Copper Imbalance in Alzheimer's Disease and Its Link with the Amyloid Hypothesis: Towards a Combined Clinical, Chemical, and Genetic Etiology

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CLINICAL, BIOCHEMICAL AND GENETIC ASPECTS OF COPPER IMBALANCE IN ALZHEIMER'S DISEASE: TOWARDS AN ETIOLOGY

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ABSTRACT

The cause of Alzheimer's disease (AD) is incompletely defined: To date, no mono-causal treatment has so far reached its primary clinical endpoints, probably due to the complexity and diverse neuropathology contributing to the neurodegenerative process. In the present paper we describe the plausible etiological role of copper (Cu) imbalance in the disease. Cu imbalance is strongly associated with neurodegeneration in dementia, but a complete biochemical etiology consistent with the clinical, chemical and genetic data is required to support that this association is causative, rather than just a correlator of disease. We hypothesize that a Cu imbalance in the aging human brain evolves as a gradual shift from bound metal ion pools, associated with both loss of energy production and antioxidant function, to pools of loosely bound metal ions, involved in gain-of-function oxidative stress, a shift that may be aggravated by chemical aging. We explain how this may cause mitochondrial deficits, energy depletion of high-energy demanding neurons, and aggravated protein misfolding/oligomerization to produce different clinical consequences shaped by the severity of risk factors, additional comorbidities, and combinations with other types of pathology. Cu imbalance should be viewed and integrated with concomitant genetic risk factors, aging, metabolic abnormalities, energetic deficits, neuroinflammation, and the relation to Tau, prion proteins, α-synuclein, TAR DNA binding protein-43 (TDP-43) as well as systemic comorbidity. Specifically, the Amyloid Hypothesis is strongly intertwined with Cu imbalance because amyloid-β protein precursor (AβPP)/Aβ are Cu/Zn binding proteins with a potential role as natural Cu/Zn buffering proteins (loss of function), and due to toxic functions of Cu-Aβ.

INTRODUCTION

In Alzheimer's disease (AD) and dementia in general, a change is needed to overcome the current dogmatic view of the disease and achieve a better understanding of its etiology to develop effective treatments [1]. AD is a multifactorial condition in which amyloid-β (Aβ) accumulation and misfolding of other proteins converge with many other genetic, environmental, vascular, metabolic, and inflammatory factors promoting the disease state [2]. In this complex interplay, aging constitutes the leading risk factor and the orchestrator of the neurodegenerative process [1].

The objective of this hypothesis article is to provide a focused overview of the role of copper (Cu) as supported by preclinical, genetic, biochemical, clinical, epidemiological, and meta-analytic data, and its relation to aging, and to discuss a consistent biochemical etiology of the disease. Our hypothesis posits that age-aggravated Cu imbalance involves a gradual shift from protein-bound metal ion pools, associated with both loss of energy production and antioxidant function [3], to pools of loosely bound metal ions that produce gain-of-function oxidative toxicity contributing to dementia risk. The proposed mechanism is particularly relevant in a subset of individuals, defined as CuAD, in which Cu dysregulation is modulated by *ATP7B* genetic risk variants [4-6].

While the dysregulation of calcium (Ca^{2+}), zinc (Zn^{2+}), iron ($Fe^{2+/3+}$), and $Cu^{+/2+}$ has been known for decades to participate in the (onset and) progression of AD [7], we propose an updated model centered on processes deriving from the shift of Cu from strongly protein-bound pools with essential function, to loosely-bound toxic pools [8].

We will also discuss cellular mechanisms of metal-related brain damage occurring at different sites (glia, neurons, synapses) as well as metal-related alteration of Aβ metabolism.

MANIFESTATIONS AND BRIEF HISTORY OF ALZHEIMER'S DISEASE ETIOLOGY

Recent studies have shown that many neurodegenerative determinants act synergistically to produce neuronal loss, which in AD ends with severe cognitive and behavioral impairment [9]. A

brief description of AD, its histopathological and biochemical manifestations, the history of the disease etiology, and the emerging role of metal ion imbalance, is reported herein.

Alzheimer's disease affects approximately 30-35 million people worldwide (50 million dementia cases, with 60-70% being AD cases) [10]. The disease produces a gradual deficit of episodic memory; deficits are then extended to all the cognitive domains, and behavioral disorders eventually ensue, thereby deeply impacting on activities of daily living.

The detrimental effects of AD span beyond the diagnosed individual and pose challenges also to caregivers and healthcare providers.

The histopathological hallmarks include neurofibrillary tangles of hyperphosphorylated tau protein common to many diseases, and extracellular insoluble deposits of senile plaques consisting of metal-enriched, oxidized, and various isoforms of Aβ. There is broad consensus that age is the primary risk factor for AD onset, making developed countries with a high life expectancy particularly vulnerable to a dementia outbreak [11], while the pathology is emerging in developing countries with a dramatic incidence.

Although the disease has a multifactorial etiology there are causal genetic variants in AD. Aβ is generated by the proteolytic cleavage of the Amyloid-β Precursor Protein (AβPP), a protein involved in important physiological functions like synapse maturation, neural plasticity, and metal-export activity [12]. Fully penetrant mutations on the *AβPP* and on the *PSEN1* and *PSEN2* genes, which encode for the catalytic subunit of the γ-secretase enzyme that produces Aβ from AβPP, strongly contribute to the early-onset, familiar form of AD (< 65 years) (see [13] for a recent review of the topic). Genetics also play a role in the late-onset, sporadic forms of dementia. In that regard, harboring the *APOE4* allele, encoding the Apolipoprotein E4 isoform involved in cholesterol and Aβ metabolism, is a common high genetic risk factor in individuals > 65 years [14].

Growing evidence supports the notion that many patients present with neuropathological heterogeneity and that mixed neuropathology is typical of the cognitive decline [9]. Thus, dementia results from person-specific combinations of many molecular determinants that work in synergy to

produce different clinical entities, modulated by additional comorbidities including, but not limited to, cardiovascular disorders, Type 2 diabetes, dyslipidemia. Accordingly, more effort is required to explore the molecular granularity of dementia subtypes. For instance, besides the different array of neurotoxic proteins [Aβ, Tau, prion proteins (PrP), α-synuclein, TAR DNA binding protein-43 (TDP-43)], the AD brain is also characterized by bioenergetic abnormalities, oxidative stress, inflammation, Ca dyshomeostasis, and heterogeneously disturbed Zn, Fe and Cu levels [2, 15, 16].

Drugs have been mainly developed within the Amyloid Hypothesis. The construct posits that the aberrant accumulation of Aβ assemblies is the critical initial step of the AD process [17]. The Hypothesis is supported by genetic risk factors of familial AD (*PSEN1/2* and *APP* genes) as well as preclinical studies [18]. Therapeutic efforts have focused on either limiting the Aβ accumulation and/or formation of toxic oligomers by antibodies or the peptide production by enzyme inhibitors or modulators, but it appears clear that addressing a single molecular determinant is not a sufficient approach [19]. Indeed, no mono-causal treatment has so far reached its primary clinical endpoints, and critics claim that this is because the Amyloid Hypothesis is too simplistic [20].

The scenario is further complicated by several studies arguing for a protective role played by Aβ deposits. Amyloid enriched plaques can be, in fact, envisioned like net traps in which toxic oligomeric species [21], infectious agents [22], or dysregulated, and thus cytotoxic metals are entrapped [23-26]. It is well-established that plaques contain substantial amounts of essential trace metals such as Zn, Fe, and Cu and that metal transport and storage protein [i.e.; metallothioneins (MTs), ferritin, Zn transporters] are consistently affected in AD [2, 27]. In addition, chemical and biochemical studies have demonstrated that Aβ is a metallopeptide and that Cu^{2+} (as well as other biologically relevant metal ions, like Zn and $Fe^{2+/3+}$) binding dramatically changes the peptide aggregation propensity, structure, and toxicity [27-29]. There is broad consensus that Aβ has a well-defined medium-affinity Cu-binding site (K_d ~0.1 nM) [27, 28]. These and other facts put this metal ion imbalance as one central player of AD etiology and in close relationship with the Amyloid Hypothesis.

THE CuAD HYPOTHESIS

The brain is particularly sensitive to aging; our Hypothesis stems from the assumption that aging produces, among other disrupting processes, oxidative stress [30], which is enhanced by the dysregulation of metal ions and particularly to Cu imbalance [2, 27]. We also explore the concept that some AD individuals are particularly susceptible to disturbances in Cu ion balance, a phenomenon that accelerates neuronal aging, increases the cost of neuronal maintenance, exhausts neurons and thereby reduce the energy available for their primary function as 50% of the neuronal energy budget is spent on cognitive processing [31].

The Metal Ion Hypothesis [7] stresses that the homeostasis of d-transition metals like Zn, Fe, and Cu is perturbed in AD, and that phenomenon plays a contributing causative role, rather than being a side phenomenon [2, 7]. Here, we sharpen this hypothesis into a specific etiology for Cu. The primary role played by the metal is in line with the Amyloid Hypothesis, since AβPP/Aβ are well-established Cu (and Zn) binding proteins (*in vitro*) [27, 28] **(Figure 1).** The importance of Cu dysregulation is supported by recent studies showing the different activity played by the diverse species (bound and non-bound to proteins) of peripheral Cu [2] as well as by growing genetic evidence [32]. About 75-95% of total serum Cu binds strongly and inertly to ceruloplasmin, while about 5-10% circulates in a weaker and more labile form, being exchanged among various protein compounds [33]. On this basis, these Cu complexes have been defined as non-ceruloplasmin Cu (non-Cp Cu), a clinical biomarker applied to Wilson disease (WD), a paradigmatic disorder of Cu toxicosis and accumulation [34]. Non-Cp Cu has been historically defined as 'free' Cu, yet really 'free' Cu does not exist (it is probably at the attomolar levels at best). This non-Cp Cu pool is likely superimposable with the exchangeable Cu-pool, i.e. the pool that exchanges with added Cu (radioactive tracer or stable isotope) or can be withdrawn with a chelator such as EDTA in minutes/hours time frame [35]. The main constituent of this non-Cp pool is serum albumin [35]. It has a N-terminal Cu-binding site with a K_d of 0.1 pM [36] and Cu can be removed with a stronger

chelator in minutes to hours [37]. Thus Cu(II)-binding is moderately strong and kinetically labile compared to Cp [38]. The terminology of non-Cp Cu is tissue specific and refers to serum/plasma. It represents the primary specie responsible for Cu transport from blood into the brain, crossing the blood brain barrier (BBB) [39]. To maintain the link with its historical and clinical application in WD, we will use the term non-Cp Cu referring to serum/plasma and 'labile' Cu for the same biological entity in the brain.

