

Agents Complexing Copper as a Therapeutic Strategy for the Treatment of Alzheimer's Disease

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Abstract: The notion that a copper dysfunction is implicated in Alzheimer's disease (AD) is based on a number of observations from *in vitro* and clinical studies, as well as animal models. However, there is still significant controversy over whether it is an excess or a deficiency of copper to be involved in the pathogenesis of AD. Numerous studies support the hypothesis that an excess of copper contributes to AD, but experimental evidence in transgenic mouse models seems to suggest the contrary, and at least one clinical study shows that cognitive decline correlates positively with low copper levels. We have recently reported on a deregulation of the ceruloplasmin-copper relationship, specific to AD patients, consisting of an elevation of the copper pool not bound to ceruloplasmin, i.e. 'free' copper. This phenomenon could provide an explanation of the contrasting results obtained in clinical studies. Several clinical trials have been attempted in search of an anti-metal effect counteracting AD progression. Some of them have delivered encouraging results indicating that "metal protein attenuating compounds" can indeed alter positively the progression of the disease. This review summarizes these clinical studies and provides an overview of those in progress and in preparation.

Keywords: Copper, ceruloplasmin, Alzheimer disease, metal protein attenuating compounds, therapy, 'free' copper.

INTRODUCTION

Copper Essentiality and Toxicity

Copper, though an essential nutrient for humans, is potentially toxic when its levels in the body become too high. As a transition metal, it takes part in a variety of biological reduction and oxidation (redox) processes, which make it an important cofactor of many redox enzymes. An overload of this metal can easily lead to oxidative reactions resulting in cell damage and death.

Humans ingest copper via food, although it has been recently discovered that varying degrees of intake originate from drinking water piped through copper plumbing [1]. For this reason, despite the general possibility of a widespread mild copper deficiency, many regulatory agencies, such as the Californian Environmental Protection Agency, are paradoxically concerned about potential local copper overexposures [1].

Occasional cases of acute copper poisoning have been reported due to beverage contamination from copper-containing dispensers, or due to suicide attempts. Chronic copper toxicosis can develop under certain conditions, depending on specific factors such as species, genetics, age and diet [2], which determine differences in the efficiency of copper absorption and excretion. Sheep, for example, are not able to increase biliary copper excretion in response to an increased intake and are consequently easily subject to copper toxicosis, as opposed to pigs which instead tolerate cop-

per very well. Also, newborn animals are more susceptible to copper poisoning than their adult counterparts, probably because of the high efficiency of copper absorption and the immaturity of the biliary excretory mechanisms at early times in life [2]. Copper toxicosis can also have a genetic origin manifesting itself in the failure to express a specific copper transporter protein, particularly in the liver (see below). Sometimes, although infrequently, a copper excess is due to a clinical disease, such as an unexplained liver cirrhosis. However, toxicity always stems from the ability of copper to catalyze the production of compounds that generate oxidative stress [2].

COPPER HOMEOSTASIS: LIVER COPPER AND REGULATION OF PLASMA COPPER LEVELS

Copper status in the body is regulated by both duodenal absorption (intestine) and biliary excretion (liver). A high copper exposure in healthy adults, as long as it remains within the homeostatic range, results in a down-regulation of copper uptake in the duodenum and an up-regulation of biliary excretion. As a result, a high copper intake does not necessarily cause an equivalent body 'copper load'. After crossing the intestinal lumen, copper is transported to the liver via portal circulation. Here, copper is partly stored and partly redistributed to other organs. In the hepatocytes, in particular, copper is incorporated into ceruloplasmin, other copper proteins and low-molecular-weight compounds, and then routed into peripheral circulation or secreted into the bile for excretion. About 95% of copper tightly binds to ceruloplasmin, whereas the remainder loosely binds to and is exchanged among albumin, amino acids, peptides and several micronutrients. We will refer to the portion that binds to

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ceruloplasmin as 'bound' copper, while to the portion that binds to the loose compounds as 'free' copper [3]. A key difference between the two pools lies in the fact that the low-molecular-weight compounds allow 'free' copper to easily cross the Blood-Brain-Barrier (BBB) [4]. Serum copper is very tightly regulated and does not represent the body copper status, which is rather represented by liver copper. Nevertheless, ceruloplasmin is considered a useful marker of body copper status since its plasma levels begin to decrease when copper supply in the liver is depleted [5]. Ceruloplasmin oxidizes Fe(II) to Fe(III) without releasing reactive oxygen species (ROS), a capacity that gives this protein an antioxidative power: in fact reduced iron, as well as copper, easily enters Fenton reactions with H_2O_2 , resulting in the production of the hydroxyl radical $\cdot\text{OH}$, the most reactive and vicious of all ROS species. Therefore, lowering the ceruloplasmin content or efficiency induces iron-dependent oxidative damage to brain tissues [6].

COPPER CHAPERONES

Copper import into intestinal epithelial cells is mediated by Ctr1, an integral membrane protein conserved from yeast to humans [7]. Based on its structural and biochemical properties, Ctr1 could be thought of as a Cu^+ -specific pore [7].

In hepatocytes, intracellular copper is carried by specific chaperone proteins to Cu-dependent enzymes. So far, three copper chaperones have been identified, although new candidates are being investigated: the Human Atox 1 Homologue (HAH1), homologous to the yeast Antioxidant protein 1 (Atox1); the Copper Chaperone for Superoxide Dismutase (CCS); the Cytochrome C Oxidase Assembly Homologue (COX17). HAH1 delivers copper to the two mammalian P-type Cu-transporting ATPases, ATP7A and ATP7B. CCS delivers copper to the metal-binding site of Copper-Zinc superoxide dismutase (Cu/Zn SOD). COX17 delivers copper to the mitochondria, where it is ultimately incorporated into the Cytochrome C Oxidase. This ensures 'safe' copper trafficking, making sure that, in a healthy cell and in a physiological environment, virtually no copper remains unbound. Chaperones are not present in plasma [7].

