohorts, 11 studies were included. This analysis resulted in a significant pooled estimate (SMD = 0.527, $p < 0.001$) with a moderate heterogeneity across the studies ($I^2 = 70.3\%$), indicating that AD subjects had higher Adj-Cp copper than controls, also when considering the weighed AFaR Fatenenefratelli study (SMD = 0.370; $p = 0.042$; $I^2 = 85.1\%$). Finally, also taking into account the moderate to high heterogeneity across all the studies (I^2 ranging from 70% to 85%), a geographic effect seemed unlikely.

On the basis of the observed asymmetry in the funnel plot, we applied the trim-and-fill procedure to verify possible bias due to publication. The procedure estimated four missing studies and after adjusting the analysis for these missing studies, we obtained a no significant pooled estimate (SMD = 0.207, $p = 0.128$).

Meta-analyses of total copper

To complete our investigation we re-ran the meta-analyses published by Ventriglia et al. [12], including all the additional studies published until March 2013. Demographic data of the 27 studies included in our

meta-analysis are listed in Table 3. The subject samples obtained from these studies reached a pooled total of 1,393 AD subjects and 2,159 controls.

The results shown in Fig. 5 confirmed higher levels of total copper in AD subjects than in healthy controls ($p < 0.0001$). Even when we ran an additional analysis collapsing all the studies from AFaR Fatebenefratelli in a sole study with a weighted mean value, the results confirmed higher levels of total copper in AD subjects than in healthy controls ($p = 0.05$).

On the basis of the observed asymmetry in the funnel plot we applied the trim-and-fill procedure to verify possible bias due to publication. Meta-regression revealed that neither gender nor age had an effect on the analysis.

DISCUSSION

The main result of our meta-analyses is that copper not bound to ceruloplasmin appears significantly increased in AD subjects when compared to healthy controls. This result was sustained when; i) the meta-analysis was carried out using Non-Cp as well as the % of Non-Cp copper; ii) when using Adj-Cp copper, a clinically equivalent measure of copper not bound to ceruloplasmin (although less rigorous), iii) when total copper was evaluated, and iv) when all the studies from AFaR Fatebenefratelli were grouped in a sole weighted study. Moreover, studies from the AFaR Fatebenefratelli group containing some redundant data were excluded at the beginning (see Methods), and consequently the meta-analyses presented here has no overlapping samples, reducing the bias of our analysis. Methods for ceruloplasmin measurements have been changed during the course of our studies started in 2002; specifically, we have employed Roche, as well as Horiba ABX and Futura System ceruloplasmin assays. Even when considering the additional check of replacing all the studies by AFaR with a sole weighted study, Non-Cp copper, % of Non-Cp copper, and total copper in general circulation remained higher in AD when compared to healthy controls, also taking into account that the random-effects estimate gives greater relative weight to a smaller study. In fact, the weight of the AFaR Fatebenefratelli studies, when collapsed in a sole study, is heavily underestimated. For example, the estimated weight of Non-Cp copper of the AFaR study was 23.73% but this study accounted for a number of AD patients 3.4 times higher than the second main larger study, which is the Arnal et al. study [15] that has a weight of 21.08% (Fig. 3). Similarly, the weight of the AFaR Fatebenefratelli study in terms of total copper has been estimated as 5.93%, but this study accounted for a number of AD subjects 4 times higher than the second main larger study, which is the Rembach et al. study [19] and has a weight of 5.86%.

Meta-regression revealed there was no significant effect from age or gender in the analysis.

Generalizing the results of current meta-analyses, it can be inferred that the increase in Non-Cp copper can account for the increase in total copper observed in AD subjects. This increase is slight, ranging around 10% of Non-Cp copper, but it is significant.

Results of the meta-analyses of Non-Cp copper were univocal, with none of the studies reporting Non-Cp copper decreases in AD compared to healthy controls, and only two studies [18, 21] reporting no variation. However, in one of these, the Snaedal and colleagues study [21], the authors reported a significant decrease in ceruloplasmin activity in AD subjects, suggesting there is a decrease in concentration of the holo-form of ceruloplasmin and consequently higher levels of Non-Cp copper, even though not quantifiable.

Among the others considered in the Adj-Cp copper evaluation, superimposable results were achieved by the Brewer et al. study [19]. In fact, these authors also found a significant decrease in ceruloplasmin activity in AD subjects than in controls. The study by Agarwal et al. [14] was the only one reporting decreased levels of Adj-Cp copper in AD. However, in their original study, Agarwal and colleagues [14] reported higher levels of total copper in AD than in healthy controls. Moreover, in this study, close to 30% of healthy controls had higher copper levels than normal reference range values [48]. This finding is consistent with an increase in total copper linked to ceruloplasmin upregulation, possibly due to inflammatory processes, suggesting not negligible additional inflammatory conditions affecting at least 30% of the control subjects. Also the study by Brewer et al. [16] resulted in no change in Adj-Cp copper, even though the authors reported a significant increase in terms of % of Non-Cp copper in AD. These data illustrates how copper status indices address the diverse biochemical relationship linking copper to ceruloplasmin. Furthermore, this data suggests there is an altered relationship between copper and ceruloplasmin in AD in most of them, despite an additional study reporting no difference in Adj-Cp copper from the Australian Imaging Biomarkers and Lifestyle Study of Aging (AIBL) [19]. At the same time, these four studies [14, 16, 19, 21] highlight the limitations of immunological methods which appear to impact the quality of their ceruloplasmin

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Table 3
Indices of serum copper status in all the 27 studies analyzed

