

Metals in alzheimer's disease: a systemic perspective

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1. ABSTRACT

Many results from *in vitro* and animal studies have highlighted the important role played by specific metals, such as copper, iron and zinc, in the diverse toxic pathways on which Alzheimer's disease (AD) develops. Metals seem to mediate the aggregation and neurotoxicity of amyloid-beta (ABeta), the main constituent of the amyloid plaques, commonly seen in AD (1). The link between metals and AD has been mostly investigated with a focus on their local accumulation in defined areas of the brain critical for AD. In the present review, I have instead approached the issue from the different perspective of a systemic, rather than local, alteration of copper and iron status. This view is supported by the results of a series of *in vivo* studies demonstrating that abnormalities of metals homeostasis correlate with the main deficits and specific markers of AD, such as ABeta and Tau proteins in the cerebrospinal fluid. These findings clearly suggest that local metals accumulation in brain areas critical for AD should be viewed within a wider framework of metals systemic alteration.

2. INTRODUCTION

There is a general agreement on the existence of a link between Alzheimer's disease (AD) and oxidative stress phenomena triggered by transition metals (2). However, these phenomena have been traditionally viewed as originating locally within the brain, and autonomously, i.e., independently of systemic influences. More recently, a wider and somewhat complementary view has emerged suggesting a relationship between AD and systemic changes of metal metabolism, rather than local and autonomous alterations. I have based my presentation on this view. After reviewing the evidence of metals involvement in AD, I will present results clearly linking the major characteristics of the disease to systemic variations of markers of copper and iron metabolism, such as levels of serum copper, iron, ceruloplasmin, ferritin, transferrin and transferrin saturation, as well as effects of the ceruloplasmin-transferrin (Cp-Tf) system, transferrin C2 (TF-C2) and hemochromatosis (HFE) genes mutations.

3. COPPER HOMEOSTASIS

Copper, though an essential nutrient for humans, is potentially toxic when its levels in the body are disarranged. As a transition metal, it takes part in a variety of biological reduction and oxidation (redox) reactions, 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.

3.1. Copper Essentiality and Toxicity

Humans ingest copper via food, although it has been recently discovered that varying degrees of intake can originate from drinking water piped through copper plumbing (3). Paradoxically, despite the general possibility of a widespread copper deficiency, some regulatory agencies, such as for example the Californian Environmental Protection Agency, are now concerned about potential local copper overexposures (3).

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 generated by different genetic and/or environmental factors (4), 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, contrary to pigs which instead tolerate copper 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 (4).

The genetic origin of copper toxicosis manifests 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 for example a liver cirrhosis. However, toxicity always stems from the ability of copper to catalyze the production of compounds that generate oxidative stress (4).

3.2. 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 85-95% of copper tightly binds to ceruloplasmin, whereas the remainder loosely binds to and is exchanged among

albumin, alfa2 macroglobulin, amino acids, peptides and several micronutrients. I will refer to the portion that binds to ceruloplasmin as 'bound' copper, while to the portion that binds to the loose compounds as 'free' copper (5). 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) (6). A recent study confirmed the evidence that the copper transport into the brain is mainly achieved through the BBB as free copper ion, and the blood-cerebrospinal fluid barrier may serve as a main regulatory site of copper in the CSF (7).

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 (8). Ceruloplasmin is a ferroxidase. It 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 H₂O₂, 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 (9). Ceruloplasmin represents the first of several proteins of connection linking copper to iron metabolism, which appears disarranged in AD.

3.3. Copper chaperones

Copper import into intestinal epithelial cells is mediated by Ctr1 (Figure 1), an integral membrane protein conserved from yeast to humans (10). Based on its structural and biochemical properties, Ctr1 could be thought of as a Cu⁺-specific pore (10). In hepatocytes, intracellular copper is carried by specific chaperone proteins to Cu-dependent enzymes (10).. 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 (Figure 1). Chaperones are not present in plasma (10). Chaperones are the main defense system against copper excess. This system also includes metallothioneins, a family of proteins, whose expression is regulated by copper and other metals (11) 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 AD (12-14). It was recently proven that this protein protects cultured