Besides chaperones, the defense system against copper excess also includes metallothioneins, a family of proteins, whose expression is regulated by copper and other metals [8] and which are active in trapping metals in excess. It has been reported that Metallothionein-3, a member of the family expressed exclusively in the central nervous system and involved in neuronal damage repair through its neuro-inhibitory activity, is significantly down-regulated in Alzheimer disease (AD) [9]. It was recently proven that this protein protects cultured neurons from toxicity generated by beta amyloid ($\text{A}\beta$), a 39- to 42-amino acid peptide, whose amyloid fibrillar form is the primary component of the amyloid plaques found in the brain of AD patients. A metal swap between Metallothionein-3 and soluble aggregated $\text{A}\beta$ 1-40-Cu(II) avoids ROS production and consequent cellular toxicity. This clearly opens new therapeutic strategies for AD treatment [10].

Cu/Zn SOD also plays a role as a buffer of intracellular 'free' copper, since it is stable in its copper-free form [11].

Recently, the amyloid precursor protein (APP) has been shown to be implicated in copper metabolism. APP is an integral membrane protein expressed in many tissues and concentrated in neuron synapses. Its primary function is still unknown, but it has been proven to be a regulator of synapse formation [12], and to be implicated in neuronal plasticity [13] and copper homeostasis [14-18]. APP is best known and most commonly studied as the precursor molecule whose proteolysis generates $\text{A}\beta$. APP binds copper in two domains, one located in the extracellular N-terminal region and the other in the C-terminal region within the $\text{A}\beta$ peptide [16]. Upon coordination, APP reduces Cu(II) to Cu(I), thus promoting the non-amyloidogenic cleavage pathway. Studies on animal models, performed to validate the function of APP as a copper detoxification/efflux transporter *in vivo*, have demonstrated that APP over-expression reduces the levels of brain copper [17]. APP knockout mice have shown increased levels of copper in the liver and in the cerebral cortex [14].

COPPER IN NORMAL AND IN ALTERED CONDITIONS

Normal ranges, within which 95% of the normal population falls and which are normally taken as reference values, are 11-22.4 $\mu\text{mol/L}$ for serum copper (corresponding to 70-142.7 $\mu\text{g/dL}$), 20-60 mg/dL for ceruloplasmin, and 0-1.6 $\mu\text{mol/L}$ for 'free' copper (corresponding to 10 $\mu\text{g/dL}$) [19]. It is generally assumed that normal values are 'healthy', although some authors suggest caution in this regard considering that natural selection works to optimize health and survival only during the reproductive period. Consequently, 'normal' values may not be necessarily optimal after age 50 [19]. When out of its normal ranges, copper easily catalyses Haber-Weiss and Fenton reactions producing $\cdot\text{OH}$, against which the body has no defences [20, 21].

In general circulation, 'free' copper is exchanged among albumin, peptides or amino acids, whereas 'bound' copper is not easily released by ceruloplasmin and virtually does not cross the BBB [5]. It has been calculated that ceruloplasmin-bound copper represents less than 1% of the brain's 'bound' copper [22], and we have recently reported the presence of a conspicuous amount of apo-ceruloplasmin in the cerebrospinal fluid (CSF) of AD patients [23]. In cells of all types, a considerable amount of 'free' copper is stored in metallothionein [9].

The impairment of some components of the system controlling copper homeostasis has serious consequences for the health and development of the brain. This is well exemplified by two genetic disorders, showing either excess - Wilson's disease - or shortage - Menkes' disease - of systemic copper, but both resulting in neurodegeneration. In these diseases, the genes coding for the two membrane copper transport proteins ATP7A and ATP7B are mutated. These proteins are highly homologous, but with different patterns of tissue expression: ATP7A (Menkes' protein) is mainly expressed in the intestine and in the BBB, while ATP7B (Wilson's protein) is primarily found in the liver [24]. The mutation impairs the function of the ATP7A pump and prevents copper absorption at the intestinal level. This causes copper deficiency and reduces the levels of cuproenzymes [24]. The location of ATP7A at the choroid plexes makes

this pump crucial for controlling the copper flux into the ventricles of the brain [25]. Indeed, the copper deficiency in the brain of Menkes' patients is particularly severe because of a decrease in the activity of ATP7A at the BBB. Defects of ATP7B in Wilson's disease individuals cause impairment of copper incorporation into ceruloplasmin, with consequent failure of copper release into the bile canalicula for excretion [26]. This produces a copper overload into the hepatocytes, inducing liver cirrhosis. At the death of the cirrhotic hepatocytes, copper – actually 'free' copper - is released into general circulation and reaches all tissues, including the brain [26].

The holo active form of ceruloplasmin depends on the ATP7B activity, mediating the incorporation of copper atoms into ceruloplasmin during its biosynthesis [5]. ATP7B absence or impairment prevents copper translocation to the secretory pathway, resulting in 1) secretion of unstable apo-ceruloplasmin which is rapidly degraded in the blood [5]; 2) alteration of copper excretion through the bile via ceruloplasmin [26]; 3) severe hypo-function, which can even cause death, of hepatocytes; 4) release of 'free' copper in general circulation and tissue copper overload or intoxication [5].

During aging, control over copper homeostasis undergoes progressive failure and also "normal" copper values could result in an altered copper burden in the aged brain [27]. No conclusive information on copper content in the human aging brain is available, although data obtained in mice speak in favour of an increase [17]. However, results from the few clinical reports on copper status in the central nervous system of AD patients show a decrease of this metal in the brain [27, 28] and an increase in the plaques [29], indicating that an alteration of the brain copper distribution occurs in this disease.

ALZHEIMER DISEASE

Alzheimer disease is an irreversible, progressive neurodegenerative disorder, characterized by a gradual appearance of cognitive deficits accompanied by abnormal behaviour and personality changes, ultimately leading to full dementia. These deficiencies are related to a loss of neurons and the presence of dystrophic synapses and neuritis. On average, AD patients live 8 to 10 years after diagnosis. So far, only symptomatic therapies have been available and development of effective therapeutic strategies is hampered by the paucity of information about the biological mechanisms underlying the disease pathogenesis.

Overt pathological alterations in the AD brain are diverse and include neuron loss, synapse loss, amyloid plaques, neurofibrillary tangles and microgliosis, as well as functional changes such as metal imbalance, oxidative stress and changes in the mediators of cell cycle. In a schematic representation, four primary functional and anatomical features characterize the AD brain: (a) the density and distribution of extracellular amyloid plaques, composed mainly of aggregated, insoluble A β peptide in the entorhinal cortex, hippocampus and the nucleus basalis Meynert; (b) the presence of intracellular neurofibrillary tangles containing hyperphosphorylated tau; (c) increased oxidative damage of lipids,

proteins and nucleic acids; (d) loss of biometal homeostasis [30].