References		AD								Controls							
		N	Female (%)	Mean Age	Age (SD)	Copper (mean) (μmol/l)	Copper (SD)	Cp (mean) (mg/dl)	Cp (SD)	N	Female (%)	Mean Age	Age (SD)	Copper (mean) (μmol/l)	Copper (SD)	Cp (mean) (mg/dl)	Cp (SD)
Shore et al (1984)	[41]	10		63.70	8.40	18.25	4.25			10		61.90	8.00	18.57	1.60		
Jeandel et al (1989)	[35]	55	72.7	81.70	5.70	22.03	6.13	33.00	7.00	24	79.2	76.40	6.10	21.24	4.10		
Kapaki et al (1989)	[36]	5	20.0	54.00		14.15	3.77			28	35.7	46.00		16.20	2.20		
Basun et al (1991)	[31]	24	70.8	75.00	8.00	17.00	3.90			28	57.1	78.00	3.00	16.70	3.00		
Mattiello et al (1993)	[37]	21	71.4	81.00	6.00	18.80	0.16			10	50.0	82.00	2.00	18.80	0.16		
Molaschi et al (1996)	[18]	31		72.00		18.87	3.41			421		77.60	2.30	19.30	3.80		
Molina et al (1998)	[38]	26	46.2	73.10	8.20	15.10	3.46			28	42.9	70.80	7.30	14.47	4.10		
Gonzalez et al (1999)	[34]	51	35.3	70.30	4.00	16.63	3.55			40	90.0	74.50	2.30	15.37	2.58		
Squitti et al (2002)	[43]	79	68.4	74.50	7.40	18.35	5.70			76	56.6	70.10	10.00	13.70	2.60	27.70	2.20
Ozcankaya et al (2002)	[39]	27	29.6	72.30	6.50	11.97	1.30			25	36.0	64.40	7.20	12.11	1.50	28.20	2.40
Smorgon et al (2004)	[42]	8				22.92	3.90			11				16.66	1.27		
Bocca et al (2005)	[33]	60	66.7	74.60	6.39	15.21	3.80			44	25.0			14.31	3.10		
Squitti et al (2005)	[24]	47	114.9	75.60	7.70	17.20	5.90	30.10	5.90	44	45.5	71.10	11.00	12.60	2.50	28.10	1.30
Sedighi et al (2006)	[20]	50	48.0	76.40		21.70	3.11	27.70	9.60	50		67.80		20.80	2.50	25.80	1.50
Squitti et al (2006)	[22]	28	71.4	71.40	8.60	16.20	3.20			25	68.0	70.00	9.60	12.80	2.30	28.40	1.20
Sevym et al (2007)	[40]	98	66.3	72.10	6.70	16.70	2.90			76	59.2	70.30	5.70	15.40	2.10		
Squitti et al (2007)	[26]	51				16.10	5.30	28.40	4.70	53				13.00	2.80		
Agarwal et al (2008)	[14]	50	38.0	59.96	11.57	24.57	4.76	41.60	9.63	50	34.0	55.32	10.88	21.15	4.90		
Zappasodi et al (2008)	[27]	54	81.5	73.70	8.70	15.10	3.40	27.60	5.90	20	65.0	71.55	9.20	12.90	3.00	28.00	1.70
Arnal et al (2009)	[15]	110	56.4	74.50	7.60	15.62	3.08	25.27	1.76	79	48.1	77.80	3.70	12.74	0.20	27.80	1.10
Vural et al (2010)	[44]	50		71.90	6.80	20.67	2.88			50		65.10	7.10	22.50	2.80		
Baum et al (2010)	[32]	44		74.30	8.70	16.20	3.50			41		79.10	6.00	15.30	2.70		
Brewer et al (2010)	[16]	28		76.20		16.90	2.40			29				18.40	3.10		
Squitti et al (2011)	[23]	105	78.0	74.00	8.00	16.00	3.70	28.00	5.40	100	57.0	69.00	9.70	14.00	2.70		
Rembach et al (2013)	[19]	152		77.00	7.09	13.90	2.70			716		69.00	6.80	14.32	2.90	28.90	1.20
Lopez et al (2013)	[36]	36		77.75	6.31	15.83	2.80			33		74.00	5.03	13.82	3.70	28.90	1.30
Squitti (2013)	[25]	93	77.0	75.14	8.69	15.82	5.13	27.78	5.74	48	48.0	70.29	8.98	12.75	2.13		
AFAR		457		66.03	7.23	16.41	4.68	21.61	4.24	366		59.87	8.39	13.32	2.58	12.60	0.79

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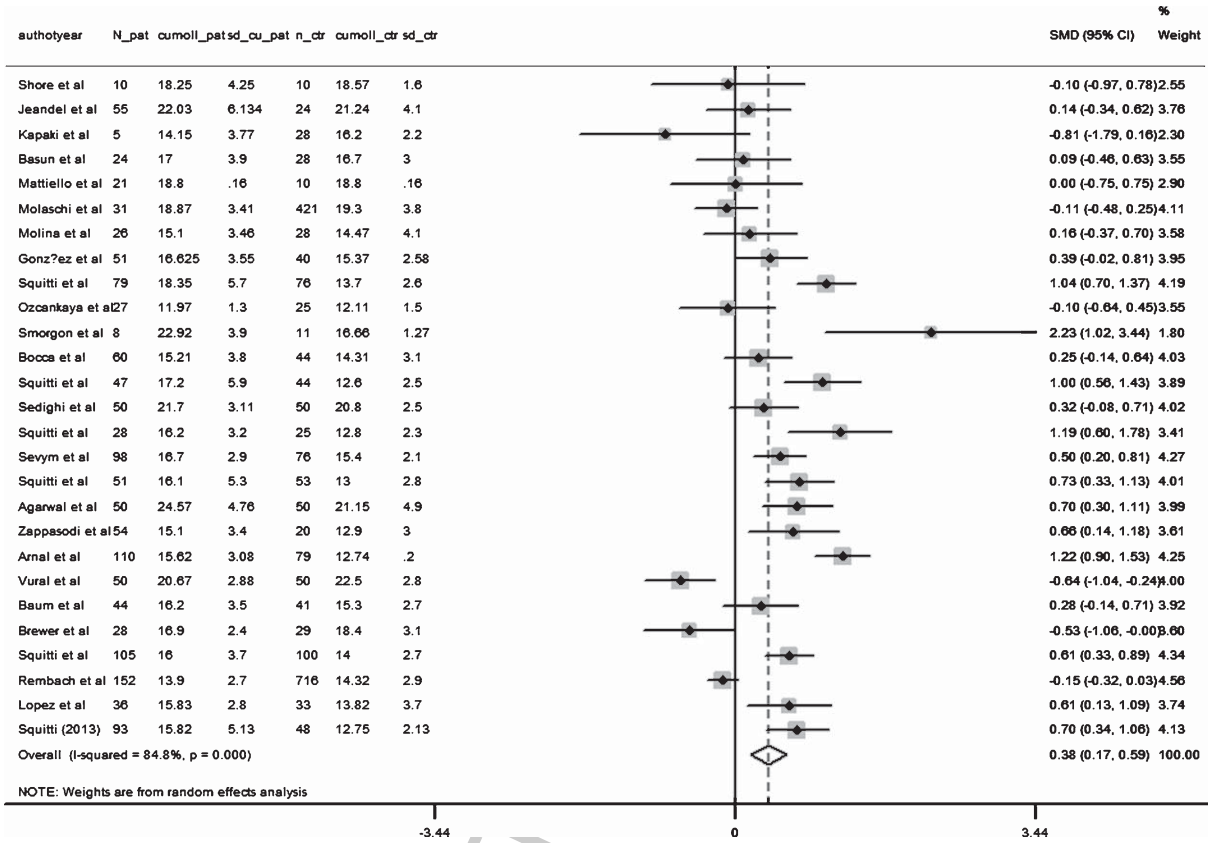