Cu IN PHYSIOLOGY: FOCUS ON THE BRAIN

Cu is an essential metal indispensable for brain development and physiology. Severe Cu deficiencies are associated with cardiac, immune, bone, and central-nervous-system conditions, while Cu chronic excess can be mostly associated with liver damage. As Fe, Cu is a transition metal, that can receive and donate electrons and, as a redox catalyst, is necessary for many enzymes' activities. Cells use Cu for mitochondria respiration, blood cell line maturation, immune responses, wound heling, myelin sheath formation, ant it is an important mediator for neurotransmitter synthesis and synaptic activity modulation.

Cu balance is determined by the equilibrium between the rates of dietary absorption from food, supplements and drinking water, and excretion through stools and bile and it is tightly regulated [2]. Its absorption, distribution, and homeostasis in the brain are tightly controlled, with the neurovascular unit and the BBB playing an essential role in the process (**Figure 2**). Human Cu transporter 1 (hCTR1), Cu-transporting P-type ATPase 7A (ATPase7A), and ATPase7B Cu-transporting P-type (ATPase 7B) regulate brain Cu levels. *The choroid plexus* harvests and releases Cu in the cerebrospinal fluid (CSF), the fluid that surrounds the brain (range between 0.5 and 2.5 µmol/L). In rat *choroid plexus,* Cu is up-taken from the non-Cp Cu(II)-pool in the blood into the brain in its ionic Cu(I) form. Hence a reduction step from Cu(II) to Cu(I) is needed. [39]. hCTR1 transports Cu(I) from the bloodstream to endothelial cells and astrocytes. On the contrary, ATPase7A and ATPase7B extrude Cu from endothelial cells to the interstitial fluid or the

bloodstream, respectively. ATPase7B also contributes to Cu loading into glycosylphosphatidylinositol-linked ceruloplasmin (GPI-Cp), thereby keeping intracellular Cu concentrations under control [2, 40] **(Figure 2)**.

Cu DYSHOMEOSTASIS: HUMAN GENETIC DISORDERS AND COMPLEX DISEASES

Mutations in the genes encoding for proteins involved in Cu pathway results in several hereditary diseases **(Table 1)**. Menkes disease, typified by Cu deficiency and WD, featured by Cu excess, are caused by mutations in *ATP7A* and gene *ATP7B,* respectively. They represent the human genetic disorders in Cu transport that reveal the importance of maintaining an appropriate Cu homeostasis and provide insight on abnormalities in Cu handling in AD **(Figure 3)**.

Abnormalities in Cu and ceruloplasmin and non-Cp Cu levels are also associated to several complex diseases, spanning from myocardial infarction, stroke, cardiovascular death {Arenas de Larriva, 2020 #1168}, hearth failure {Huang, 2019 #1169}, ischemic heart {Li, 2018 #1153}, acute aortic dissection {Ma, 2021 #1170} and diabetes mellitus type 2 {Squitti, 2017 #898}.

Aβ-DYSREGULATION, AND Cu TARGETING TREATMENTS IN AD

Many findings indicate that Cu dyshomeostasis plays a critical part in AD [2, 8]. The metal has a direct role in amyloid pathology by promoting Aβ aggregation. On the other hand, Cu sequestration by plaques may render the cation unavailable for key biological functions [25]. This scenario is further complicated by Cu-related loss and gain of function mechanisms.

An early and seminal discovery was that Cu can enhance Aβ-driven oxidative stress [41], and promote Aβ aggregation under conditions of acidic pH (i.e., < pH 6) [41]. At physiological pH, Cu can also inhibit the Zn-mediated aggregation of Aβ by competing with Zn for the peptide histidine residues [42]. Furthermore, Aβ can trigger neurotoxic effects by promoting deficits of intracellular Cu ($[Cu]_i$). Very importantly, it has been found that AβPP, from which Aβ is produced, can bind

Cu(I) and Cu(II) with picomolar affinity *in vitro* [43]. AβPP-KO mice exhibit increased Cu levels in the cerebral cortex, whereas the over-expression of AβPP leads to significantly reduced brain levels of Cu in a preclinical model of AD [44]. Moreover, Cu levels can also significantly affect the neuronal redox state, thereby indicating a pathogenic link between Aβ dysmetabolism, oxidative stress, and Cu dyshomeostasis [45].

In line with the Amyloid Hypothesis, β-site AβPP-cleaving enzyme (BACE1), the enzyme that catalyzes the rate-limiting step in the amyloidogenic processing of AβPP, binds strongly to the Cu Chaperone for Superoxide Dismutase (CCS) [46]. CCS is the chaperone that delivers Cu to superoxide dismutase-1 (SOD-1) a Cu-dependent cytosolic scavenging enzyme; therefore, high levels of BACE1, by binding to CCS, can decrease the amount of CCS available for SOD1 activation [47], thereby reducing the antioxidant capability of neurons.

A Cu-enriched diet was initially reported to increase the metal brain levels and counteract the decreased SOD-1 activity observed in AβPP transgenic mice [48]. In the same transgenic animals, Cu treatment lowers brain Aβ production long before the induction of detectable reductions in Aβ plaques, implying that AβPP functions/assists in Cu export [48]. However, a phase II clinical trial supplying AD patients with Cu ([Cu-(II)-orotate-dihydrate; 8 mg Cu daily) failed to meet its primary outcomes [49]. Treatment with the Cu/Zn ionophore clioquinol (CQ) inhibited amyloid plaque formation [50]. PBT2, a second generation Cu/Zn binding molecule intended to prevent Cu-facilitated Aβ aggregation has been tested in a phase II clinical trial that met its primary endpoints of safety and tolerability and showed a reduction of Aβ in the CSF and beneficial effect in a subset of cognitive tests [51, 52]. A recent phase II molecular imaging study evaluating Aβ levels in PBT2-treated patients did not show beneficial effects on amyloid deposition [53]. The study was underpowered and the smaller placebo group showed large variability, and unexpectedly remain stable over the 12-month observation period. This precluded evaluation towards the efficacy of the drug.

Together, these studies suggest that AβPP is involved in Cu transport [54], and that Cu promotes amyloid aggregation, but that exogenic Cu control has little effect on Cu balance, likely because the Cu homeostatic machinery is quite robust. Still, Cu levels in the brain can be altered long-term as implied by a study taking into account the different species of peripheral Cu [55]. AβPP transgenic mice treated with 0.13 mg/L of Cu sulfate in drinking water for 90 days doubled their non-Cp Cu level [2, 55]. The treated transgenic mice also showed a reduced CSF clearance of Aβ across the BBB and an increased Aβ production [55].

Previous results on the toxic effect of Cu on Aβ plaques and learning deficits [56, 57] reinforced the association between the production of cognitive impairments and the presence of excess non-Cp Cu as shown by previous clinical studies in AD patients (reviewed in [2]). The discovery that genetic variants of *ATP7B*, the gene encoding for ATPase7B, a Cu pump located in hepatocytes, and endothelial cells of the BBB is involved in AD, unraveled a more complex scenario related to the Cu imbalance that occurs in the disease [32]. The ATPase7B pump is essential for proper Cu homeostasis (reviewed in [8]) and defects in the process can lead to a buildup of non-Cp Cu in the blood and its transport across the BBB [26, 55], and activate cell-damaging oxidative events in the brain [58]. This mechanism is centered on the shift towards a prevalent fraction of Cu that is not firmly bound to proteins, and in this form, promotes cytotoxic effects [8]. We believe that the clinical, biochemical, and genetic data cited above are consistent with this etiology.

CHEMICAL FORMALIZATION OF THE CuAD HYPOTHESIS

The data indicate that Cu-related dysregulation in a subset of AD patients manifests as a shift towards a labile (and weaker bound) Cu pool that is made available outside neurons and non-neuronal cells. Reduced total Cu levels in the brain are associated with the soluble fraction (bound strongly and inertly to proteins), while its content within insoluble plaques is increased [25, 59, 60], as well as that labile Cu [58]. The Hypothesis has been chemically formalized by using a location-

dependent Cu dissociation constant (K_{dc}) that identifies the shift from the pool of functional Cu that is strongly bound to proteins to pools of loosely bound, toxic Cu (e.g. non-Cp Cu and labile Cu) [8]. This shift has strong causal implications as discussed below. We also note that partial pathways, e.g. Cu deficiency resembling only the loss of function pathway, or Cu toxicity representing mainly the gain of function pathway, is consistent with our hypothesis under some conditions (**Figure 3)**. In this sense, the *Cu-Hypothesis* by G.J Brewer may represent a source of some of the gain of function etiology via exogenous oxidized Cu(II) excess [61, 62], as supported by some studies linking Cu excess to cholesterol and amyloid pathology in rabbit and mice [55, 56].

Cu brain deficiency may involve deposition of Cu outside the neuron together with amyloid sorting and segregation within the lipid drafts [63], or excess zinc as illustrated by some studies [2]. Furthermore, ischemic episodes in the brain might trigger mechanisms of exporting Cu from the brain to the blood mediated by *COMMD1* as recently depicted in myocardial infarction [64]. However, the main hypothesis explaining Cu imbalance as it emerges from the meta-analysis data **(Figure 3)** is consistent with WD. AD and WD have diverse etiologies with WD being a monogenic disease of fairly clear Cu-related etiology [65], but complex diseases such as AD have a global susceptibility that is influenced by genetic heterogeneity. The heterogeneity can explain 'portions' of susceptibility. The evidence that genetic variants identified on *ATP7B* gene are statistically associated with an increased AD risk [66] in a subset of patients [6, 66], is consistent with a genetic heterogeneity in AD that might explain Cu susceptibility.