The deposition of aggregated A β and the hyperphosphorylation of tau have been shown to cause neuronal damage and contribute substantially to AD pathology.

COPPER IMPLICATION IN ALZHEIMER DISEASE

Copper may be involved in the pathogenesis of AD at many stages. For example, A β is generated from APP cleavage by β - and γ -secretases. β -secretase (BACE1) cleaves at the N terminus, while γ -secretase cleaves at the C terminus of the A β sequence. BACE1 is an aspartic protease and may function as a dimer, whereas γ -secretase is a complex of presenilin-1/2, Aph1, Pen2, and Nicastrin [31]. It was recently reported that BACE1 modulates APP processing and the release of A β by interacting with CCS. The concept that copper content can modulate BACE1, i.e. the rate-limiting enzyme in the production of A β [32], is supported by the recent evidence that copper (Cu²⁺) and manganese (Mn²⁺) potentially increase the expression of both APP and BACE1 in a time- and concentration-dependent manner, whereas zinc (Zn²⁺), iron (Fe²⁺) and aluminum (Al³⁺) do not [33]. APP has a copper binding domain which reduces Cu(II) to Cu(I) and produces oxidative damage [21] through the formation of H₂O₂ [34]. A β binds copper efficiently and precipitates in plaques which are known to contain high levels of metals [29]. A β has been shown to be toxic to neuronal cells *in vitro* and this toxicity seems to be mediated by the interaction with Cu(II) and its reduction by physiological molecules (cholesterol, catecholamines, ascorbate) [34]. *In vitro* studies have demonstrated that different variants of A β (1-40, 1-42) have different affinity for copper, and that higher affinity generates higher toxicity [30]. The results of recent *in vitro* studies suggest that Cytochrome C Oxidase, and therefore the energy production of the cell, may be one of the targets of the Cu-A β toxic effect [35].

Copper plays a role also in the basal regulation of the APP gene. This has been shown in human fibroblasts overexpressing ATP7A, a condition that causes copper intracellular depletion. Depletion of intracellular copper in these cells results in significant reduction of APP gene expression [18, 36]. Moreover, a study of cDNA microarray technology demonstrated an up-regulation of APP and of the normal cellular prion protein (PrP^c) in two genetic models of chronic copper overload mutants – fibroblasts from C57BL/6-Atp7aMobr and C57BL/6-Atp7aModap – and in a nutritional model of chronic copper overload [37]. Overall, this evidence shows that the APP gene harbours a copper response element within its promoter, such that copper depletion leads to a marked decrease in APP expression and supports the notion that APP is involved in copper homeostasis as a copper detoxification/efflux protein [18, 36, 37].

The Tau protein is a major component of the neurofibrillary tangles and also binds copper, which appears to promote aggregation [38].

A second line of evidence is the effectiveness of metal chelators in hindering redox and toxic activities of amyloid plaques and neurofibrillary tangles, whereas a replacement with copper or iron restores those activities [39].

Regarding genetic risk factors, the Apolipoprotein E4 (APOE4), which is the only established risk factor for AD, was demonstrated to interact with copper metabolism at different levels. It has been shown that ApoE possesses antioxidant properties which depend on bound copper [41, 42]. ApoE4 has a lower antioxidant power than ApoE2 or ApoE3, and is therefore the least effective in protecting neurons from the oxidative damage caused by A β [40-42]. In fact, absolute and 'free' copper concentrations are higher in carriers of the APOE4 allele than in non-carriers [43-45]. In addition, the correlation between typical electroencephalographic (EEG) spectral abnormalities of the AD brain and higher-than-normal serum levels of 'free' copper is stronger in APOE4 carriers than in non-carriers [46, 47].

The level of plasma homocysteine is a risk factor for AD, and it's known that copper mediates LDL oxidation by homocysteine [48].

A β binds copper and cholesterol, facilitating copper oxidation of cholesterol to 7-OH cholesterol and to 4-cholestenone [49], which are extremely toxic to neurons [50]. Trace amounts of copper, well below the levels considered safe for humans, given to cholesterol-fed rabbits have induced accumulation of A β in senile plaques and impaired the animals' learning capability [51]. Thus, cholesterol increases A β formation and copper promotes A β aggregation and toxicity. Furthermore, cholesterol can catalyze copper-A β redox cycling [34]. The results from the cholesterol-fed rabbit model have been confirmed by a large community prospective study showing a strong correlation between copper intake from a diet rich in saturated and trans fats and mental decline [52]. As a result, serious concerns have been raised about a possible relationship between copper overexposure from food or drinking water and cognitive disturbances. The belief that copper toxicity is involved in the evolution of cognitive disturbances is also supported by a recent investigation demonstrating an inverse correlation between cognitive performance and serum copper levels in a large cohort of healthy aged women [53].

The notion that relative copper availability contributes to AD is also sustained by the fact that copper dyshomeostasis in the brain, present in normal aging [17], is substantially more pronounced in the aged AD brain [29]. Lovell *et al.* [29] demonstrated that copper levels in the plaque-free neuropil of an AD brain are approximately 400% higher than in the neuropil of a healthy brain, and that, within the AD brain itself, copper levels are approximately 30% higher in the amyloid plaques than in plaque-free regions. These data indicate that an accumulation of copper within the brain, no matter whether a cause or a consequence, is consistent with the development of AD. Despite gross increases of cerebral extracellular copper, intracellular copper appears to be deficient in the AD brain [54].

Our research group has contributed to collecting evidence of copper implication in AD, primarily via clinical studies. In recent years, our laboratories have been involved in a series of studies aimed at assessing whether alterations of copper homeostasis and oxidative stress may represent markers of AD in tissues other than the brain. In particular, we have focused on identifying these alterations in serum and CSF, given that copper concentrations in the brain depend on cir-

culating and CSF copper levels [55]. We have attempted to assess whether abnormal copper could be representative of the main deficits and markers of the disease. We have measured copper, iron, total hydro-peroxides, transferrin, ceruloplasmin levels and the antioxidant capacity (Total Radical Trapping Antioxidant capacity or TRAP) in the sera of patients affected by AD and vascular dementia (VAD) [44, 56, 57]. Comparison with values obtained from normal subjects indicated that copper levels are higher in AD patients than in normal elderly controls [43, 44] and correlate with the main cognitive, neuro-anatomical, electrophysiological deficits [44-47, 56, 57], with accepted markers, such as CSF A β and Tau proteins [58] (Table 1), and with known risk factors, such as APOE4 allele [44, 45], of the disease.