Fig. 5. Standardized mean difference (SMD) computed from the studies on total copper (μmol/L). SMDs between AD subjects and controls are represented by squares, whose sizes are proportional to the sample size of the relative study. The whiskers represent the 95% confidence interval (CI). The diamond represents the pooled estimate based on the random effects model, with the center representing the point estimate and the width the associated 95% CI. Heterogeneity Chi-squared = 171.11 (d.f. = 26), $p = 0.000$. I^2 (variation in SMD attributable to heterogeneity) = 84.8%. Estimate of between-study variance Tau-squared = 0.24; Test of SMD = 0: z = 3.59, $p = 0.0001$.

measurements, as exemplified by their Cu:Cp ratio (see Table 2). Two additional considerations can be made for the study of Snaedal et al. [21] and for the AIBL study [19]. The Snaedal et al. [21] study indicates that despite even reaching a valid Cu:Cp ratio, immunological detection still overestimates ceruloplasmin quantification, since these authors reported decreased levels of ceruloplasmin activity in their AD cohort. Thus, Non-Cp values showed in current meta-analyses are very conservative, pointing out the need to overcome calculations developing new devices to allow a direct measure of Non-Cp copper in serum [49, 50]. Moreover, the AIBL study is the only one with a Cu:Cp ratio below 6. The speculation that genetic factors may account for diverse ceruloplasmin estimations in the Australian cohort, cannot be discharged aprioristically. In particular, ethnicity may explain this result [47]. Serum ceruloplasmin levels are affected by genetics [51], and among human populations, Australians show a very complex genetic distribution [47]. This is borne out by considering the results of the meta-analyses run excluding the AIBL study data [19], whereby exclusion of ethnic heterogeneity resulted in decreases from $I^2 = 91.8\%$ to $I^2 = 70\%$. Additional metal studies on Australians are needed to confirm or discharge this hypothesis.

To further address the evidence of copper dyshomeostasis in AD, we have re-run the meta-analysis of total copper considering all the studies published on this matter from 1983 to March 2013, updating our previous meta-analysis of 2012 [12]. This meta-analysis revealed that total copper is higher in AD subjects than in healthy controls, even when all the studies from AFaR Fatebenefratelli were grouped in one study with a weighted mean value.

Some considerations about heterogeneity of the results can be done at this point. Even though it was still higher if considered as whole of the data pre-

sented, Non-Cp copper heterogeneity, as expressed by Higgins [52], fell in the moderate range ($I^2 = 64.6\%$). Conversely, I^2 values higher than 75%, i.e., falling in the high range of heterogeneity were obtained for Adj-Cp copper ($I^2 = 88.5\%$) and total copper ($I^2 = 84.8\%$) (Figs. 4 and 5). Even though methodological weakness can account for a % of this heterogeneity, additional factor have to be taken into account, for example, genetics which can affect metal levels [53]. Moreover, a heterogeneity associated with disease can be taken into account. Expressively, in the Italian population copper dysfunction was estimated in about 60% of subjects with AD [48]. This is not in contrast with Australian AD subjects cohort analyzed in the AIBL study. In fact, recent investigations on complex diseases, such as AD, proposed that multiple and independent pathogenic pathways can modulate the same pathologic condition [54], therefore numerous factors may account for different percentage of disease risk in populations with diverse ethnicity or living in varied environments.

Data presented in our study concern meta-analyses of systemic measurements of copper and some its related indices in AD subjects and controls. Recently, a meta-analysis of copper levels in the AD brain has been performed [55], demonstrating decreased levels of total copper in the more affected areas of the AD brain. This result has recently been replicated [50], demonstrating lower copper concentration in Brodman Area 46 (BA46) and in the temporal lobe (BA22) of the AD brain. However, in the same study, these authors demonstrated that labile copper, which resembles Non-Cp copper in serum, is increased in BA46 and BA22 areas of 25% of AD subjects compared to healthy controls. Results from the James et al. study [50] confirm the results of current meta-analyses supporting the notion of an increased concentration of Non-Cp or labile copper in serum and in the brain of AD subjects, even though levels of total copper appear unchanged or decreased in the brain. Yet some of this data is in disagreement with data achieved from studies on total copper measurements in serum or plasma, which agreed with an increase of total copper. However, it should be considered that at autopsy, AD neurodegenerative processes, not excluding those based on oxidative stress via metals in Fenton and Haber Weiss chemistry, have already damaged the brain, and unfortunately, no information about copper concentration in the areas close to death is feasible, and after death what remains is atrophy. In this framework, information from living subjects are still critically valuable and appear reliable to demonstrate a toxic impact of some metals especially when associated with clinical and functional information about the cognitive status of the patient. Numerous studies have demonstrated a link between copper toxicity and cognitive decline in AD subjects (reviewed in [56]). Moreover, the supplementation of an oral therapy of copper to subjects with AD in a phase 2, proof of concept, clinical trial by Kessler et al., 2008 was unsuccessful [57]. Conversely, an extensive number of studies since 1990 demonstrated that the hypermetallation of amyloid-β peptides can cause oxidative stress, H_2O_2 production, and Aβ plaques (reviewed in [56]).

In conclusion, meta-analytic strategies largely conclude there is a systemic copper dysfunction in AD. This evidence is supported by observed increased levels of labile copper within the brain [50], which can account for cognitive decline as demonstrated by studies of systemic copper studies on AD living subjects [56]. Recent additional results from genetic studies sustain this evidence [58, 59], supporting the holistic perspective of a copper dysfunction as a risk factor affecting a percentage of AD subjects. A positive implication of this finding is that perturbation in copper homeostasis may be amenable to treatment with anti-copper agents in those AD subjects showing a copper altered metabolism.

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RESEARCH ARTICLE

Patients with Increased Non-Ceruloplasmin Copper Appear a Distinct Sub-Group of Alzheimer's Disease: A Neuroimaging Study

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Abstract: ***Background:*** Meta-analyses show that copper non-bound to ceruloplasmin (non-Cp Cu, also known as 'free' copper) in serum is higher in a percentage of Alzheimer's disease (AD) patients. Genetic heterogeneity in AD patients stratified on the basis of non-Cp Cu cut-off sustains the existence of a copper AD metabolic subtype.