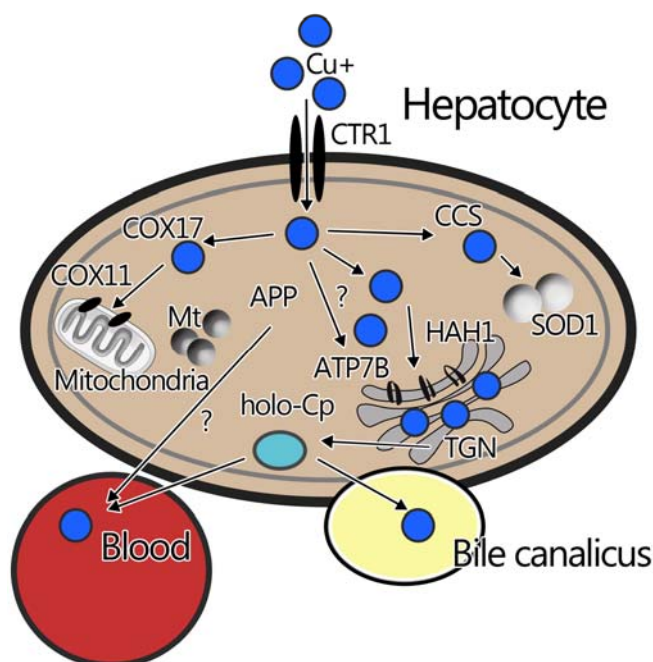


Figure 1. Copper is metabolically finely regulated by the organism. Once copper crosses the intestinal lumen, it is transported into the liver via portal circulation. In the hepatocytes copper is incorporated into ceruloplasmin, other copper proteins and compounds and then routed into peripheral circulation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

neurons from toxicity generated by ABeta 39-42 amino acid peptides whose amyloid fibrillar form is the primary component of the amyloid plaques. A metal swap between Metallothionein-3 and soluble aggregated ABeta 1-40-Cu(II) avoids ROS production and consequent cellular toxicity. Cu/Zn superoxide dismutase, beside its primary antioxidant function of superoxide dismutation, also plays a role as a buffer of intracellular free copper, since it is stable in its copper-free form (15).

Recently, the amyloid precursor protein (APP) - the precursor of the ABeta peptide - has been shown to be implicated in copper as well as iron metabolism (see below). APP is an integral membrane protein expressed in many tissues (platelets, liver, heart and kidney) and concentrated in neuron synapses which can undergo two diverse cleavage pathways, one non- amyloidogenic and one amyloidogenic, resulting in ABeta peptide production. Its primary function is still unknown, but it has been proven to be a regulator of synapse formation, to be implicated in neuronal plasticity (16, 17) and in copper as well as iron homeostasis (18, 19). APP binds copper in two domains, one located in the extracellular N-terminal region and the other in the C-terminal region within the ABeta peptide (20). 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 (21, 22). APP knockout mice have shown increased levels of copper in the liver and in the cerebral cortex (19).

3.4. 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 $\mu\text{g/dL}$) (23). 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 (24). In fact, ranges of plasma/serum copper values of healthy elderly individuals reported in the literature are very heterogeneous, arising complexity in the interpretation of their results. This statement appears immediately true when looking at the data recently collected by us in a meta-analysis which evaluated all the studies carried out to investigate serum/plasma copper results of AD and healthy cohorts since 1983 (Table 1) (25). Table 1 shows the high heterogeneity of the value of serum copper reported in healthy subjects in the 21 studies included in the meta-

Table 1. High heterogeneity of the values of serum copper reported for healthy subjects in all the studies carried out since 1983 till today