Furthermore, we investigated the possible effects in AD of copper-enzymes Cu/Zn SOD [59] and ceruloplasmin [57]. AD patients showed higher Cu/Zn SOD activity than controls [59]. The slight increase of ceruloplasmin that we initially detected in AD patients may reflect the inflammatory component of the disease, despite the fact that we found no changes in other peripheral marker of inflammation (e.g. Erythrocyte Sedimentation Rate (ESR), albumin, electrophoresis alpha1 and alpha2 fractions, interleukin-1 beta, Tumor Necrosis Factor alpha) [57]. However, this slight ceruloplasmin increase cannot account for the pronounced rise of serum copper. In fact, when we performed a deeper analysis of our data we could distinguish two pools of copper, related and unrelated to ceruloplasmin, and we could associate the increase in copper level that best characterized AD patients with the pool 'not explained' by the protein [57]. In particular, in our 2005 study [57], about 56% of serum copper variability in healthy controls could be explained by the ceruloplasmin variation. On this basis, we initially computed two copper measures: the first, copper 'explained' by ceruloplasmin, corresponded to the expected level of copper, given a ceruloplasmin level; the second, copper 'not explained' by ceruloplasmin, corresponded to the residual copper [57]. AD patients had higher 'unexplained' copper than both VAD patients and controls [57]. In a subsequent study [58], we have established that the 'unexplained' copper could be referred to as non-ceruloplasmin copper, i.e. 'free' copper [58, 60]. Moreover, we performed ultrafiltration experiments aimed at finding filterable 'free' copper in AD sera, which revealed a concentration of 'free' copper 3.7 times higher in patients than in controls [58]. These observations were recently confirmed by two other research groups, one in the Netherlands [61] and one in the US [62]. In particular, hard data from the latter indicated that 'free' copper levels in AD sera nearly doubled those in normal sera (AD 2.27 +/-0.16 $\mu\text{mol/L}$; normal 1.56 +/- 0.29 $\mu\text{mol/L}$). Interestingly, even though the results of previous AD studies regarding absolute serum copper (i.e., total of serum copper, both bound and not bound to ceruloplasmin) appear controversial [43, 44, 63-66], the controversy does not dispute the ceruloplasmin-copper relationship, i.e. the involvement of 'free' copper in AD (Table 2). All authors who investigated both copper and ceruloplasmin confirm our finding of a copper/ceruloplasmin dyshomeostasis in AD [57, 61-63, 65, 66]. In particular, Snaedal *et al.* [63] found similar levels of absolute serum copper in AD and controls, but significantly lower concentrations of ceruloplasmin, which means a

Table 1. Serum Copper, Peroxides, TRAP and AD Markers in the CSF of AD Patients and Controls

Biological Sample	Biological variables	AD patients (n=28) [Mean (SD)]	Controls (n=25) [Mean (SD)]	ANOVA
Serum	Total Copper ($\mu\text{mol/L}$)	16.2 (3.2)	12.8 (2.3)	$p < 0.0001$
	Ceruloplasmin (mg/dL)	27.9 (5.3)	24.6 (2.9)	$p = 0.009$
	'Free' copper ($\mu\text{mol/L}$)	3.04 (1.8)	1.2 (1.6)	$p = 0.001$
	Peroxides (U.CARR)	335 (68)	266 (54)	$p = 0.002$
	TRAP (mmol/L)	1.26 (0.20)	1.4 (0.22)	$p = 0.03$
CSF	A β (ng/mL)	0.26 (0.11)	0.5 (0.25)	$p < 0.0001$
	h-tau (ng/mL)	0.48 (0.27)	0.27 (0.14)	$p = 0.001$
	P-tau (ng/mL)	0.05 (0.03)	0.06 (0.06)	$p = 0.3$
	Copper ($\mu\text{mol/L}$)	0.4 (0.14)	0.44 (0.15)	* $p = 0.35$

Comparison of biological variables of copper metabolism, oxidative stress and CSF markers of AD between AD patients and controls. AD patients show evidence of disease markers in the CSF as well as a copper and oxidative stress dysfunction.

*Number of AD patients = 24; number of controls = 20.

Table 2. Results from Different Studies on Serum Copper in AD Appear Controversial, but Values of 'Free' Copper are Coherent Across Studies

Published literature	'Free' copper in AD patients	'Free' copper in controls	Comparison between 'free' copper in AD patients and controls
Serum or plasma copper is higher in AD patients than controls	In the AD group of a study of ours ¹ , having a mean value of serum total copper concentration of 16.1 $\mu\text{mol/L}$ and a serum ceruloplasmin concentration of 28.4 mg/dL, the bound copper concentration resulted $28.4 * 10 * 0.0472 = 13.04 \mu\text{mol/L}$ and the 'free' copper concentration resulted $16.1 - 13.04 = 2.6 \mu\text{mol/L}$ In additional studies of ours, carried out on different panels of AD patients, we estimated 'free' copper values of 2.1 $\mu\text{mol/L}$ ² , 3.00 $\mu\text{mol/L}$ ³ and 3.04 $\mu\text{mol/L}$ ⁴	In the controls included in the same study ¹ , having a mean value of serum total copper concentration of 13 $\mu\text{mol/L}$ and a serum ceruloplasmin concentration of 26.8 mg/dL, the bound copper concentration resulted $26.8 * 10 * 0.0472 = 12.6 \mu\text{mol/L}$ and the 'free' copper concentration resulted $13 - 12.6 = 0.4 \mu\text{mol/L}$ In the different panels of controls evaluated for 'free' copper concentrations in the corresponding study reported in column 1, we found values of 'free' copper of 0.2 $\mu\text{mol/L}$ ² 1.2 $\mu\text{mol/L}$ ³ and 0.7 $\mu\text{mol/L}$ ⁴	In all our studies ^{1,2,3,4} , total and 'free' serum copper were higher in AD patients than in controls. Values for controls are in the range of the healthy population ($< 1.6 \mu\text{mol/L}$) ⁵
Serum or plasma copper is the same in AD patients and controls	In the AD patients studied by Sedighi and colleagues ⁶ , having a mean value of serum total copper concentration of 137.8 $\mu\text{g/dL}$, corresponding to 21.7 $\mu\text{mol/L}$, and a serum ceruloplasmin concentration of 27.7 mg/dL, the bound copper concentration was $27.7 * 10 * 0.0472 = 13.07 \mu\text{mol/L}$ and the 'free' copper concentration was $21.7 - 13.07 = 8.6 \mu\text{mol/L}$	In the controls studied in the same study ⁶ , having a mean value of serum total copper concentration of 132.5 $\mu\text{g/dL}$, corresponding to 20.8 $\mu\text{mol/L}$, and a serum ceruloplasmin concentration of 31.1 mg/dL the bound copper concentration was $31.1 * 10 * 0.0472 = 14.68 \mu\text{mol/L}$ and the 'free' copper concentration = $20.8 - 14.7 = 6.1 \mu\text{mol/L}$	Even though in this study ⁶ total copper and ceruloplasmin did not differ between AD and controls, 'free' copper is 1.4 times higher in AD than in controls. Moreover, 'free' copper is consistently higher than normative values.