Objective: In order to find evidence of the existence of a detectable metabolic subtype of AD associated to copper abnormalities, we explore the hypothesis of a neuroimaging pattern heterogeneity in an homogenous and well characterized AD population classified in two groups by the stratification of patients on the basis non-Cp Cu cut-off.

Method: We assessed levels of copper, ceruloplasmin, non-Cp Cu, cerebrospinal levels of total Tau protein (h-tau), Thr 181 phosphorylated tau protein (P-tau) and β-amyloid 1-42, and *APOE4* genotype in 66 AD patients and compared neuroimaging indices of a visual rating scale of cerebral atrophy and neurovascular burden in AD patients stratified in 'Normal' and 'High' non-Cp Cu groups.

Results: The stratification for non-Cp Cu originated AD groups which did not differ for medial temporal lobe atrophy, periventricular hyperintensities, deeper hyperintensities (including frontal, parieto-occipital and temporal white matter hyperintensities), infratentorial hyperintensities indices, while they differed for global atrophy. More specifically, AD patients within the high non-Cp Cu group had a less severe burden of global atrophy (p=0.042).

Conclusion: This neuroimaging heterogeneity between AD groups is suggestive of the existence of a copper metabolic subtype of AD; non-Cp Cu appears a good marker of this copper AD.

Keywords: Neuroimaging heterogeneity, copper not bound to ceruloplasmin (non-Cp Cu), Alzheimer's disease, free copper, global atrophy.

1. INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia in the elderly. Based on the evidence of the monogenic familial forms, the key mediator of AD pathology has been settled as brain amyloid-β peptides (Aβ) that form dimers and oligomers, leading to aggregates, responsible for the altered function of neural cells and neurodegeneration, accompanied by the intracellular aggregates of phosphorylated tau protein forming neurofibrillary tangles. These autosomal-dominant forms of AD accounts for about 5% of cases, at difference from the sporadic late onset form (95%), which exists primarily as a complex disease. In this form, polygenic and epigenetic factors, which interact with the environment, contribute to increase the risk of the disease onset. The major risk factors are age and familiarity, and modifiable factors include diabetes mellitus, midlife hypertension, midlife obesity, physical inactivity, depression, lifetime prevalence of major depressive disorder, smoking, low educational attainment. The evidence of the impact of essential, primarily copper, and some environmental metals, as for example lead (Pb), on the risk of having AD in relation to genetic and epigenetic regulation of key genes connected to neurodegeneration and oxidative stress is growing in the latest years [1; 2].

The discovery of a copper binding domain on the amyloid precursor Protein (APP) in the latest '90 started research investigating the interaction of this essential metal with Aβ

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[3]. Enhanced concentrations of copper have been reported to co-localize within both Aβ and neurofibrillary tangles [4], and to regulate *APP* expression [5]. More recently, meta-analyses, large population [6, 7] and longitudinal cohort [8, 9] studies demonstrated copper imbalance as a risk factor for AD. The robust and consistent findings provided by meta-analyses pointed out to the breakdown of copper homeostasis, consisting in a copper decrease in the AD brain [10], a total copper increase in the blood [11, 12], and an increase of the component of copper in serum not bound to ceruloplasmin (non-Cp Cu) [11]. In the same line, large population studies demonstrated that copper in the soil correlated with an increased rate of mortality for AD in China [7], and that increased consumption of copper supplements increased the rate of total mortality [13], as well as the risk for cognitive decline, estimated in 19 years of further aging, when associated with a high trans-saturated fat diet [6].

At the cell level copper homeostasis is mainly regulated by Human copper transporter (hCtr)1, which facilitates copper influx within the cell and the ATP7A and ATP7B efflux pumps. In the liver, the ATP7B pump loads copper into nascent ceruloplasmin at the trans-Golgi network and releases excess of intracellular copper in to the bile.

Mutations in the *ATP7B* gene are responsible of Wilson's disease, which is characterized by a drop of ceruloplasmin, the spillage of toxic low molecular weight non-Cp Cu into the blood-stream and its accumulation in tissues and organs, including the brain. The blood-serum fraction of non-Cp Cu is redox active, and makes up 5-10% of total copper in serum in normal condition [14]. If the non-Cp Cu pool becomes expanded, this copper becomes toxic, as exemplified by Wilson's disease [15] and reported in AD [11].

hCTR1 mutation cause striking neonatal defects, mimicking a copper deficiency, while Menkes's and Wilson's disease are copper metabolic disorders, which show serious neurological symptoms [16]. They are the result of sex inheritance mutations of the *ATP7A* gene on chromosome X (Menkes' disease) and autosomal recessive inheritance of *ATP7B* mutations on chromosome 13 (Wilson's disease). The copper deficiency due to the impaired copper transport into and within the developing brain related to the Menkes' disease results in demyelination and neurodegeneration, whereas brain non-Cp Cu accumulation in Wilson's disease is associated with dystonia, dysarthria, depression, cognitive deterioration, personality change, psychosis and schizophrenia [17].

Since 2002 we studied metal status in AD observing copper alterations in AD patients resembling copper metabolic abnormalities typical of Wilson's disease [14]. In two different follow-up studies, we reported that subjects with higher levels of non-Cp Cu had higher risk for a more severe cognitive decline and a faster rate of deterioration with respect to subjects with normal non-Cp Cu [8, 9]. Recently we demonstrated that AD patients have different clinical and genetic features when they are stratified on the basis of a non-Cp Cu cut-off. More precisely, specific *ATP7B* gene variants have single-locus and haplotype frequency differently distributed in AD patients with higher levels of non-Cp Cu than in AD patients with normal values of this copper marker [2]. The same heterogeneity has been demonstrated when comparing another AD population for electroencephalographic (EEG)-derived brain activity alterations [18]. As a whole the heterogeneities found, especially that in genetics, speak in favor of the existence of a copper subtype of AD that is detectable by a copper marker (i.e. non-Cp Cu), which typifies a subgroup of AD individuals. Moreover, a peculiar case-report of an AD patient with 11C-labeled Pittsburgh Compound-B PET positive and a [18F] fluorodeoxyglucose PET altered with a typical AD topography and carrying Kayser-Fleischer rings - pathognomonic of Wilson's Disease - strongly support the existence of a copper phenotype of AD [19]. Current study was exactly designed to explore the hypothesis of a neuroimaging pattern heterogeneity in an homogenous and well characterized AD population classified in two groups by the stratification of the patients on the basis non-Cp Cu cut-off to find further evidence of the existence of a detectable metabolic subtype of AD associated to copper abnormalities.