IMPLICATIONS OF NON-CP Cu EXCESS

The Hypothesis posits that an age-driven Cu imbalance resulting in a gradual shift from protein-bound metal ion pools to pools of loosely bound metal ions drives a bloodstream non-Cp Cu excess in AD [8]. Non-Cp Cu is bound with moderate affinity (Kd ~ 0.1 pM) and relatively labile mainly to albumin, but also α-macroglobulin, peptides (45 kDa proteins with unknown identity referred as small Cu carriers), and amino acids and exchanged among them [2]. This Cu can be

redox-active, and if expanded (> 1.6 µmol/L) becomes toxic, crossing the BBB, as exemplified in WD **(Figure 3)** {Sensi, 2018 #411;Hoogenraad, 2001 #207}. In AD patients, levels of non-Cp Cu reach values that are commonly found in WD [67] (**Table 2**), and the amount of Cu in the CSF appears modulated by the non-Cp Cu concentrations (1 µM non-Cp Cu accounted for 0.03 µM increase of Cu content in the CSF [26]). We can then hypothesize a blood-to-brain inward flux of Cu, fueled by non-Cp Cu that can diffuse [39, 68] or be transported across the BBB by Cu transporter hCTR1, and by ATPase7A (labile Cu) and an opposite brain-to-blood outward flux driven by ATP7aseB or by AβPP, based on AβPP properties as a regulator of neuronal Cu homeostasis [54]: the Cu efflux associated to AβPP function can explain the reduction of Aβ concentrations in the CSF [54], and also the decrease levels of Cu observed in the brain **(Figure 4)**. Aβ buffering for Cu labile excess - binding and precipitating the metal in amyloid plaques - removing it from the CSF [23-26], and decreasing Aβ levels in the CSF can be envisaged as an additional process that might restrain the increase in labile Cu if the inward Cu flux in the AD brain would be considered an enduring condition, mirroring WD, resulting in a continuous supply of the Cu brain reservoir.

Of note, Cu dysregulation also affects Fe metabolism via crosstalk with ceruloplasmin as the protein manages both metal ions [2]. In support of this view, variant alleles in the functional single nucleotide polymorphisms (SNPs) *ATP7B* rs732774 and rs1061472 [4], and still unknown *ATP7B* variants might contribute to the global susceptibility to AD [69], also altering the Fe/Cu balance and triggering cell death. According to these new findings, a shift from bound to labile Cu may be pathogenic in several ways:

i. By its participation in uncontrolled redox-cycling reactions, Cu-Aβ complex promotes oxidative stress: Cu-Aβ has the ability to produce reactive oxygen species (ROS) on its own, [70] due to an ill-controlled environment, while Cu in enzymes such as Cu,Zn SOD has a tightly controlled coordination sphere and thus participate in ROS detoxification. Hence, Cu-

Aβ complex participates in oxidative stress via Fenton-type chemistry, driving the generation of ROS formation, including the highly toxic hydroxyl radical (HO•), a hallmark of both AD [70], and aging of the human brain [30];

ii. By reducing energy production in mitochondria. Cu loss may impair the electron transport chain function (cytochrome C oxidase requires Cu) and deplete neuronal energy. Loss of Cu from SOD-1 impairs oxidative stress defenses with an impact on aging and mitochondrial efficiency. Conversely, abnormal accumulation of labile Cu can increase ROS generation and damage mitochondria [8, 15];

iii. By affecting the aggregation of amyloid peptides [27] with possible stabilization of oligomeric species [71], as well as increasing intracellular accumulation of phosphorylated Tau [72], even though only *in vitro* evidence have been collected so far;

iv. By altering synaptic function: labile Cu is released in the synaptic cleft (up to 100 μmol/L) and may have a dual role at the glutamatergic synapse: labile Cu can inhibit the activity of the glutamate receptor NMDA (N-methyl-D-aspartate), thereby protecting neurons from glutamatergic excitotoxicity (extreme stimulation of glutamatergic signaling that leads to neuronal cell death [73]), or catalyze Fenton-type reaction thus producing ROS. Furthermore AβPP, α-synuclein and PrP have been proposed to modulate neurotransmission as buffering proteins that control Cu(II) within the synaptic space] [8];

v. By affecting neuroinflammation: excess Cu can drive abnormal pro-inflammatory microglial activation; alternately, limiting Cu can lead to impaired early (beneficial) responses of microglia and astrocytes [74];

vi. By accelerating advanced glycation end-products (AGEs) formation. AGEs formation is a known feature of AD. Fe and Cu accelerate AGEs formation that also promotes protein glycoxidation. AGEs damage the arterial walls in diabetes and facilitate progressive Cu-trapping. In AD, most of the Aβ in plaques is found in the form of AGEs [75, 76].

OBSERVATIONAL DATA SUPPORTING THE CuAD HYPOTHESIS

The extent of Cu imbalance in AD can be appreciated through meta-analysis of the multiple AD studies performed on Cu in serum/plasma and brain specimens from 1984 till July 2020 **(Figure 4)**. The meta-analysis showed decreased values of Cu in the brain (pooled total of 182 AD and 166 healthy controls, **Figure 4A**), coexisting with increased values of non-Cp Cu in the blood (pooled total of 985 AD and 1325 healthy controls, **Figure 4C**), a phenomenon that can explain the reported increased serum values of total Cu (pooled total of 2749 AD and 3394 healthy controls, **Figure 4B**). On average, data extrapolated from the literature indicate a decrease of Cu in the brain by about 24% and an increase of Cu in the blood by about 7%, while non-Cp Cu doubles, consistent with an overall Cu imbalance that supports our Hypothesis of a shift away from functional protein-bound Cu to pools of loosely bound metal ions (i.e. non-Cp Cu) that is toxic and should almost certainly have a biological effect. Interestingly, these alterations resemble those occurring in WD (**Table 2)** [2]. Some WD-like Cu-related alterations are present in a subset of AD patients who can be set apart from the general pool of AD subjects by taking into account non-Cp Cu levels that are higher than 1.6 μmol/L, the designed cut-off (**Table 2)** [2]. CuAD patients, when compared to AD patients, show distinct electroencephalographic (EEG)-derived cortical brain rhythms, further supporting the idea of an AD subtype characterized by Cu abnormalities. Moreover, CuAD individuals display less severe burden of global atrophy, and increased frequency of *ATP7B* rs732774 and rs1061472 [2].

MAIN CHALLENGES FOR THE CuAD HYPOTHESIS

In recent years, we have acquired new experimental and theoretical tools to address several of the challenges of the Hypothesis, even though a lot of questions still remain open.

Addressing current knowledge gaps in metal imbalance: *future experiments and validation studies.* Meta-analysis is a quantitative, formal, epidemiological study design tool employed to systematically assess the results of previous research and infer conclusions about that body of

research. Future meta-analysis studies focused on Zn and Fe in human serum/plasma and brain specimens may expand further the imbalance of these two metals that act in close synergy with Cu i.e., via ceruloplasmin and MTs [27].

Another gap relies in the exact role played by the genes encoding for enzymes/transporters that control Cu balance. The metal gene scouting of patients exhibiting Cu imbalance, using a sequencing hypothesis-driven approach can be a valid tool to disentangle their real prevalence in the disease, weighing for the relative frequencies of more and less pathogenic (and penetrant) variants. Our Hypothesis posits that variants in *ATP7B* and other gene pertaining to the Cu pathway may infer a percentage of the risk of sporadic AD [69]. Previous genome-wide association studies (GWAS) [77], failed to identify a significant association between AD and the above-mentioned SNPs in the *ATP7B* chromosomal region. This can be at least partly explained by the tendency of GWAS to be less effective in detecting these types of multiple rare variants, yet these are variants plausible exist as they are required to account for the missing hereditability of complex diseases, as discussed elsewhere for the *ATP7B* gene [69].

We also expect that chemical and biochemical studies focused on the interplay between Cu and Aβ will continue to expand our knowledge of the link between these two molecular AD drivers. We expect Cu to bind to many peptides with moderate affinity as those reported for Aβ (in the nM range) due to flexible environment provided by peptides. However, Aβ is a known Cu-binding peptide in amyloid plaques and tends to localize in the synapses where much of the pathology takes place, which supports a possible (although still hypothetical) Cu-Aβ relationship *in vivo* [27, 78]. Developing Cu-targeting molecules can also document, although indirectly, on the relation between Aβ and Cu.

Disease progression and biomarkers. AD is a heterogeneous disorder with a complex disease etiology that challenges any simple etiology. A key, and still unmet challenge in AD is the identification of biomarkers for early diagnosis or for patient stratification as a personalized medicine approach aimed to *stratify individuals for tailoring the right therapeutic strategy for the*

right person at the right time [79]. Metabolic defects are among the first events in AD, according to current biomarker models [80], with early glucose utilization and positron emission tomography (PET) anomalies, followed later by Tau dysfunction. We propose that metal imbalance may be part of this sequence of events. However, the temporal dynamics remain largely unexplored. It is still unclear if metal ion dyshomeostasis precedes amyloid pathology as well as other AD-related abnormalities. To monitor metal imbalance, we assume a continuous disease progress from a state in which almost all Cu is bound as functional pools to a state in which more Cu becomes "free" and toxic (e. g. non-Cp Cu and labile Cu). Longitudinal studies evaluating Aβ pathology together with metal biomarkers such as non-Cp Cu, Cu/Cp ratio, Cu/SOD-1, and MTs levels are warranted. Along this line, alterations of ceruloplasmin levels in the CSF have been shown to predict cognitive decline and brain atrophy in people with underlying Aβ pathology [81]. Furthermore, previous studies reported that non-Cp Cu is a stratification biomarker [82], and can be employed as a prognostic biomarker for conversion from mild cognitive impairment (MCI) to symptomatic AD [2]. On this basis non-Cp Cu can serve as an inclusion criterion for eligibility assessment in early clinical trials testing anti-Cu based therapy.