(Table 2) contd....

Published literature	'Free' copper in AD patients	'Free' copper in controls	Comparison between 'free' copper in AD patients and controls
	The AD patients studied by Snaedel and colleagues ⁷ had a mean value of serum total copper concentration of 19.1 μmol/L and a serum ceruloplasmin detected by enzymatic activity of the protein of 89 D/ml.	The controls studied in the same study ⁷ had a mean value of serum total copper concentration of 19.4 μmol/L and a serum ceruloplasmin detected by the same method of 136 D/ml.	In this study ⁷ , total copper did not differ between AD and controls, contrary to ceruloplasmin. The usual expression of ceruloplasmin values in D/ml prevents the calculation of 'free' copper. However, it is possible to calculate the ratio Cu/Cp, which is 0.21 in AD and 0.14 in controls. The Cu/Cp ratio is 1.5 times higher in AD patients than in controls.
Serum or plasma copper is lower in AD patients than controls	In AD patients with evidence of Aβ and Tau proteins markers in their CSF studied by Kessler and colleagues ⁸ and having a mean value of serum total copper concentration of 100.25 μg/dL, corresponding to 16 μmol/L, and a serum ceruloplasmin concentration of 25 mg/dL, the bound copper concentration was $25 \times 10 \times 0.0472 = 12 \mu\text{mol/L}$ and the 'free' copper concentration was $16 - 12 = 4 \mu\text{mol/L}$	The authors of this study ⁸ used a control group of AD patients with no evidence of alteration of Aβ and Tau proteins in the CSF. For this control group having a mean value of serum total copper concentration of 115 μg/dL, corresponding to 18 μmol/L, and a serum ceruloplasmin concentration of 29 mg/dL, the bound copper concentration was $29 \times 10 \times 0.0472 = 14 \mu\text{mol/L}$ and the 'free' copper concentration was $18 - 14 = 4 \mu\text{mol/L}$	Even though AD patients with no evidence of AD markers in their CSF have higher total serum copper than patients showing AD markers in their CSF ⁸ , both AD groups have the same higher-than-normal level of 'free' copper.

The table shows the controversial data from the literature about 'free' copper in AD. Studies are classified on the basis of the role of total copper in the disease (column 1). Data of total copper and ceruloplasmin and calculations of 'free' copper are reported in column 2 for AD patients and in column 3 for controls. Comparisons between AD and controls are in column 4. Calculation of 'free' copper is based on standard procedures⁵ based on the notion that ceruloplasmin contains 0.3% of copper. Briefly: ceruloplasmin bound copper (CB) = ceruloplasmin (mg/dL) * 10* n; n = 0.0472 (μmol/mg); 'free' copper = total serum copper – CB⁵.

¹Ref [45] in the text; ²Ref [47] in the text; ³Ref [58] in the text; ⁴Ref [57] in the text; ⁵Ref [60] in the text; ⁶Ref [66] in the text; ⁷Ref [63] in the text; ⁸Ref [65] in the text

higher concentration of 'free' copper in AD than in controls; Kessler and colleagues [65] reported data coherent with a 2.5 fold increase of 'free' copper in AD with respect to normal values (< 1.6 μmol/L [60]); Sedighi *et al.* [66] found a 40% increase of 'free' copper in AD patients with respect to controls versus similar levels of copper and ceruloplasmin. Table 2 reports calculations aimed at clarifying this point.

The copper condition in the AD serum resembles the one observed in Wilson's disease, where a micronutrient-associated copper fraction exists, independent of ceruloplasmin. It must be noted, though, that in Wilson's disease this fraction is much higher than we estimated in AD.

Ceruloplasmin is routinely measured in clinical chemistry as an immunoreactive protein, comprising both the active holo-form and the inactive and copper-free apo-form. In a dedicated study, we have recently shown that a conspicuous amount of apo-ceruloplasmin is present in the CSF of AD patients [23]. We have also reported the presence of low-molecular-weight fragments (<50 KDa) of ceruloplasmin in AD samples from patients with higher-than-normal levels of 'free' copper [67], which suggests an impairment in the incorporation of copper into the protein during the biosynthesis (see the "copper chaperone" section). Ceruloplasmin fragmentation, together with the 'free' copper rise in AD, could be the cause or the result of a disturbed hepatocyte function,

possibly resulting in liver hypo-metabolism. This notion is supported by the results of a previous clinical study of ours which demonstrated that AD patients with no evidence of additional pathological conditions – including liver diseases – have higher 'free' copper, longer prothrombin time and lower albumin levels than controls matched for age, sex and risk factors for cardiovascular diseases and medication intake [45]. The hypothesis that copper in AD might be related to liver hypofunction linked to APP metabolism at the hepatocyte level is strongly supported by the evidence that, in the APP knock-out mouse model, the APP ablation causes a massive (80%) copper increase in the liver [14]. More precisely, it could be speculated that, in this mouse model, a perturbation of copper efflux linked to APP at the liver level disrupts normal copper transport producing a reduction of the liver's efficiency to excrete copper through the bile. This would explain the elevated (40%) copper level found in the brain [14]. A hint towards this interpretation comes from another study of ours, in which we estimated - via a clearance measure – that about 3% of serum 'free' copper crosses the BBB in AD patients and possibly interacts with Aβ [58]. This evidence fits very well with the results in a mouse model of brain uptake of radiocopper [⁶⁷Cu(II)] [4]. In this model, a net brain copper uptake occurs and parallels the 'free' copper increase in the injectate, starting from a con-

centration of Cu(II) of 3.2 ng/ml, corresponding to 0.05 $\mu\text{mol/L}$, much lower than the 2.5 $\mu\text{mol/L}$ value estimated in AD. The belief that there is a direct interaction between copper and A β , based on the strong negative correlation between 'free' copper in serum and A β in the CSF [58], is sustained by the recently demonstrated negative correlation between copper and A β in the CSF [68].