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

The subject sample consisted of 66 AD patients (NINCDS-ADRDA criteria) [20-22] which a Mini-Mental State Examination (MMSE) score of 25 or less [23]. Patients underwent a structured clinical interview, a neurological examination, brain magnetic resonance imaging (MRI); an extensive neuropsychological assessment, which included the Mental Deterioration Battery (MDB) [25] the Digit Span [26] and the test of Corsi [25], as well as routine laboratory tests [26].

Sixty three % of this sample patients has been analyzed in a previous study [27].

2.3. Biochemical and Molecular Investigations

Total serum copper was measured by spectrometry, using an A Analyst 600 Perkin Elmer atomic absorption spectrophotometer [9]. Ceruloplasmin concentration was measured with an immunoturbidimetry assay (Horiba ABX, Montpellier, France). Ceruloplasmin concentration measures were automated on a Cobas Mira Plus (Horiba ABX, Montpellier, France), and performed in duplicate. 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 1: "Calculation of 'free copper' concentration") [28]. Briefly: CB = ceruloplasmin (mg/dL) * 10* n; n=0.0472 (µmol/mg); 'free' copper = absolute serum copper – CB [28]. This calculation expresses non-Cp 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. CSF levels of total Tau protein (h-tau), Thr 181 phosphorylated tau protein (P-tau) and β-amyloid 1-42 were determined by ELISA (INNOGENETICS, Belgium) [29]. *APOE* genotyping was performed according to established methods [30].

2.4. MRI Evaluation

Brain MRI was performed using a 1.5 Tesla superconductor magnet. The imaging protocol consisted of axial T2 W double Spin Echo (SE) sequences and T1 W SE images in axial, coronal, and sagittal planes, with 5 mm slice thickness and intersection gap = 0.5 mm. MR images were evaluated by two experienced neuroradiologists, blind to the patients' diagnoses and laboratory results. Atrophy and white matter lesions were graded following standardized visual rating scales on plain MRI [31-33]. The degree of medial temporal lobe atrophy (MTA) was evaluated with a ranking procedure and validated by linear measurements of the medial temporal lobe including the hippocampal formation and surrounding spaces occupied by CSF, following standardized criteria (five point rating scale of MTA) [27; 32]. Generalized brain atrophy (ventricular and sulcal atrophy) was rated as present (=1) or absent (=0; global atrophy; Fig. **1**). The visual rating scale of white matter changes included the anatomical distribution as well as the severity of the lesions. Based on anatomical distribution, a distinction was made between areas of periventricular hyperintensities (PVH) (caps and rims) and deeper hyperintensities (including frontal, parieto-occipital and temporal white matter hyperintensities - DWMH; basal ganglia hyperintensities - BGH; and infratentorial hyperintensities - ITH). Large vessel cortical infarcts in the anterior, posterior and medial cerebral artery were also evaluated. Presence of mass lesions and lobar hemorrhages were exclusion criteria from the study [27, 31, 34]. MR images were evaluated by two experienced neuroradiologists, blind to the patients' diagnoses and laboratory results. The agreement between the two neuroradiologists was >93% measured calculating inter-rate agreement kappa coefficient (MTA: k=0.98 (Standard Error=0.02), p<0.001; PVH: k=0.93% (SE=0.04), p<0.001; DWMH: k=0.93 (SE=0.04), p<0.001; BGH: k=0.94 (SE=0.04), p<0.001; ITH: k=1 (SE=0), p<0.001; Global Atrophy: k=1 (SE=0), p<0.001.

Our study pertains *biomarker science* and poses the working hypothesis that an homogeneous population of AD patients display neuroimaging heterogeneity when they are stratified in two groups on the basis of a copper marker.

To test this hypothesis, we have considered a cut-off of non-Cp Cu of 1.6 µmol/L (normal values 0-1.6 µmol/L [28]) and divided the AD patients into two subgroups: a 'Normal non-Cp Cu' group, composed of 27 AD patients with non-Cp Cu≤1.6 µmol/L, and a 'High non-Cp Cu' group, of 39 patients (who have non-Cp Cu >1.6 µmol/L).

2.5. Statistical Analyses

Normality of distributions was tested with Shapiro-Wilk test. Categorical data are expressed as percentages, and continuous variables as mean ± SD or median (interquartile range, 25th-75th percentiles), as appropriate. To compare High non-Cp Cu AD and normal non-Cp Cu AD, parametric (Student's t-test) and non-parametric (Chi-square- $\chi 2$) tests were applied. A p value < 0.05 was considered statistically significant. All analyses were performed with SPSS 16.0 (SPSS Inc., Chicago). To demonstrate the existence of a subtype of a disease four strategies can be taken into account: 1. identification of a bimodal distribution of the biomarker of interest in the patient population; 2. the response to a specific therapy; 3. cluster analysis; 4. the identification of a specific biomarker that distinguishes the cases, classifying them in sub-groups that are then compared for demographic, clinical, genetic, or biological variables. If groups are different and heterogeneity between them can be detected, then this speaks in favor of a disease subtype.

3. RESULTS

Sixty-six AD patients were included in this study. Demographic and biological variables of the AD patients under study are reported in Table **1**.

The patients were stratified on the basis of a non-Cp Cu cut-off, 27 AD patients were included in the normal non-Cp Cu group [non-Cp Cu mean (standard deviation, sd): 0.26 (1.5)] and 39 in the high non-Cp Cu group [non-Cp Cu mean (sd): 3.5 (1.6); p<0.001].

The two AD subgroups did not differ for age, sex, MMSE score, and *APOE4* frequency allele (Table **2**), while they differed for copper (p<0.001), which was increased in AD patients of the high non-Cp Cu group in comparison with those of the normal non-Cp Cu AD.

The neuroimaging evaluations revealed that MTA was observed in 41% of normal non-Cp Cu AD, and in 59% of high non-Cp Cu AD, without significant differences between the two groups (p = 0.14). Table **3** reports the comparisons between the two copper AD groups for the neuroimaging indices evaluated. The normal and high non-Cp Cu AD groups were similar for MTA, PVH, DWMH scores, while they differed for presence of global atrophy (p=0.042, Fig. **2**). About ITH, 100% of patients of the high non-Cp Cu AD group presented a score equals to 0 and 96.3% of the normal non-Cp Cu AD patients. More specifically, AD patients within the high non-Cp Cu group had a less severe burden of global atrophy and BGH, even though the differences in the BGH did not reach the statistical threshold (p= 0.052).

4. DISCUSSION

There is a growing recognition of the impact of either essential or environmental metals as risk factors contributing to the global susceptibility for AD.