Treatment. The ultimate challenge in AD is to treat causally. Mirroring WD, a Zn-based therapy could be used to correct the Cu imbalance: a proof-of-concept phase II clinical in MCI trial using non-Cp Cu > than 1.6 µmol/L as an inclusion criterion for eligibility is started in April 2021 (ZINCAiD, EudraCT 2019-000604-15, funded by the Alzheimer's Association). An additional benefit associated with the administration of Zn is that the metal is a powerful driver for neurotrophic signaling and neuronal plasticity as it promoted the maturation of the Brain-Derived Neurotrophic Factor (BDNF) from pro-BDNF through the activation of Zn-dependent Matrix metallopeptidases [83, 84]. It has also become clear that in it is important to leave the Zn in place and hence in chelation therapy targeting weakly and Aβ-bound Cu, selective Cu chelators are required [85, 86]. Other approaches envisage the normalization of brain cell Cu homeostasis through the development of targeted Cu delivery drugs. High-affinity, cell-permeable Cu chelators

have the potential to enter brain cells and interfere with normal metal homeostasis [87]. The major challenge of any such therapeutics is to control and to fine-tune the localization of effects like, for instance, avoiding unwanted stripping of Cu from essential proteins or mitochondria or the delivery of Cu to amyloid.

Despite plausible answers to these critical questions, the new Hypothesis is not able to provide a definitive answer for the selective vulnerability that implies an anatomical brain region specificity associated with the syndrome, although we note that the hippocampus is one of the most vulnerable brain regions, particularly fragile to ischemia insults [88] and tends to be enriched in metal ions. Furthermore, the hippocampal formation. The same holds for the pattern of progression of the disease and for how and when the clinical manifestations become related to the pathophysiology; these questions remain hypothetical at this point and substantial work is required to explore them.

LINKAGE TO OTHER MAJOR THEORIES

We have argued that metal ion and amyloid dyshomeostasis are manifestations of the same underlying etiology. As we discussed above, they are, in fact, strongly intertwined because of the key interaction between AβPP/Aβ and Cu and Zn. As metal ions need to be tightly controlled, it is not surprising that the delicate AβPP/Aβ balance is the connecting link between the Amyloid and the Metal Ion Hypotheses.

The Metal Hypothesis. As previously reported Aβ has the ability to bind Cu and *in vivo* Cu is bound to Aβ in the amyloid-plaques **(Figure 1)** [28], and mutations in the genes involved in its buildup and processing, AβPP, PSEN1/PSEN2 coalesce with other molecular determinants to favor the disease development. We propose that mutations in these risk genes would disturb the metal-buffering AβPP/Aβ system, which implies both loss and gain of function risks [27, 89].

The Amyloid Hypothesis. The self-assembly of Aβ has been regarded as an important process in AD etiology and the origin of the toxicity has shifted from the amyloid plaques to intermediate-

size aggregates via a variety of mechanisms [90]: Cu might stabilize such oligomeric species [71, 91]. This might provide such an explanation of the failure of clinical trials aiming at eliminating the peptide since the disappearance of amyloid plaques could drive the equilibrium between deposits and the soluble state towards the oligomeric species regarded as more toxic.

Relation to Tau, prions, α-synuclein, and TDP-43. Tau dysfunction severely affects many key aspects of neuronal functioning like axonal transport, mitochondria respiration, synapse integrity and protein turnover [92]. Cu can also contribute to Tau pathology. In that regard, Cu dysfunction alters Tau phosphorylation through several mechanisms and primarily by chronic Cu exposure that accelerates Tau phosphorylation by inducing hydrogen peroxide production [93].

Cu is also involved in prion-like mechanisms of disease progression. The PrP is a glycoprotein located at synapses. PrP^{Sc} is the aggregated form and considered the agent of prion disease [94]. PrP can bind up to five Cu(II) atoms, and the Cu(II)-PrP complex facilitates PrP internalization within the cell as well as PrP conversion to PrP^{Sc}. At glutamatergic synapses, the Cu-PrP complex modulates neurotransmission and also binds to Aβ [8]. Furthermore, Cu can affect α–synuclein functioning, the protein is localized at the synapse, and Cu^{2+}, via oxidative stress, can contribute to its oligomerization and fibrillation as well as the formation of Lewy's bodies [95].

Finally, TDP-43 is a pathological protein associated with sporadic amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration with ubiquitinated inclusions (FLTD-U), and the Limbic-predominant Age-related TDP-43 Encephalopathy, or LATE, a newly discovered form of dementia [96]. The abnormal C-terminal fragments of TDP-43 are ubiquitinated, hyperphosphorylated, and accumulate as cellular inclusions in neurons and glia, thereby helping the production of neurodegenerative processes. Changes in metal homeostasis of Zn, Cu, and Mn (manganese) have been described in the TDP-43A315T mouse model that exhibits increased metal levels, specifically in the spinal cord [97].

The aging factor. Accumulation of Cu, as well as Zn and Fe, upon aging, can affect a variety of concurring pathogenic pathways, including the antioxidant stress defense, synaptic plasticity,

vesicular transport, and mitochondrial function [30]. Age driven loss of function is implied in the Hypothesis from improper recruitment of Cu and Zn into the key protein SOD-1, the scavenging enzyme protecting cells from oxidative metabolism byproduct generated by mitochondria and one of the few proteins known to enhance rodent life span [98-100]. Impaired anti-aging defenses, exemplified by direct loss of SOD-1 function, is an appealing, simple etiology that immediately includes the age risk factor [27, 31].

Our hypothesis may also help to find a causative link between disease progression and oxidative stress: In addition to the loss of SOD-1 function, labile Cu can also directly trigger oxidative stress by Fenton-type reactions, and thus the proposed shift from functional protein-bound Cu to labile Cu is a two-edged sword against oxidative stress balance, a key feature of aging. The axonal terminals and secretory granules, as well as the synaptic cleft, is highly enriched with Cu (up to 100 µmol/L) upon glutamatergic neurotransmission and is a primary and early target of AD-related pathogenic mechanisms [101]. These processes have been linked to non-Cp Cu excess associated neurophysiological abnormalities {Sensi, 2018 #411;Tecchio, 2016 #961}.

Systemic commonalities to other diseases. As mentioned above, most sporadic cases of AD are probably driven by a combination of neuropathological, vascular, metabolic, and neuroinflammatory pathogenic pathways, which may have an age-aggravated systemic feature in common: energy deficits. Neurons are among the most energy-demanding cells in the body. Thus, if Cu and Fe are lost from cytochrome C oxidase and SOD-1, mitochondrial energy production can be severely impaired, thereby leaving less energy available for protein turnover, which could explain both the presence of various protein lesions as well as the accumulation of lysosomal proteins in AD and related diseases, but also provide a causal relationship to the ultimate culprit of AD which may be the extremely energy-demanding neuronal execution [102, 103].

The main "zero hypothesis" against our construct/ better proposal/hypothesis??? would be that the Amyloid Hypothesis is correct and that Cu imbalance is not causative but rather a downstream consequence of a global neurodegenerative cascade. We note that there are many theories (oxidative

stress, tau, amyloid, different metal ions, metabolism, inflammation) and our view is that none of them is entirely wrong or right, but reflect a complex pathology with multifaceted clinical manifestations; we expect the underlying biochemical pathways to overlap and be aggravated by aging. Cu plays a role in at least a subset of these cases but not necessarily all. A direct counterargument could be that no Cu transporters are direct genetic risk factors of AD. We argue that this is not ruling out the hypothesis because *ATP7B* variants do confer risk in a subset of patients and APP/PS1 variants relate to the processing of a peptide that could be a Cu binding peptide if the hypothesis is right – indeed the work by Multhaup and coworkers suggests that APP functions as a Cu transporter so that would counter the "lack of genetic support argument" as APP variants are a main cause of familial AD [41].

In summary, the accumulated body of evidence supports the idea that metal dysregulation is a crucial player in the neurodegeneration associated with dementia, not just as a consequence, although much remains to be done to explore this hypothesis further. Metal ion imbalances, energy depletion of high-energy demand neurons, oxidative stress, and protein misfolding work in concert to produce disease phenotypes **(Figure 5)**. Cu imbalance has a very strong and appealing explanatory power both in terms of loss of physiological function and gain of pathological function etiologies. We expect substantial individual variations in clinical presentation and etiology, depending on the pathology that first occurs and confounding risk modifiers, but the already observed strong evidence for Cu imbalance cannot reasonably be assumed to have zero effect on the already vulnerable patient, regardless of the exact contribution to overall pathogenesis. It is our hope that future research will define this contribution much more precisely, as outlined above.

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CONFLICT OF INTEREST

RS is Chief Scientific Officer and has some shares in IGEA Pharma N.V.; other authors declare no commercial or noncommercial conflicts of interest relating to this work.

AUTHOR CONTRIBUTION

All authors contributed significantly in the writing processes and literature review.