Our interpretation of the etiopathology of AD, restricted to patients carrying the 'higher-than-normal free copper' phenotype of the disease (about 65%), is illustrated in Fig. (1): copper is metabolically finely regulated by the organism, and this is why even a small increase of the serum low-molecular-weight component can be significant, particularly over a long period of time. In fact, when bound to low-molecular-weight compounds, copper can be easily exchanged among albumin and micronutrients, such as peptides or amino acids. This makes copper potentially more toxic and promotes oxidative stress, a characteristic made even more significant by the fact that these compounds can easily cross the BBB, exacerbating A β toxicity to the brain [34, 69, 70].

So far, our findings have a statistical connotation, even though we started to collect also hard data [23, 58, 67]; the next step will be to investigate 'free' copper levels in AD sera with a solid biochemical approach. Of course, one must keep in mind that association does not prove cause and effect and this is the reason why there is still a significant controversy over whether an excess of copper is involved in the pathogenesis of AD. Data suggesting otherwise include animal and human studies: for example, a model analysing the mutation ATPase7b transporter of the toxic-milk (txJ) mice - favoring elevated Cu levels - in combination with the transgenic (Tg) CRND8 APP - exhibiting robust A β deposition - demonstrated a reduction of A β in the brain [71]. Coherently, supplementation of copper lowered A β production and increased longevity [72]. Also, some clinical AD studies have shown cognitive decline correlating positively with low plasma levels of copper [65, 73], although these studies raised some concerns for not including healthy controls and AD patients with higher serum copper concentrations in the analysis [73]. In 2005, Bayer and colleagues [73; http://www.alzheimer-bayer.de/alzh_st1.html] started a phase II placebo-controlled double-blind therapeutic trial on mild to moderate AD patients, who received 8 mg Cu/day as an add-on therapy for 12 months. Preliminary results indicate no effects of the treatment on the patients' neuropsychological parameters.

Data proving that copper supplementation has no efficacy in slowing AD progression [http://www.alzheimer-bayer.de/alzh_st1.html], together with the very encouraging results obtained with PBT-2, a new therapeutic metal protein attenuating compounds (MPAC) of the iodochlorhydroxyquin (clioquinol, PBT1) drug class [74, 75], should contribute to resolve the confrontation between those who believe in a toxic effect and those who believe in a beneficial effect of copper. Some research groups - and ours among them - are starting new clinical trials based on the concept that a metal attenuation approach could have such a beneficial effect on AD progression.

In the following paragraphs we will discuss our choices of drug type, dosage and administration, as well as inclusion and exclusion criteria in our clinical trial after a brief sum-

mary of the evidence collected in previous pilot clinical trials based on the same concepts.

METAL PROTEIN ATTENUATING COMPOUNDS (MPAC) AS A THERAPEUTIC STRATEGY FOR THE TREATMENT OF ALZHEIMER'S DISEASE

Desferrioxamine

A clinical trial of the iron chelator desferrioxamine - a MPAC - given for two years to AD patients slowed the clinical progression of dementia [76]. The target metal was aluminum and iron was administered together with desferrioxamine throughout the whole trial period, with the aim of attenuating the bioavailability of aluminum, but not of iron. Thus, direct effects of a desferrioxamine treatment on the metabolism of metals other than aluminum and iron, as for example copper, cannot be ruled out.

Dementia was measured by a video recorder home-behavioral assessment, which had three parts and measured 44 tasks, mostly samples of daily living. The statistically better performance of the treated group versus the untreated one means that tasks of daily living were significantly better preserved in the treated group. A major point of the study was the two-year duration of the drug administration period. However, the study had some limitations, as for example the fact that patients were not blind to the treatment, since desferrioxamine was injected in the active drug arm while the placebo was administered orally. Moreover, patients endured many adverse events.

D-Penicillamine

In a pilot study we started a clinical trial with D-penicillamine [59, 77]. D-penicillamine is a well-established MPAC used for treatment of Wilson's disease and functions as a copper chelator, controlling both the reactivity and bioavailability of that metal, ultimately facilitating its disposal through the urine. The study was interrupted for ethical reasons before finishing recruitment, due to adverse events, including the death from cardiac arrest of one patient under treatment [77]. Although a causal relationship between the active drug and the patient's death could not be proven, we stopped the trial on the basis of a literature review listing death as a potential, though extremely rare, side-effect of D-penicillamine. At that point, 34 patients had completed the 24-week treatment period. Their levels of serum peroxides, representing the extent of oxidative stress, had decreased with respect to both their own levels before starting the treatment and the levels of the AD patients who had taken the placebo [77]. Furthermore, erythrocytes from patients treated with D-penicillamine revealed a decrease in the activity of Cu/Zn SOD with respect to the level found in untreated AD patients [59]. The level of Cu/Zn SOD in the D-penicillamine-treated patients reached values even lower than those of healthy controls [59]. These results suggest that Cu/Zn SOD is a reliable sensor of bioavailable copper and that the measurement of its activity may represent a sensitive test to monitor pharmacological trials based on copper attenuation, aimed at modulating drug dosage and length of treatment, and prevent undesired adverse events. This trial had numerous limitations, such as 1) the choice of the drug - known to produce severe adverse events including a wors-