study	Serum copper (mean, $\mu\text{mol/L}$)	Serum copper (SD, $\mu\text{mol/L}$)	Upper limit 95% ($\mu\text{mol/L}$)	% above 24 $\mu\text{mol/L}$	% above 24.4 $\mu\text{mol/L}$
Vural <i>et al.</i> , 2010 (27)	22.50	2.80	27.11	29.61%	24.87%
Jeandel <i>et al.</i> , 1989 (89)	21.24	4.10	27.98	25.04%	22.04%
Agarwall <i>et al.</i> , 2008 (78)	21.15	4.90	29.21	28.04%	25.36%
Sedighi <i>et al.</i> , 2006 (93)	20.80	2.50	24.91	10.03%	7.49%
Mattiello <i>et al.</i> , 2003 (91)	18.80	0.16	19.06	0.00%	0.00%
Brewer <i>et al.</i> , 2010 (95)	18.40	3.10	23.50	3.54%	2.65%
Basun <i>et al.</i> , 1991 (86)	16.70	3.00	21.64	0.75%	0.51%
Smorgon <i>et al.</i> , 2004 (82)	16.66	1.27	18.75	0.00%	0.00%
Kapaki <i>et al.</i> , 1989 (90)	16.20	2.20	19.82	0.02%	0.01%
Sevym <i>et al.</i> , 2007 (81)	15.40	2.10	18.85	0.00%	0.00%
Gonzales <i>et al.</i> , 1999 (61)	15.37	2.58	19.61	0.04%	0.02%
Baum <i>et al.</i> , 2010 (87)	15.30	2.70	19.74	0.06%	0.04%
Molina <i>et al.</i> , 1998 (92)	14.47	4.10	21.21	1.01%	0.77%
Bocca <i>et al.</i> , 2005 (80)	14.31	3.10	19.41	0.09%	0.06%
Squitti <i>et al.</i> , 2002 (62)	13.70	2.60	17.98	0.00%	0.00%
Squitti <i>et al.</i> , 2007 (63)	13.00	2.80	17.61	0.00%	0.00%
Zappasodi <i>et al.</i> , 2008 (65)	12.90	3.00	17.84	0.01%	0.01%
Squitti <i>et al.</i> , 2006 (83)	12.80	2.30	16.58	0.00%	0.00%
Arnal <i>et al.</i> , 2009 (79)	12.74	0.20	13.07	0.00%	0.00%
Squitti <i>et al.</i> , 2005 (100)	12.60	2.50	16.71	0.00%	0.00%
Ozcankaya <i>et al.</i> , 2002 (26)	12.11	1.50	14.58	0.00%	0.00%
Average	16.05	2.55	20.25	0.09%	0.05%

The Table shows here the percentage of the healthy subjects having copper values above the upper limit of the normal reference interval (specificity = 95%) for each study. This can be calculated on the basis of the mean and standard deviation (SD) of the specific study, assuming that data have a Gaussian distribution. The studies reported in Table 1 are ordered by decreasing serum copper mean values. Taking 24 $\mu\text{mol/L}$ for men and 24.4 $\mu\text{mol/L}$ for women as the upper reference values of healthy population (23), we calculated (assuming gaussianity) the percentage of healthy subjects with levels of serum copper higher than the upper limit of the reference range. This analysis revealed that the heterogeneity of the results is very high. Even when the studies were considered as a whole (last line of the Table; average of the means of all the studies = 16.05; % of subjects above the upper value of 24.4 = 0.05) the result of 0.05% is very far from the expected Gaussian 5%.

analysis ranging from [mean (SD) value 12.11 (1.5) $\mu\text{mol/L}$ in (26) to 22.50 (2.8) $\mu\text{mol/L}$ in (27)]. If on the one hand this result indicates the need for each laboratory to set up a control group to be compared with a pathological sample, on the other hand, it suggests that efforts should be made to reduce such heterogeneity and to obtain more comparable reference intervals.

When out of its normal ranges, copper easily catalyses Haber-Weiss and Fenton reactions producing $\cdot\text{OH}$, against which the body has no defenses (28). In general circulation, free copper levels are very low and it is exchanged among albumin, α_2 macroglobulin, peptides or amino acids, whereas bound copper is not easily released by ceruloplasmin and virtually does not cross the BBB (7, 8). It has been calculated that ceruloplasmin-bound copper represents less than 1% of the brain's bound copper (29), and we have recently reported the presence of a conspicuous amount of apo-ceruloplasmin in the CSF of AD patients (29), indicating an higher percentage of CSF free copper in AD patients than in controls. In cells of all types, a considerable amount of copper is stored in metallothionein (12-14).

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 shortage - Menkes' disease - or excess - Wilson's

disease (WD) - 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 (30). 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 (30). The location of ATP7A at the choroid plexus makes this pump crucial for controlling the copper flux into the ventricles of the brain (31). 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 (32). This produces a copper overload into the hepatocytes, inducing liver cirrhosis as well as slight increased levels of free copper. At the death of the cirrhotic hepatocytes, copper - actually free copper - is released into general circulation in huge amounts and reaches all organs and tissues, including the brain (7, 32, 33). The holo-active form of ceruloplasmin depends on the ATP7B activity, which mediates the incorporation of copper atoms into ceruloplasmin during its biosynthesis (8). 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 (8); 2) alteration of copper excretion through the bile via ceruloplasmin (32); 3) severe hypofunction, which can even cause death, of hepatocytes; 4) release of free copper in general circulation and tissue copper overload or intoxication (8).

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 (34). No conclusive information on copper content in the human aging brain is available, although data obtained in mice speak in favour of an increase (35). 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 and an increase in the plaques (34, 36, 37), indicating that an alteration of the brain copper distribution occurs in this disease.