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Table 1. Hereditary diseases associated to genes encoding for proteins involved in Cu pathway

Copper disease	**Gene (inheritance)**	**Protein function**	**Cu status**	**Symptoms**
Wilson disease [104]	*ATP7B* (autosomal recessive inheritance)	Cu transporter/ metallochaperone	Low levels of ceruloplasmin, Low level serum Cu, high levels of non-Cp Cu	Jaundice, Dystonic rigidity Dysarthria, dysphagia, Fatigue, Tremor
MENDIK Syndrome [105]	*AP1S1* (autosomal recessive inheritance)	Trafficking of ATP7aseA or ATPase7B	Low levels of serum Cu and ceruloplasmin	Brain atrophy, mental retardation enteropathy, deafness, keratoderma, peripheral neuropathy, ichthyosis, and cholestatic hepatopathy
Menkes disease [106]	*ATP7A* (X-linked recessive inheritance)	Cu transporter/ metallochaperone	Low levels of serum copper and ceruloplasmin	Intellectual disability and developmental delay, seizures, lack of muscle tone, floppiness, kinky hair
Occipital Horn Syndrome [107]	*ATP7A* (X-linked recessive inheritance)	Cu transporter/ metallochaperone	Low levels of serum copper and ceruloplasmin	Cutis laxa, coarse hair, cerebral calcification exostoses, hyperextensible skin, mild cognitive deficits, global developmental delay, and loose joints
Huppke-Brendel Syndrome [108]	*SLC33A1* (autosomal recessive inheritance)	Acetyl CoA transporter protein	Very low serum copper and ceruloplasmin levels	Cataracts, developmental delay, cerebral atrophy, hypacusis, hearing loss, and nystagmus

X-Linked Distal Hereditary Motor Neuropathy [109]	*ATP7A* (X-linked recessive inheritance)	ATPase7A	Low levels of serum copper and ceruloplasmin	Weakness of distal muscles, motor neuron syndrome, muscle atrophy, and abnormal sensory examination affected peripheral nerves
Infantile Cardioencephalomyopathy with severe deficiency of cytochrome C oxidase in heart, brain, and muscle [110]	SCO2 (SCO1) (autosomal recessive inheritance)	Metallochaperones involved in the assembly and Cu delivery to the catalytic core (CuA site) of cytochrome C oxidase, complex IV of the mitochondrial respiratory chain and result in cytochrome C oxidase deficiency	Severe Cu deficiency	Abnormalities in the nervous system, heart, and skeletal muscle (including Leigh syndrome), hypertrophic cardiomyopathy, lactic acidosis, stridor with ventilator insufficiency, and a spinal muscular atrophy

Table 2. A subset of Alzheimer's disease patients shares biochemical features/clinical traits with WD

Biochemical features/clinical traits	**WD**	**Cu Alzheimer's disease subset of patients**
Non-Cp Cu in serum; normal reference range: 0.1-1.6 µM	> 1.6 µmol/L; a cut-off of 2.3 µmol/L is diagnostic of probable WD; a cut-off of 3.9 µmol/L is diagnostic of WD [111]	> 1.6 µmol/L; a cut-off of 1.61- 2.4 µM is supportive of MCI and AD patients with CuAD subtype [2, 67]
ATP7B gene variants	more than 700 *ATP7B* variations are disease-causing mutations; additional 800 SNPs have been described [111]	Increased frequencies of the functional SNPs rs732774 and rs1061472 and of rs1801243, rs2147363, rs7334118 associated with an increased risk of AD [4]
Ceruloplasmin; ***normal reference range: 23-50 mg/dL***	< 20 mg/dL is suggestive of probable WD [111]	Decreased values of ceruloplasmin specific activity associated with an increased risk of AD [112]
Apo-ceruloplasmin[a] fragmentation	Increased values	Increased values in CSF,[113] and in serum [114]
Liver disease	Highly variable, ranging from asymptomatic, with only biochemical abnormalities, to hepatic cirrhosis [111]	Biochemical abnormalities: decreased values of albumin, longer prothrombin time (PT) associated with Non-Cp Cu [115]

***Cu excretion in the urine*; *normal reference range:* < 40 µg/24h**	> 40 µg/24h (ULN)[b].[111]	AD patients have 24h urinary excretion higher than healthy control [67]
***D-penicillamine test; Cut-off* 200 µg/24 h**	87% [67]	78% [67]
***Kayser-Fleischer rings*[d]**	Present in 44-62% [111]	Present in an AD patient positive to 11C-labeled Pittsburgh Compound-B PET[e] and [18F] fluorodeoxyglucose PET[e] [116]
***Paradoxical effect under D-penicillamine treatment*[f]**	Present in 10-20% WD [111]	Present in < 50% [2]
Copper in the brain	Preclinical model of WD Long Evans Cinnamon rat, and toxic milk show decreased or normal levels of Cu in the brain coexisting with Non-Cp Cu excess in the bloodstream and excess labile Cu in the brain [2]	AD have 20% increase in brain labile Cu, coexisting with an overall decreased level of Cu in the AD brain [58], and Non-Cp Cu excess (Figure 4)

[a]Apo-ceruloplasmin: The incorrect loading of Cu into nascent hepatic ceruloplasmin because of *ATP7B* mutations causing a defective ATPase7B protein generates the inactive serum apo-form of ceruloplasmin that is rapidly fragmented;

[b]UNL: upper limit of normal

[c]D-penicillamine challenge test: is a screening test for asymptomatic WD patients (generally in pediatric age); cut-off of 200 μg/24 h (5x ULN).

[d]Kayser-Fleischer rings*:* Cu deposition in the Descemet's membrane of the cornea pathognomonic of WD;

[e]PET: Positron emission tomography;

[f]Paradoxical effect upon D-penicillamine treatment: serious "iatrogenic" deterioration with increase of the neurological symptoms, thought to be caused by a frantic mobilization and redistribution of Cu which results in high Cu level in the brain and in the blood.

FIGURE LEGENDS

Figure 1. Examples of structurally established Cu- and Zn-binding sites in AβPP and Aβ. (A) Zn2+ site in E2 domain of AβPP (3UMI) and zoom on the Zn2+ binding site (made of three His residues and one water molecule). (B) Copper-Binding Domain (CuBD) of AβPP (2FK1) and zoom on the Cu2+ binding site (made of two His, one Tyr residues and two water molecules). (C) Zn2+ binding site in Aβ (made of two His residues, and two carboxylate-containing residues) and (D) Cu2+ binding site in Aβ (made of two His residues, the N-terminal amine and the adjacent CO form the peptide bond). Green dot: Zn2+, purple dot: Cu2+ (models proposed based on spectroscopic studies as recently reviewed in {Atrián-Blasco, 2018 #1156}).

Figure 2. Mechanisms of Cu absorption and distribution in physiology. The pictogram illustrates a concise overview of Cu metabolism in humans.

Small intestine absorption (left box). Cu ingested through the diet is absorbed by small intestine enterocyte. The integral membrane protein hCTR1 imports Cu. The metal is then loaded onto copper enzymes via several chaperone proteins (not shown). ATPase7A (ATP7A) pumps Cu out of the enterocyte basolateral membrane. Cu, bound to albumin, α2 macroglobulin, or amino acids, is then transported to the liver through the portal vein.

Liver absorption (middle box). The liver plays an essential role in Cu storage, metabolism, and distribution. hCTR1 promotes hepatocytes Cu uptake. In the liver, ATPase7B (ATP7B), the homologue of enterocyte ATPase7A, incorporates Cu into ceruloplasmin (Cp). Under physiological conditions 85-95% of total Cu is bound to Cp. Cp-Cu is then released in the bloodstream for systemic distribution.10-15% of Cu is released in bloodstream as non-Cp Cu.

Brain metabolism (right box). The intersection between the blood-brain barrier (BBB) and the neurovascular unit (NU) is critical for brain Cu regulation. In close analogy with the small intestine and the liver, hCTR1 controls Cu absorption into endothelial cells and astrocytes. Conversely, ATPase7A and ATPase7B regulate metal efflux from endothelial cells to the interstitial fluid or the blood flow, respectively. Within astrocytic feet, ATPase7B loads Cu into glycosylphosphatidylinositol-linked ceruloplasmin (GPI-Cp), a maneuver instrumental for maintaining intracellular Cu concentrations under control. The pump is also involved in Cu extrusion from astrocytes for metal distribution to neurons.

Figure 3. **Model of differences in Cu (red dots) pathway among normal, Wilson, Menkes and Alzheimer's disease**. Under healthy conditions, a biological system (black rectangle, e. g. a cell, an organism or organ) needs Cu bound to proteins (target protein, purple) for essential functions. An uptake and secretion system assures the correct Cu concentration in the system and correct copper trafficking (plain arrows). In Wilson disease, the secretion is reduced. An accumulation of Cu in the system occurs and Cu ions bind to non-target proteins (hollow green square), where it gains function, e .g. Cu catalysed production of reactive oxygen species (ROS). In

Menkes disease, Cu deficiency occurs due to impaired Cu-uptake. No Cu arrives on the target proteins and a loss of essential function is observed. In Alzheimer's disease, Cu uptake and secretion seem to be less affected and keep a certain control over total bulk Cu-content of the system. But a Cu-imbalance occurs in the system, by moving Cu from target proteins (loss of essential function) to pools of loosely bound Cu ions (gain of toxic function, e.g. ROS production). The therapeutic approach would be to re-equilibrate by transferring Cu back to the essential Cu-target proteins.

Figure 4 Meta-analyses of Cu in AD. The table depicts standardized mean difference (SMD) computed from studies performed in AD patients and healthy controls on Cu brain specimens (μg/g; panel A); Cu serum/plasma levels (μmol/L; panel B); and serum non-Cp Cu (μmol/L; panel C). SMDs between patients 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. **Panel A:** Results indicate that AD subjects had lower levels of Cu in the brain than healthy controls [SMD=-0.77 (95% CI -1.09, -0.44); $p<0.001$]; there was substantial heterogeneity among the included studies (I^2=58.97%; $p<0.001$). **Panel B:** AD subjects had higher levels of Cu in serum than healthy controls [SMD =0.64 (95% CI 0.30, 0.98); $p< 0.001$]; there was considerable heterogeneity among the included studies (I^2=95.96%; $p<0.001$). Studies from the Fatebenefratelli research group [82, 112, 117, 118] were pooled together and considered as a single study. **Panel C:** Results indicate that AD subjects had higher levels of non-Cp Cu than healthy controls (SMD =0.60 (95% CI 0.36, 0.83; $p<0.001$); there was considerable heterogeneity among the included studies (I^2=81.5%; $p<0.001$).