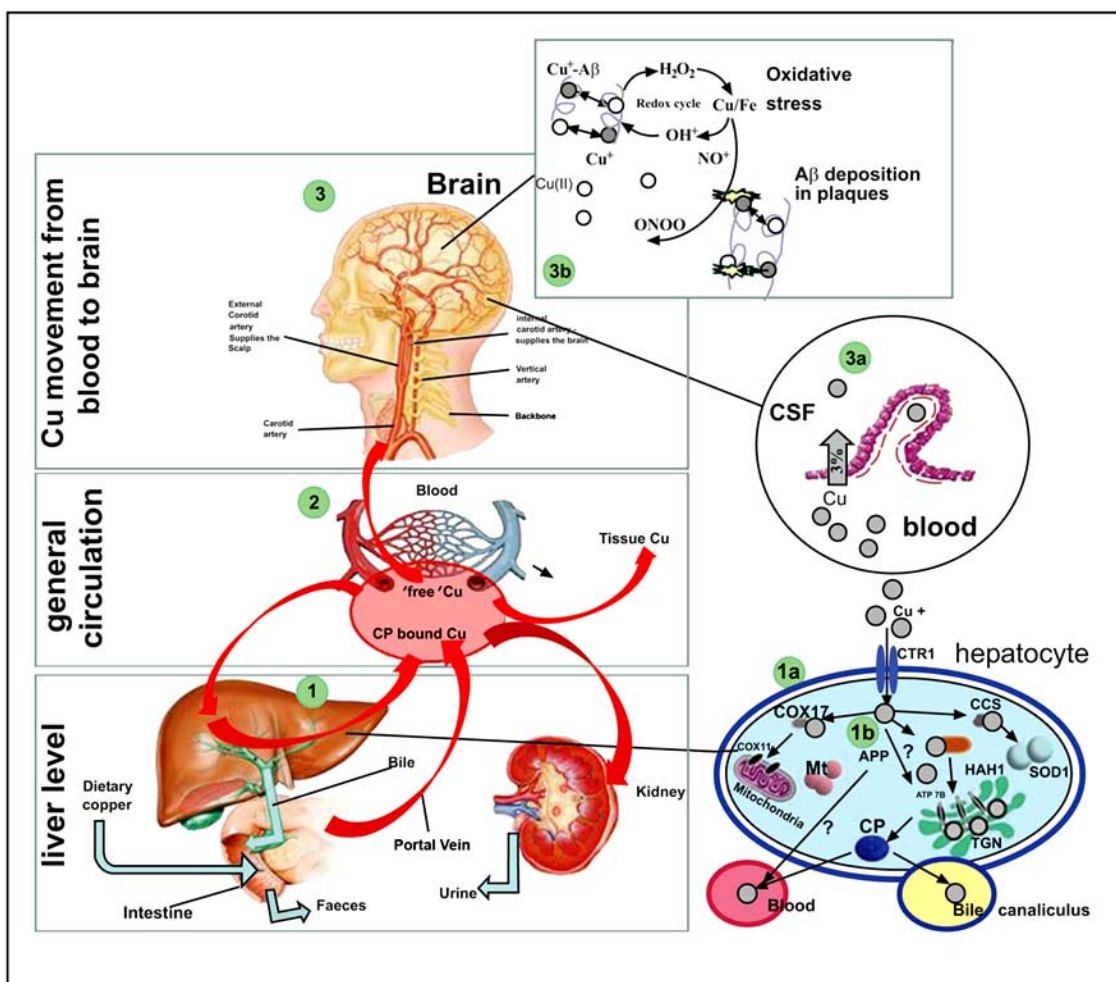


Fig. (1). 1. Liver level. Copper metabolism in normal condition: Copper is metabolically finely regulated by the organism. Once copper crosses the intestinal lumen, it is transported into the liver via portal circulation. In the hepatocytes copper is incorporated into ceruloplasmin, other copper proteins and compounds and then routed into peripheral circulation or secreted into the bile for excretion. Copper in excess from general circulation is excreted through the kidney, in urine.

1a. In the hepatocytes, intracellular copper is carried by specific chaperone proteins to Cu-dependent enzymes. Three copper chaperones have been identified to date: the Human Atox 1 Homologue (HAH1), homologous to the yeast Antioxidant protein 1 (Atox1); the Copper Chaperone for Superoxide Dismutase (CCS); the Cytochrome C Oxidase assembly homologue (COX17). The amyloid precursor protein (APP) is deemed to be a new copper chaperone.

1b. Proposed model for 'free' copper implication in AD. The evidence in support of a role of APP in copper homeostasis implies a function of APP in copper efflux/detoxification at the plasma membrane of neuronal cells [14-18]. Alternatively, one can consider a model of APP perturbation of copper efflux from the hepatocytes linked to copper translocation to the secretory pathway, resembling what happens in the case of ATP7B absence or impairment. In this case, copper translocation to the secretory pathway is prevented, resulting in i) secretion of unstable apo-ceruloplasmin which is rapidly degraded in the blood [5]; ii) altered excretion of copper via the bile through ceruloplasmin [26]; iii) hypofunction causing even death of hepatocytes; iv) release of 'free' copper in general circulation and tissue copper overload or intoxication [5]. This model is based on the evidence that, in the APP knock-out mouse model, APP ablation produced an 80% copper increase in the liver, which would explain the 40% copper increase found in the mouse brain [14]. In line with this model, we detected a conspicuous amount of low-molecular-weight (<50 KDa) fragments of apo-ceruloplasmin [67], longer prothrombin time, lower albumin concentrations and higher-than-normal 'free' copper levels (normal values 1.6 μmol/L [60]) [45] in the serum of AD patients. This suggests that an impairment in the incorporation of copper into the protein probably occurs in AD during ceruloplasmin biosynthesis.

2. General circulation. This model proposes that an excess of 'free' copper [mean (SD) = 2.6 (4.6) μmol/L] [58] can enter the blood bound to low-molecular-weight micronutrients and can be transported to tissues, reaching the brain through the BBB [4, 5, 58].

3. Movement of copper from blood to brain. We hypothesize that a net brain copper uptake occurs and parallels the increase of the 'free' copper, as it has been demonstrated by injecting ⁶⁷Cu (II) in a mouse model of brain copper uptake [4].

3a. Model of movement of copper from blood to brain in AD patients. It was calculated that in AD patients a unit μM increase of 'free' copper in the serum produces an expected 0.03 μmol/L increase of copper in the CSF (linear regression model, t = 2.2, p = 0.04) [58].

3b. Free copper implication in oxidative stress and Aβ aggregation. Our model proposes that over a long time course, 'free' copper can feed the brain with a continuous flux of noxious redox copper, playing a role in Aβ-mediated toxicity [34, 58, 69, 70].

ened evolution of the neurologic symptoms in line with what is reported in Wilson's disease [77]; 2) The very short duration of the drug administration period. Indeed, no cognitive decline was observed in the placebo group in the 24 weeks follow-up period, thus precluding any comparison with the D-penicillamine-treated patients; 3) The lack of patients inclusion criteria based on evidence of individual serum copper dysfunction. 4) The lack of markers evaluation to monitor copper bioavailability, as for example through ceruloplasmin or Cu/Zn SOD activity, that could have possibly prevented the severe adverse events that occurred.