The main result of current study consists in reporting that a homogeneous population of AD patients display neuroimaging heterogeneity when they are stratified in two groups on the basis of a copper marker, namely non-Cp Cu, supporting the existence of a copper metabolic subtype of AD. Even though the differences observed are small and need to be confirmed in bigger patients' samples, they are instrumental to show heterogeneity within a population representative of a homogeneous clinical entity [22], with a similar range of MMSE. Coherently with this clinical picture, the MRI profile appears homogenous, but differences appear when the

1A	GLOBAL ATROPHY = 0		
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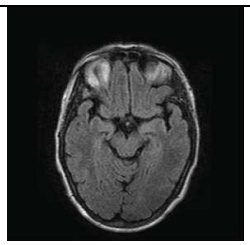
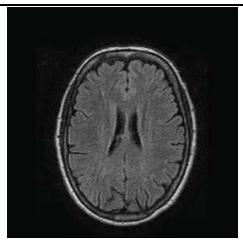
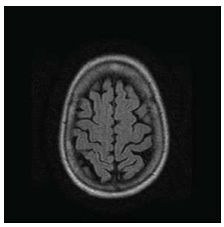
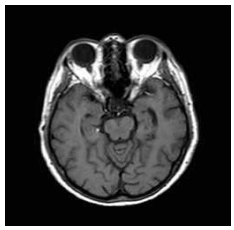
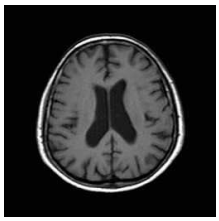
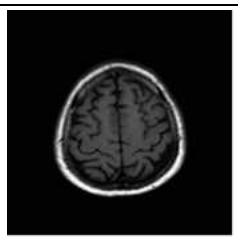
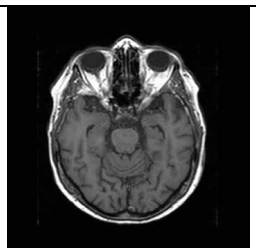
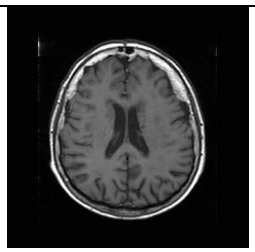
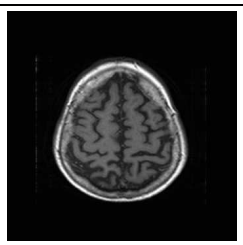
NO ATROPHY			
VENTRICLES'S ECTASIA COMPATIBLE WITH AGE (AMPLITUDE AT THE UPPER LIMITS OF NORMAL)			
SULCI'S ECTASIA COMPATIBLE WITH AGE (AMPLITUDE AT THE UPPER LIMITS OF NORMAL)			
1B	**GLOBAL ATROPHY = 1**		
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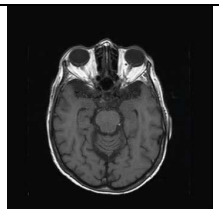
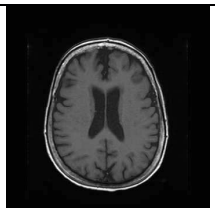
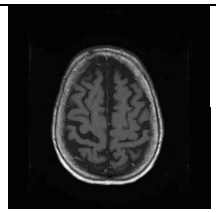
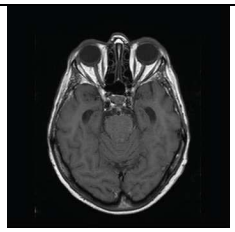
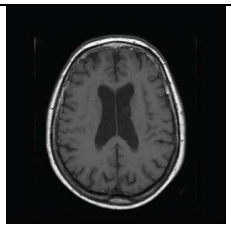
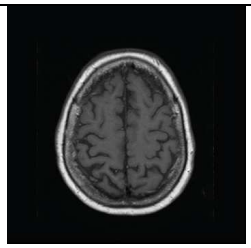
ECTASIA OF SULCI OF CONVEXITY			
ECTASIA OF TEMPORAL HORNS			

Fig. (1). To assess global atrophy a 2-point rating scale was used. **A)** global atrophy = 0: no atrophy or ventricles' or sulci's ectasia compatible with age (amplitude at the upper limits of normal values). **B)** global atrophy =1: ectasia of the sulci of convexity or ectasia of the temporal horns. Images were Axial Se T1w.

stratification for copper abnormalities is applied and two subgroup are created on the basis of a non-Cp Cu cut-off, demonstrating our working hypothesis and extending previous findings on this topic [8, 9, 18, 35, 36]. Some authors [37] showed in a large, multicenter study that visual rating scales from routine acquired structural MRIs are fairly reliable and highly correlated with cerebral atrophy evaluated with volumetric indices in brain regions vulnerable to dementia pathology. Comparing the two AD groups in current study, we noted that while MRI index of MTA were similar,

Table 1. Characteristics of the overall AD patients under study.

	Whole AD group (n=66)
Sex [n of F] (F%)	53 (80.3)
Age [years] mean (Standard Deviation, SD)	75.6 (8.0)
***APOE4* allele frequency [%] (at least one copy)**	23 (34.8)
MMSE [row score] median (25th-75th percentiles)	21 (17-23)
MMSE [adjusted score*] mean (SD)	18.8 (3.54)
Education [years] mean (SD)	7.5 (4.6)
β-Amyloid (ng/mL)	0.39 (0.18)
P-Tau (ng/mL)	0.05 (0.03)
H –Tau (ng/mL)	0.467 (0.231)
non-Cp Cu [µmol/L] mean (SD)	1.9 (2.5)
Copper [µmol/L] mean (SD)	14.5 (3.0)
Ceruloplasmin [mg/dL] mean (SD)	26.8 (5.1)

*adjusted for age and education years [59].

Table 2. Characteristics of the AD groups stratified for non-Cp Cu.