Figure 5. Model of Aβ, glutamate, oxidative stress, and ionic dyshomeostasis in AD pathogenesis. The figure depicts Aβ, oxidative stress, excitotoxicity, and $Cu^{+/2+}$ dyshomeostasis

acting synergistically to promote synaptic dysfunction and neuronal loss. Excessive glutamate builds up in the synaptic cleft (**1**) leads to prolonged activation of NMDA receptor, aberrant neuronal Ca^{2+} accumulation (**2**), and induces Cu-ATPase7A/B (ATP7A/B) translocation (**3**) at synapses where vesicular Cu is released. The released Cu^{2+} (in concentrations up to 100 μmol/L) may inhibit NMDA receptors, thereby protecting neurons from glutamatergic excitotoxicity **(4)**, or catalyze Fenton-type reaction, thereby promoting reactive oxygen species (**ROS**) generation (**5**). Enhanced ROS production damages proteins, lipids, nucleic acids, and eventually leads to cell death (**5**). Ca^{2+} overload increases superoxide anion ($O_2•^-$) production from mitochondria (**6**), and nitric oxide (NO) generation via Ca^{2+}-dependent activation of NO synthase (NOS) (**7**). Reactive oxygen and nitrosative (**RNS)** species, as well as ROS, mobilize Cu from Cu-proteins [such as Atox1, metallothionein 3 (MT3)] increasing intracellular toxic Cu concentrations (**8**), and promoting mitochondrial dysfunction and the release of pro-apoptotic factors from the organelles (**9**). ROS-driven Cu mobilization from Cu-proteins and metal release from mitochondria can further aggravate oxidative stress and initiate Aβ oligomerization **(10**). Altered trafficking of APP and/or elevated Aβ oligomer secretion can generate intracellular Cu^+ deficiency, thereby causing oxidative stress by loss of SOD-1 function (**11**). Aβ, α-synuclein, and PrP can modulate neurotransmission as [Cu^{2+}] buffers within the synaptic cleft or amplify the vicious cycle by further promoting ROS generation (**12**). Excess Non-Cp Cu in the bloodstream is a source for the buildup of labile Cu^{2+} in the interstitial space promoting ATPase7A/B translocation of Cu^{2+} into vesicles of the trans-Golgi network and endoplasmic reticulum (ER) **(13)**. These processes critically occurring at the level of dendritic spines, within the synaptic cleft, and in the neurovascular unit, can be the *primum movens* of synaptic dysfunction, neuronal deafferentation, and ultimately brain cell death.

Figure 1

Figure 1

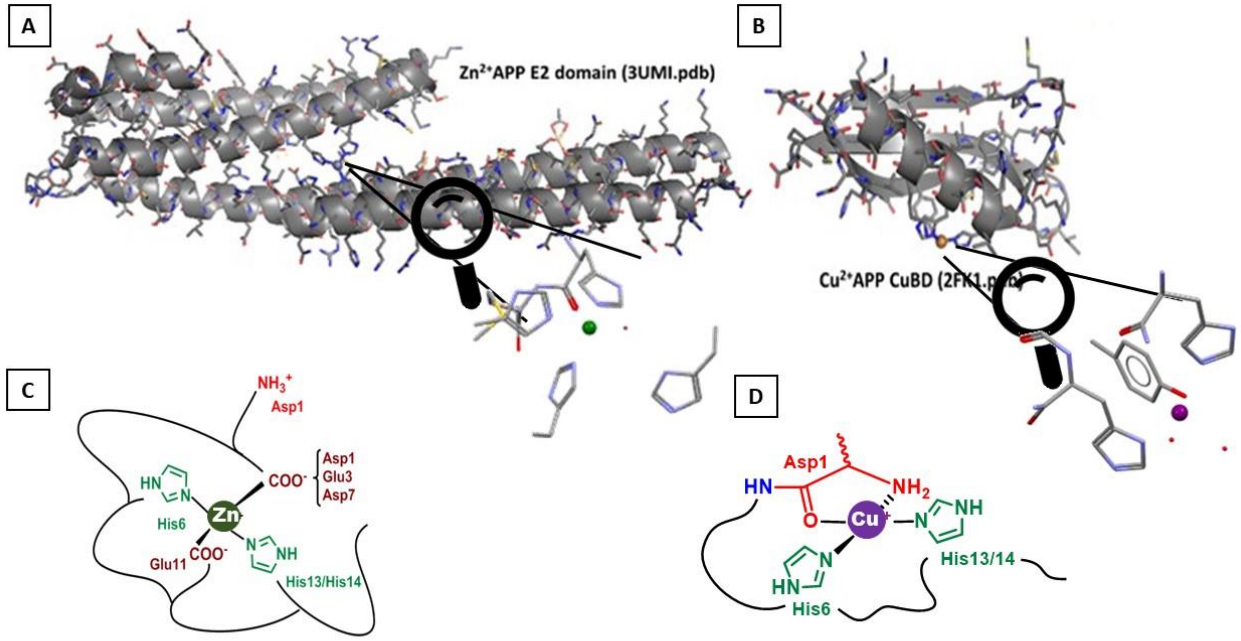


Figure 2

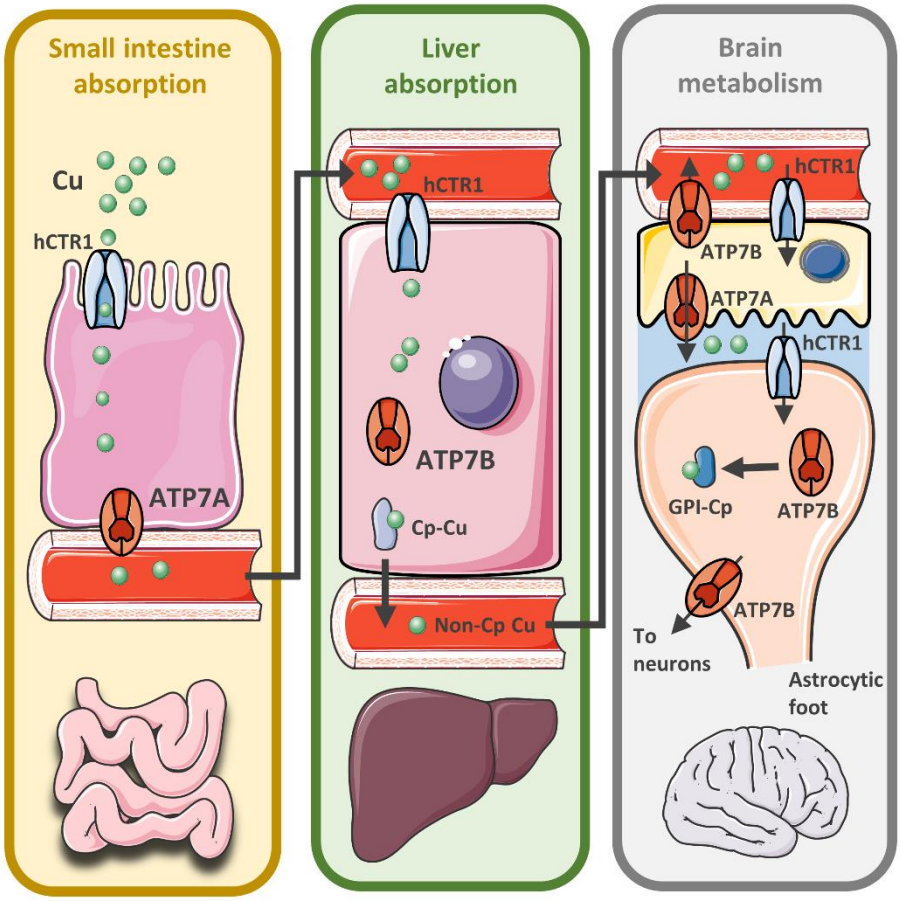

Small intestine absorption
Cu
hCTR1
ATP7A
Liver absorption
hCTR1
ATP7B
Cp-Cu
Non-Cp Cu
Brain metabolism
hCTR1
ATP7B
ATP7A
hCTR1
GPI-Cp
ATP7B
ATP7B
To neurons
Astrocytic foot

Figure 3

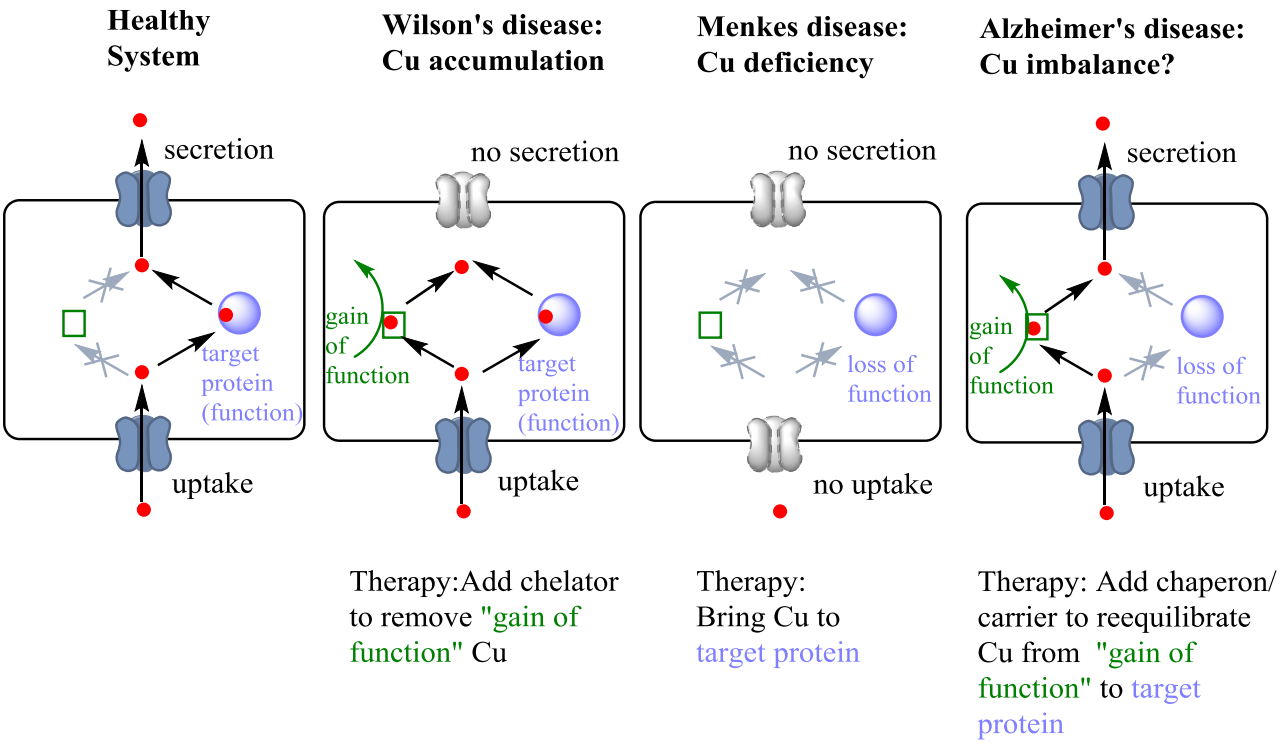

Healthy System
Wilson's disease: Cu accumulation
Menkes disease: Cu deficiency
Alzheimer's disease: Cu imbalance?
secretion
target protein (function)
uptake
no secretion
gain of function
target protein (function)
uptake
no secretion
loss of function
no uptake
secretion
gain of function
loss of function
uptake
Therapy:Add chelator to remove "gain of function" Cu
Therapy: Bring Cu to target protein
Therapy: Add chaperon/ carrier to reequilibrate Cu from "gain of function" to target protein