Clioquinol (PBT1)

The clinical trial carried out by Ritchie and colleagues [78] with the 8-hydroxyquinoline derivative clioquinol (CQ) MPAC was preceded by a salient study demonstrating that the brain amyloid plaque burden of Tg2576 mice decreased by 49% when the mice were treated orally with this metal attenuating compound [79]. CQ is capable of crossing the BBB, and it is believed that it solubilizes A β plaques by stripping them of their metal content. The trial demonstrated positive clinical effects on the cognitive loss of severe AD patients [78], supporting the *in vitro* work previously reported [79], in line with the notion that an excess of extracellular metals contribute to the amyloid pathology of AD. However, there may be also an explanation different than metal attenuation for these positive results. In fact, in a recent study relative to the soluble A β burden of the AD brain, a mechanism was described by which a decreased intracellular metal bioavailability may contribute to the accumulation of soluble A β outside the cell: White *et al.* [80] treated Chinese Hamster Ovary cells over-expressing human APP (CHO-APP) with CQ complexed to Cu or Zn; they found that the Metal-CQ treatment decreased the levels of extracellular A β not by preventing an A β -metal interaction outside the cell, but by facilitating the delivery of metals into the cell. Once inside the cell, Cu and Zn, but not Fe, activated the phosphoinositol 3-kinase mediated protein kinase pathways, which ultimately led to an increase in the secretion of matrix metalloproteinases (MMPs), which can degrade A β [80]. Therefore, treatment with CQ may have a two-fold effect on A β : it may prevent metal mediated A β aggregation and toxicity by binding extracellular metals and then, by delivering the bound metals into the cell, it may activate specific protein kinases inducing an increase in the production of A β -degrading MMPs.

Further phase II/III studies were stalled by difficulties in preventing di-iodo 8-hydroxy quinoline contamination upon large scale chemical synthesis.

PBT-2

PBT-2 is a second-generation compound of the iodochlorhydroxyquin (clioquinol, PBT1) drug class - but lacks iodine - that has replaced CQ as a therapeutic MPAC for treatment of AD. Recently it has been tested in a phase IIa, double-blind, randomised, placebo-controlled 12 weeks trial. In the 74 AD patients who completed the trial, 250 mg of PBT-2 were well tolerated and induced a significant, dose-dependent reduction of CSF A β concentrations. Moreover, PBT-2 was found to preserve two executive functions as

evaluated via category fluency test and trail making part B. This MPAC promises to achieve cognitive improvements for AD patients in larger and longer studies [74, 75].

Bis(thiosemicarbazonato) Complexes

Recently the ability of metal bis(thiosemicarbazonato) complexes - MII(btsc), where M stands for either CuII or ZnII - to affect extracellular levels of A β was examined in Chinese hamster ovary cells overexpressing APP. Treatment with engineered MII(btsc) increased levels of bioavailable intracellular copper and zinc but also resulted in a dose-dependent reduction of A β levels, demonstrating that MII(btsc) complexes have potential in AD therapy [81].

Ammonium Tetrathiomolybdate

A new potentially beneficial agent for Wilson's disease, the copper-lowering agent ammonium tetrathiomolybdate (TM), has been developed in a recent series of *in vitro* and clinical studies [19]. Ammonium tetrathiomolybdate is a MPAC that forms a stable tripartite complex with copper and proteins [19]. Given with food, TM complexes food copper with food proteins, making all copper, including the endogenously secreted copper in saliva, gastric juice and intestinal secretion, completely unabsorbable and thus causing an immediate negative copper unbalance. Given instead separate from food, TM is absorbed into the blood, where it forms the tripartite complex, bridging the freely available, and potentially toxic, copper with albumin. This complexed copper cannot be taken up by cells, and is therefore non-toxic [19], it has no known biological activity and is largely cleared in the bile.

Phase II and III clinical trials in Wilson's disease suggest that TM could be ideal for the initial therapy of the neurologically presenting patient. Ammonium tetrathiomolybdate, when given in a dose of 120 mg/day in six separate doses with and in-between meals, has shown, in about eight weeks, excellent efficacy in protecting the neurologic function of patients presenting neurological complications [19].

Ammonium tetrathiomolybdate is capable of safely producing a greater degree of copper depletion than other drugs as long as ceruloplasmin levels are monitored and kept within an intermediate range. At this level of copper depletion, copper-dependent enzymes are not affected. This drug was chosen by our group, in collaboration with Prof Brewer's group, to carry out a Phase II clinical trial aimed at evaluating the safety and efficacy of a TM dose adequate to maintain serum ceruloplasmin between 10 and 18 mg/dL, for therapy of AD patients in treatment with acetylcholinesterase inhibitors. The study population is composed of 150 mild to moderate AD patients (16-24 MMSE score), and the design is multicentered, prospective, randomized, double-blind, parallel, placebo-controlled. Duration is 52 weeks, in two phases (ceruloplasmin induction phase, ceruloplasmin maintenance phase). The principal outcome measure is ADAS-cog, and the secondary one are ADCS-CGIC, DAD, NPI, CDR, GDS, MMSE, and RUD-lite. N-acetylaspartate, myoinositol and creatine concentrations in the brain are to be evaluated with Magnetic Resonance Spectroscopy, while hippocampus volume with Magnetic Resonance Imaging.

CONCLUSIONS

Clues to a direct role of copper in the pathogenesis of AD can be detected in a number of *in vitro*, *in vivo* and clinical trials conducted during the past 20 years. Several strategies to inhibit A β production have been explored and recently MPACs have been reported to slow the rate of cognitive decline in AD patients. These trials provide preliminary and encouraging results indicating that MPACs can modify the natural history of AD for the better. It appears that the fundamental points to take into account in a well designed clinical trial are the following: a) treatment duration of at least one full year, as it is expected with curative compounds, rather than symptomatic approaches; b) a patient inclusion criterion based on evidence of individual serum copper dysfunction; c) monitoring of the bioavailability of copper throughout the study with detection of markers of copper metabolism such as ceruloplasmin or Cu/Zn SOD levels, to prevent adverse events.

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