	AD patients, high non-Cp Cu (n=39)	AD patients, normal non-Cp Cu (n=27)	p value
Sex [n of F] (F%)	31 (79.5)	22 (81.5)	p=0.840*
Age [years] mean (SD)	74.8 (8.1)	76.4 (10.2)	p=0.481
***APOE4* allele frequency [%] (at least one copy)**	15 (38.9)	8 (29.6)	p=0.446
MMSE [score] median (25th-75th percentiles)	21.0 (16.3-23)	20.0 (17-24)	p=0.999**
MMSE [adjusted score] mean (SD)	17.88 (5.83)	18.44 (3.24)	P=0.780
Education [years] mean (SD)	6.5 (4.4)	8.4 (4.3)	p=0.274
β-Amyloid (ng/mL)	0.412 (0.19)	0.367 (0.17)	p=0.313
P-Tau (ng/mL)	0.052(0.24)	0.056 (0.038)	p=0.575
H –Tau (ng/mL)	0.487 (0.27)	0.453 (0.2)	p=0.562
Copper [µmol/L] mean (SD)	16.3 (2.9)	12.7 (2.8)	p< 0.001
Ceruloplasmin [mg/dL] mean (SD)	26.6 (6.0)	27.0 (4.6)	p= 0.300

*chisquare test.**Mann-Whitney test; Standard Deviation (SD).

presence of cortical atrophy and of cerebrovascular lesions differed between the two groups, even though the latter just approaching the significant threshold. More specifically, in patients with increased levels of non-Cp Cu cortical atrophy was less frequent, and they exhibited a less severe pattern of white matter hyperintesities, specifically located in the basal ganglia structurers. White matter hyperintesities are considered to be etiologically related to cerebral small vessel disease and are important substrates for cognitive impairment and functional loss. They are characterized by partial loss of myelin, axons, and oligodendroglial cells, mild reactive astrocytic gliosis, sparsely distributed macrophages as well as

Table 3. Neuroradiological variables of the AD groups stratified for non-Cp Cu.

	AD patients, high non-Cp Cu (n=39)	AD patients, normal non-Cp Cu (n=27)	p value
MTA, mean; median (25th-75th percentiles)	1.85; 1 (0-1)	3.44; 1 (0-2)	p= 0.433
Global atrophy, present n (%)	3 (7.7%)	7 (25.9%)	**p= 0.042***
[1]PVH, mean; median (25th-75th percentiles)	1.1; 0 (0-2)	2; 0 (0-4)	p= 0.218
[2]DWMH, mean; median (25th-75th percentiles)	1.8; 0 (0-2)	3.4; 1 (0-4)	p= 0.288
[3]BGH, mean; median (25th-75th percentiles)	0.6; 0 (0-0)	1.8; 0 (0-2)	p= 0.052
[4]ITH score equals to 0 n (%)	39 (100%)	26 (96.3%)	p=0.409

All p values are referred to Mann-Whitney test. *chi square test. Table legend:

Atrophy and white matter changes lesions were graded following standardized visual rating scales on plain MRI [24-27]. Generalized brain atrophy (ventricular and sulcal atrophy) was rated as present (=1) or absent (=0; global atrophy).

The degree of medial temporal lobe atrophy (MTA) was evaluated with a ranking procedure and validated by linear measurements of the medial temporal lobe including the hippocampal formation and surrounding spaces occupied by CSF, following standardized criteria (five point rating scale, score: 0-4). The visual rating scale of white matter changes included the anatomical distribution as well as the severity of the lesions. Based on anatomical distribution, a distinction was made between areas of periventricular hyperintensities (PVH) (caps and rims [1]PVH: 0 = absent; 1 = ≤ 5 mm; 2 = 6-10 mm) and deeper hyperintensities (including frontal, parieto-occipital and temporal white matter hyperintensities - [2]DWMH: 0 = no abnormalities; 1 = ≤ 3 mm, n ≤5; 2 = ≤ 3 mm, n >5; 3 = 4-10 mm, n ≤3; 4 = 4-10 mm, n >3; 5 = 10mm, n ≥1; 6 = confluent); basal ganglia hyperintensities (including caudate nucleus, putamen, globus pallidus, thalamus - **[3]BGH**; 0 = no abnormalities; 1 = ≤ 3 mm, n ≤5; 2 = ≤ 3 mm, n >5; 3 = 4-10 mm, n ≤3; 4 = 4-10 mm, n >3; 5 = 10mm, n ≥1; 6 = confluent) and infratentorial hyperintensities (cerebellum, tronco – **[4]ITH**). Large vessel cortical infarcts in the anterior, posterior and medial cerebral artery and presence of mass lesions and lobar hemorrhages were exclusion criteria from the study.

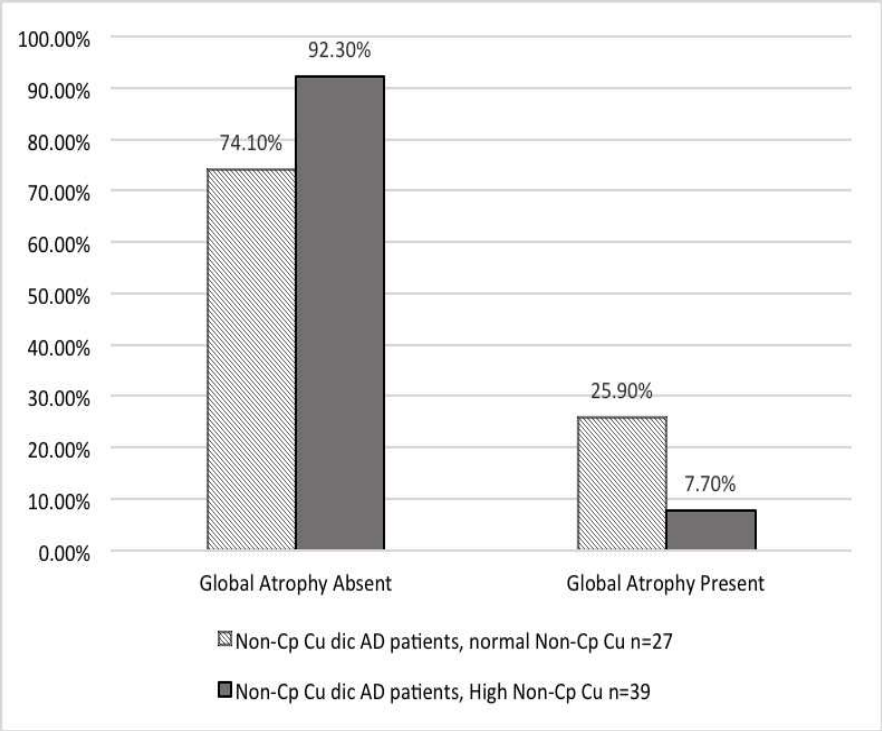


Fig. (2). Association between Global atrophy in AD patients stratified on the basis of non-Cp Cu cut-off. Patients within the group of high non-Cp Cu have a lower percentage of cortical atrophy with respect to AD patients with non-Cp Cu in the normal range.