Figure 4

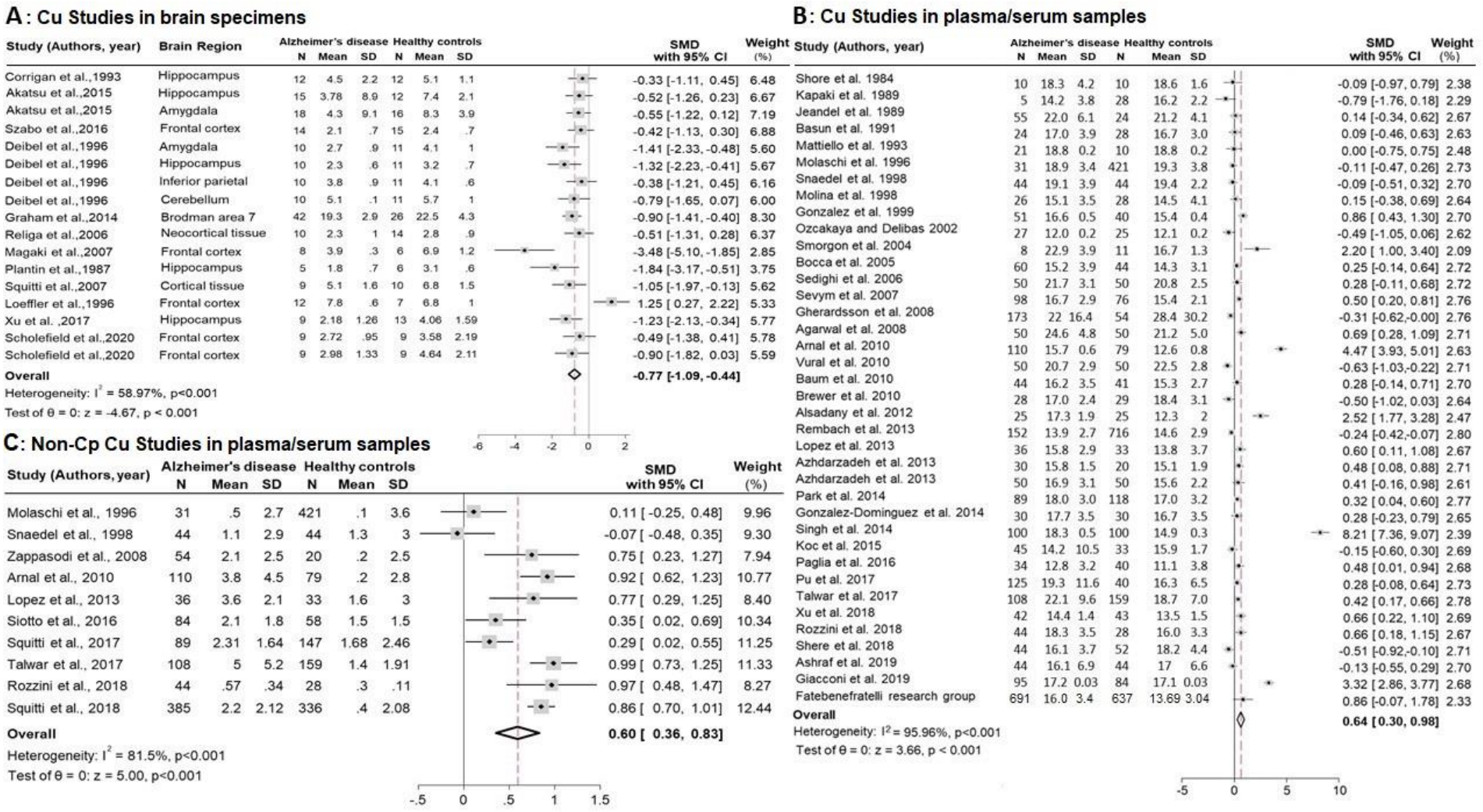
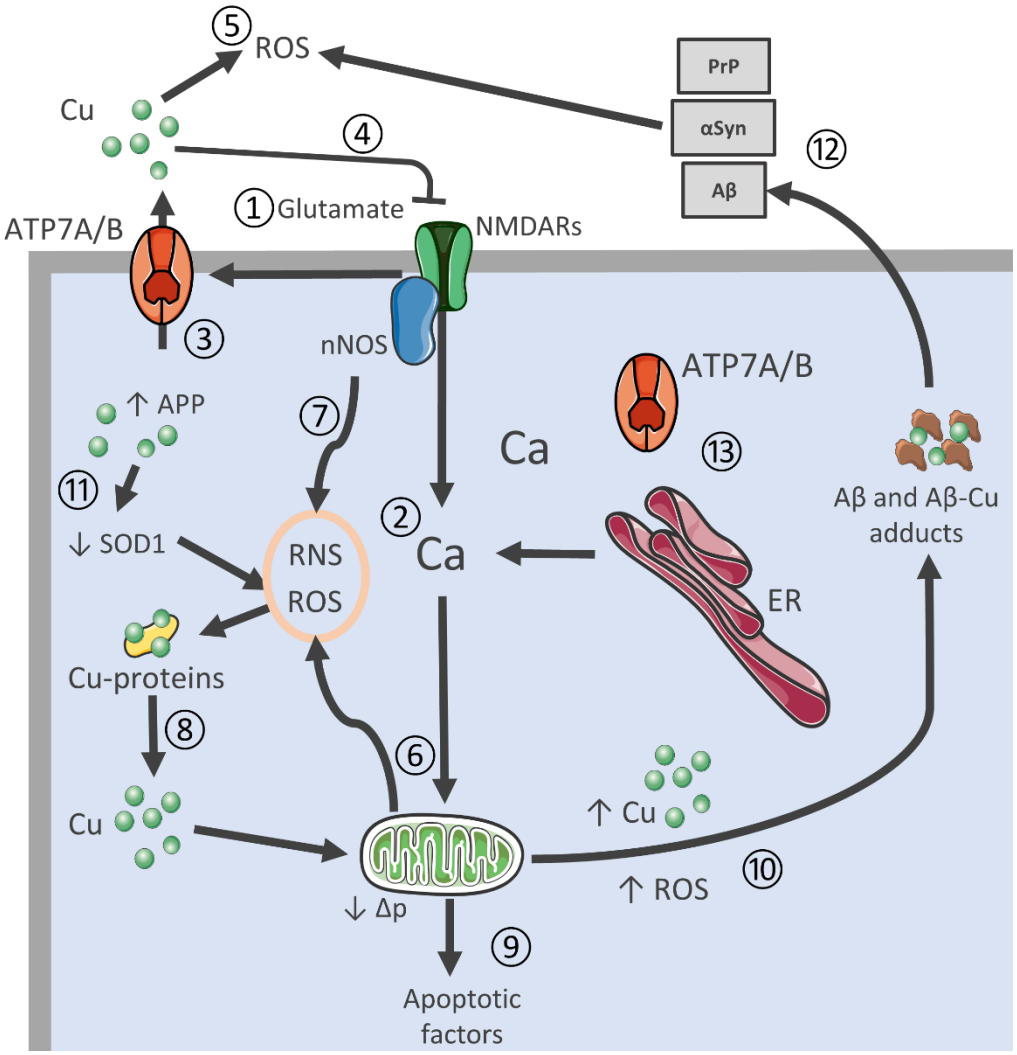