stenosis resulting from hyaline fibrosis of arterioles and smaller vessels [38]. Nowadays, the most accepted opinion is that white matter hyperintesities represents incomplete ischemia mainly related to cerebral small vessel arteriolosclerosis [38]. Data presented indicate a different degree of structural brain injury in the two AD groups, which was severer in the normal non-Cp Cu. Conversely, the two subgroups were similar from a cognitive point of view, as re-

vealed by their MMSE scores. This suggests an anticipation of the 'functional' cognitive decline with respect to the 'structural' brain injury in those subject having copper metabolic abnormalities (for the same structural damage, individuals with high non-Cp Cu would have a severer cognitive impairment). Numerous findings, indicating that oxidative stress precedes AD neurodegeneration and structural brain damage (reviewed in [39]), are in line with notion. In fact, having serum non-Cp Cu concentrations higher than normal values point out to a homeostatic copper failure, which can manifest in various biochemical defects of copper metabolism [affecting e.g. ceruloplasmin activity, iron oxidative state, activity of dopamine beta hydroxylase or Cu/Zn superoxide dismutase 1 (SOD1) [40]] as it is exemplified in Wilson's disease, and increasing knowledge demonstrates also in AD [18, 36, 41-45]. Moreover, non-Cp Cu is redox active and, in the suitable milieu, it catalyzes oxidative stress *via* Fenton like and Haber Weiss reactions. It generally crosses the brain blood barrier and it has been reported to be increased in the brain areas more damaged by the disease [42].

Our observations need verification in bigger patients' samples and future investigations to explore the biological process at the basis of the associations observed.

As a multifactorial or complex disease, AD includes a broad spectrum of phenotypes, [18, 36, 46].

As shown in other studies and confirmed in current patient sample, about a half of patients with AD have copper abnormalities [9, 18, 27, 36]. In another study on this topic, we investigated the frequencies of some specific variants of the *ATP7B* gene - informative of the structure of the gene - in AD patients with high and normal copper [36]. A risk haplotype (GT in rs1061472, rs732774) in *ATP7B* resulted more frequent in the high copper AD. However, it was present only in 66% of the patients with copper AD metabolic subtype, suggesting either the existence of other genetic variants (including Wilson's disease mutations), or additional pathways controlling copper homeostasis that can play a role in raising the risk of having AD linked to the breakdown of copper homeostasis.

Based on mass screening studies performed in Korea, data about Wilson's disease prevalence have be recently questioned, suggesting that Wilson's disease might be much more common than estimated in the past (1984), passing from 1:30,000 to 1:1500 or 1:3000 cases [17, 48, 49]. In line with this finding, a more recent study reported an unexpected rate of about 1 in 40, higher than 1:90, that was the rate previously reported, of *ATP7B* heterozygote mutation carriers in UK population, predicting a prevalence of 1 case in 7000 inhabitants [17, 48, 49]. The large discrepancy from the estimated genetic prevalence of the disease and the number of clinically diagnosed Wilson's disease patients, which is significantly lower, raises concerns about the possibility that a number of *ATP7B* mutations might account for an increased likelihood of having AD.

The regulation of copper metabolism is controlled at the homeostatic maintenance of *hCTR1* mRNA, which is regulated by the transcription factor SP1. SP1 is a copper-sensing transcriptional activator of *hCTR1* - but controls other gene expression related to AD (e.g. *APP* and *MAPT* gene) - and is regulated by copper depletion and copper-replete conditions [50]. Very recent works demonstrated the impact of SP1 transcription factor in neurodegeneration processes linked to epigenetic regulation activated by exposure to heavy metals, primarily Pb, in early life, by diet or pesticides. This line of research arose from the hypothesis that exposure to lead before '80 - when the metal was extensively used in gasoline and other commercial products - could have effects on public health, being the lead-poisoning known since ancient romans. Consistent and robust findings about the cumulative lead dose on trajectories of cognitive decline have been provided by the Baltimore Memory Study (BMS) [51], demonstrating the association of tibia lead concentration with cognitive decline in adults aged 50-70 years. Experimental models of Pb exposure in early life produced latent overexpression in old age of *SP1*, *APP* and *MAPT* mRNA, and the related increased synthesis of SP1, APP, and of the microtubule-associated protein tau (MAPT) protein [1, 52, 53]. The overexpression of *SP1* in old life might have some effects on the regulation of copper homeostasis, increasing the influx of copper in the body through activation of the biosynthesis of hCTR1. This additional epigenetic mechanism can contribute to the susceptibility to copper homeostatic abnormalities associated to AD. In line with this literature, some authors proposed that the increasing prevalence of AD can be influenced by copper plumbing use in developed countries and by the ingestion of copper (II) from the diet associated with an increase consumption of meat or with diabetes through advanced-glycation end-products (AGEs) ingestion and formation as glucose derivatives [54-56]. The combination of the findings reported support of a tie link between genetic and environmental risk factors through epigenetic modification which can account for the etiology of this specific sporadic form of AD.

The influence of environmental hits and their interaction with genetic and epigenetic factors is the focus of recent investigations aimed at disentangle the complexity of the gene-environmental interaction. Gene controlling copper metabolism are regulated by, and in turn strongly influence the levels of copper bioavailable in our body.

The complexity of these interactions prompt to the development of hypothetical models, which provides mechanistic explanation for the contribution of gene-environmental interactions contributing to the onset of sporadic form of AD. The epigenome-based latent early-life associated regulation (LEARn) is an hypothetical model focused on the effects that accumulated environmental hits can produce on epigenome [57, 58].

On the basis of the information provided, a 'two hits' latency model of disease progression appears appropriate to explain increasing evidence of cross-sectional and longitudinal studies of environmental (Pb) and essential metals (copper) associated with a worsen cognitive state, even through their combined effects .

Even though this study has the limitation of small sample size and the observations reported have to be confirmed in bigger samples, it shows the potential of non-Cp Cu classification of AD people, in terms of preventive or therapeutic interventions based of tools able to counteract copper dy-

shomeostasis, which can improve the chance on a percentage of patients, sparing expense and side effect.

CONCLUSIONS

Data presented show a mild neuroimaging heterogeneity in two subgroups of AD patients originated by the stratification of a very homogeneous population of AD patients based on a cut-off marker of copper failure, i.e. non-Cp Cu in general circulation. Differences consist in a lesser degree of global atrophy in the group with higher levels of non-Cp Cu, suggesting an anticipation of the cognitive decline with respect to the structural brain injury in those subject with a deregulation of copper homeostasis, which results in oxidative stress. Copper imbalance can be activated by *ATP7B* gene variants or by environmental insults in early life which affect *SP1* gene expression, or more likely by different degrees of their combined effects [2].

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

Rosanna Squitti was in the last three years consultant of Canox4drug SPA (IT) and IGEA Research Corporation (US). Other authors declare no conflict of interest.

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