A: Cu Studies in brain specimens
Study (Authors, year) | Brain Region | Alzheimer's disease N Mean SD | Healthy controls N Mean SD | SMD with 95% CI | Weight (%)
Corrigan et al.,1993 | Hippocampus | 12 4.5 2.2 | 12 5.1 1.1 | -0.33 [-1.11, 0.45] | 6.48
Akatsu et al.,2015 | Hippocampus | 15 3.78 8.9 | 12 7.4 2.1 | -0.52 [-1.26, 0.23] | 6.67
Akatsu et al.,2015 | Amygdala | 18 4.3 9.1 | 16 8.3 3.9 | -0.55 [-1.22, 0.12] | 7.19
Szabo et al.,2016 | Frontal cortex | 14 2.1 .7 | 15 2.4 .7 | -0.42 [-1.13, 0.30] | 6.88
Deibel et al.,1996 | Amygdala | 10 2.7 .9 | 11 4.1 1 | -1.41 [-2.33, -0.48] | 5.60
Deibel et al.,1996 | Hippocampus | 10 2.3 .6 | 11 3.2 .7 | -1.32 [-2.23, -0.41] | 5.67
Deibel et al.,1996 | Inferior parietal | 10 3.8 .9 | 11 4.1 .6 | -0.38 [-1.21, 0.45] | 6.16
Deibel et al.,1996 | Cerebellum | 10 5.1 .1 | 11 5.7 1 | -0.79 [-1.65, 0.07] | 6.00
Graham et al.,2014 | Brodman area 7 | 42 19.3 2.9 | 26 22.5 4.3 | -0.90 [-1.41, -0.40] | 8.30
Religa et al.,2006 | Neocortical tissue | 10 2.3 1 | 14 2.8 .9 | -0.51 [-1.31, 0.28] | 6.37
Magaki et al.,2007 | Frontal cortex | 8 3.9 .3 | 6 6.9 1.2 | -3.48 [-5.10, -1.85] | 2.85
Plantin et al.,1987 | Hippocampus | 5 1.8 .7 | 6 3.1 .6 | -1.84 [-3.17, -0.51] | 3.75
Squitti et al.,2007 | Cortical tissue | 9 5.1 1.6 | 10 6.8 1.5 | -1.05 [-1.97, -0.13] | 5.62
Loeffler et al.,1996 | Frontal cortex | 12 7.8 .6 | 7 6.8 1 | 1.25 [0.27, 2.22] | 5.33
Xu et al. ,2017 | Hippocampus | 9 2.18 1.26 | 13 4.06 1.59 | -1.23 [-2.13, -0.34] | 5.77
Scholefield et al.,2020 | Frontal cortex | 9 2.72 .95 | 9 3.58 2.19 | -0.49 [-1.38, 0.41] | 5.78
Scholefield et al.,2020 | Frontal cortex | 9 2.98 1.33 | 9 4.64 2.11 | -0.90 [-1.82, 0.03] | 5.59
Overall | -0.77 [-1.09, -0.44]
Heterogeneity: I^2 = 58.97%, p<0.001
Test of θ = 0: z = -4.67, p < 0.001
-6 -4 -2 0 2
B: Cu Studies in plasma/serum samples
Study (Authors, year) | Alzheimer's disease N Mean SD | Healthy controls N Mean SD | SMD with 95% CI | Weight (%)
Shore et al. 1984 | 10 18.3 4.2 | 10 18.6 1.6 | -0.09 [-0.97, 0.79] | 2.38
Kapaki et al. 1989 | 5 14.2 3.8 | 28 16.2 2.2 | -0.79 [-1.76, 0.18] | 2.29
Jeandel et al. 1989 | 55 22.0 6.1 | 24 21.2 4.1 | 0.14 [-0.34, 0.62] | 2.67
Basun et al. 1991 | 24 17.0 3.9 | 28 16.7 3.0 | 0.09 [-0.46, 0.63] | 2.63
Mattiello et al. 1993 | 21 18.8 0.2 | 10 18.8 0.2 | 0.00 [-0.75, 0.75] | 2.48
Molaschi et al. 1996 | 31 18.9 3.4 | 421 19.3 3.8 | -0.11 [-0.47, 0.26] | 2.73
Snaedel et al. 1998 | 44 19.1 3.9 | 44 19.4 2.2 | -0.09 [-0.51, 0.32] | 2.70
Molina et al. 1998 | 26 15.1 3.5 | 28 14.5 4.1 | 0.15 [-0.38, 0.69] | 2.64
Gonzalez et al. 1999 | 51 16.6 0.5 | 40 15.4 0.4 | 0.86 [0.43, 1.30] | 2.70
Ozcakaya and Delibas 2002 | 27 12.0 0.2 | 25 12.1 0.2 | -0.49 [-1.05, 0.06] | 2.62
Smorgon et al. 2004 | 8 22.9 3.9 | 11 16.7 1.3 | 2.20 [1.00, 3.40] | 2.09
Bocca et al. 2005 | 60 15.2 3.9 | 44 14.3 3.1 | 0.25 [-0.14, 0.64] | 2.72
Sedighi et al. 2006 | 50 21.7 3.1 | 50 20.8 2.5 | 0.28 [-0.11, 0.68] | 2.72
Sevym et al. 2007 | 98 16.7 2.9 | 76 15.4 2.1 | 0.50 [0.20, 0.81] | 2.76
Gherardsson et al. 2008 | 173 22 16.4 | 54 28.4 30.2 | -0.31 [-0.62,-0.00] | 2.76
Agarwal et al. 2008 | 50 24.6 4.8 | 50 21.2 5.0 | 0.69 [0.28, 1.09] | 2.71
Arnal et al. 2010 | 110 15.7 0.6 | 79 12.6 0.8 | 4.47 [3.93, 5.01] | 2.63
Vural et al. 2010 | 50 20.7 2.9 | 50 22.5 2.8 | -0.63 [-1.03,-0.22] | 2.71
Baum et al. 2010 | 44 16.2 3.5 | 41 15.3 2.7 | 0.28 [-0.14, 0.71] | 2.70
Brewer et al. 2010 | 28 17.0 2.4 | 29 18.4 3.1 | -0.50 [-1.02, 0.03] | 2.64
Alsadany et al. 2012 | 25 17.3 1.9 | 25 12.3 2 | 2.52 [1.77, 3.28] | 2.47
Rembach et al. 2013 | 152 13.9 2.7 | 716 14.6 2.9 | -0.24 [-0.42,-0.07] | 2.80
Lopez et al. 2013 | 36 15.8 2.9 | 33 13.8 3.7 | 0.60 [0.11, 1.08] | 2.67
Azhdarzadeh et al. 2013 | 30 15.8 1.5 | 20 15.1 1.9 | 0.48 [0.08, 0.88] | 2.71
Azhdarzadeh et al. 2013 | 50 16.9 3.1 | 50 15.6 2.2 | 0.41 [-0.16, 0.98] | 2.61
Park et al. 2014 | 89 18.0 3.0 | 118 17.0 3.2 | 0.32 [0.04, 0.60] | 2.77
Gonzalez-Dominguez et al. 2014 | 30 17.7 3.5 | 30 16.7 3.5 | 0.28 [-0.23, 0.79] | 2.65
Singh et al. 2014 | 100 18.3 0.5 | 100 14.9 0.3 | 8.21 [7.36, 9.07] | 2.39
Koc et al. 2015 | 45 14.2 10.5 | 33 15.9 1.7 | -0.15 [-0.60, 0.30] | 2.69
Paglia et al. 2016 | 34 12.8 3.2 | 40 11.1 3.8 | 0.48 [0.01, 0.94] | 2.68
Pu et al. 2017 | 125 19.3 11.6 | 40 16.3 6.5 | 0.28 [-0.08, 0.64] | 2.73
Talwar et al. 2017 | 108 22.1 9.6 | 159 18.7 7.0 | 0.42 [0.17, 0.66] | 2.78
Xu et al. 2018 | 42 14.4 1.4 | 43 13.5 1.5 | 0.66 [0.22, 1.10] | 2.69
Rozzini et al. 2018 | 44 18.3 3.5 | 28 16.0 3.3 | 0.66 [0.18, 1.15] | 2.67
Shere et al. 2018 | 44 16.1 3.7 | 52 18.2 4.4 | -0.51 [-0.92,-0.10] | 2.71
Ashraf et al. 2019 | 44 16.1 6.9 | 44 17 6.6 | -0.13 [-0.55, 0.29] | 2.70
Giacconi et al. 2019 | 95 17.2 0.03 | 84 17.1 0.03 | 3.32 [2.86, 3.77] | 2.68
Fatebenefratelli research group | 691 16.0 3.4 | 637 13.69 3.04 | 0.86 [-0.07, 1.78] | 2.33
Overall | 0.64 [0.30, 0.98]
Heterogeneity: I^2 = 95.96%, p<0.001
Test of θ = 0: z = 3.66, p < 0.001
-5 0 5 10
C: Non-Cp Cu Studies in plasma/serum samples
Study (Authors, year) | Alzheimer's disease N Mean SD | Healthy controls N Mean SD | SMD with 95% CI | Weight (%)
Molaschi et al., 1996 | 31 .5 2.7 | 421 .1 3.6 | 0.11 [-0.25, 0.48] | 9.96
Snaedel et al., 1998 | 44 1.1 2.9 | 44 1.3 3 | -0.07 [-0.48, 0.35] | 9.30
Zappasodi et al., 2008 | 54 2.1 2.5 | 20 .2 2.5 | 0.75 [0.23, 1.27] | 7.94
Arnal et al., 2010 | 110 3.8 4.5 | 79 .2 2.8 | 0.92 [0.62, 1.23] | 10.77
Lopez et al., 2013 | 36 3.6 2.1 | 33 1.6 3 | 0.77 [0.29, 1.25] | 8.40
Siotto et al., 2016 | 84 2.1 1.8 | 58 1.5 1.5 | 0.35 [0.02, 0.69] | 10.34
Squitti et al., 2017 | 89 2.31 1.64 | 147 1.68 2.46 | 0.29 [0.02, 0.55] | 11.25
Talwar et al., 2017 | 108 5 5.2 | 159 1.4 1.91 | 0.99 [0.73, 1.25] | 11.33
Rozzini et al., 2018 | 44 .57 .34 | 28 .3 .11 | 0.97 [0.48, 1.47] | 8.27
Squitti et al., 2018 | 385 2.2 2.12 | 336 .4 2.08 | 0.86 [0.70, 1.01] | 12.44
Overall | 0.60 [0.36, 0.83]
Heterogeneity: I^2 = 81.5%, p<0.001
Test of θ = 0: z = 5.00, p<0.001
-.5 0 .5 1 1.5

Figure 5


⑤ ROS
Cu
④
PrP
αSyn
Aβ
⑫
ATP7A/B
① Glutamate
NMDARs
③
nNOS
↑ APP
⑦
Ca
ATP7A/B
⑬
Aβ and Aβ-Cu adducts
⑪
↓ SOD1
RNS
ROS
②
Ca
ER
Cu-proteins
⑧
⑥
↑ Cu
Cu
↑ ROS
⑩
↓ Δp
⑨
Apoptotic factors