4. IRON HOMEOSTASIS

Iron is essential for life; human body contains around 4-5 grams of iron. About 2.5 g is contained in the hemoglobin and is needed for respiration. 3-4 mg circulates through the plasma, bound to transferrin. Most of the iron in the body is hoarded and recycled by the reticulo-endothelial system, which breaks down aged red blood cells.

4.1. Iron absorption and metabolism

The majority of the iron absorbed from digested food or supplements (1 mg a day for men, and 1.5–2 mg a day for women) is absorbed in the duodenum by enterocytes of the duodenal lining in its ferrous Fe^{2+} form as part of heme protein. Since Fe^{2+} is the more easily absorbed form of iron in food, expression and function of Dcytb on the enterocyte - which reduces ferric Fe^{3+} to Fe^{2+} - is modulated by iron body demand. Divalent metal transporter 1 (DMT1) transports the iron (Fe^{2+}) across the enterocyte's cell membrane and into the cell. In the intestinal lining cells iron can be stored as Fe^{3+} in ferritin (4500 atoms of iron), which is a 450 kDa protein consisting of 24 subunits present in every cell type. Conversely, iron can move into the body, transported out of the cell as Fe^{2+} by ferroportin and distributed throughout the body as Fe^{3+} by transferrin, and in this form it reaches all cells which store iron. Ceruloplasmin - the main ferroxidase protein in the plasma - catalyzes the oxidation of Fe^{2+} (ferrous iron) into Fe^{3+} (ferric iron), therefore assisting in its transport in the plasma in association with transferrin, which can only carry iron in the ferric state. Hephaestin, a ferroxidase which can oxidize Fe^{2+} to Fe^{3+} and is found mainly in the small intestine, helps ferroportin transfer iron across the basolateral end of the intestinal cells. The role of hephaestin in enterocyte iron efflux is analogous to that of ceruloplasmin in the reticulo-endothelial system. In the reticulo-endothelial system (macrophage) iron derived from heme is returned to the plasma via the membrane transporter ferroportin. Ceruloplasmin plays an essential role in determining the rate of iron efflux via oxidation, which is required for binding Fe^{3+} to transferrin.

Additional proteins are involved in iron metabolism. Of note is the human hemochromatosis (HFE) protein, encoded by the homonymous gene (38), which has been recently linked to AD. HFE is a membrane protein which controls iron absorption by regulating the affinity of transferrin receptors on cell membranes. Specific mutations of the HFE gene cause hemochromatosis, i.e., an increased absorption of dietary iron and its consequent over-deposition in tissues and organs. Hemochromatosis is inherited as an autosomal recessive trait. Severe iron overload is typical of homozygous HFE mutations. However, minor modifications of serum iron, ferritin and transferrin saturation are also observed in a fraction of heterozygous cases (39). Despite their mild nature, these modifications of the iron status can influence the severity and clinical evolution of a variety of conditions, such as hepatitis, porphyria cutanea tarda and cardiovascular disease (39). A number of studies have shown various relationships between AD and mutations of the transferrin C2 variant and HFE genes, but reported data are still controversial and far from univocal interpretation.

4.2. From the intestine to the BBB

After absorption or recycling from the reticulo-endothelial system, iron reaches the vicinities of the brain transported by transferrin; in the brain's capillaries it crosses the BBB, made of Brain Capillary Endothelial Cells (BCECs) that form the wall of the capillaries. The luminal side of the BCECs presents transferrin receptors that pick up the iron-loaded transferrin protein. An endocytosis is initiated and the transferrin receptor ($\text{Rcpt}+\text{Tf}$ [Fe^{3+}] complex is internalized into an endosome. Iron remains inside the endosome while the latter crosses through the BCEC and reaches the abluminal side, where it fuses with the external membrane, exposing the $\text{Rcpt}+\text{Tf}$ [Fe^{3+}] complex to the extracellular interstitial space, where 2 Fe^{3+} atoms are released. The apo-transferrin remains instead attached to the Rcpt and the two undergo again an endocytosis to travel back to the BCEC luminal side, where apo-transferrin is released into the capillary blood for recycling (40). In the extracellular interstitial space, the astrocytic end-foot presents ceruloplasmin molecules on its surface. Thus, when iron, after traveling through the extracellular space, reaches the vicinity of a connection between an astrocytic end-foot and a neuron, ceruloplasmin catalyzes the oxidation of Fe^{2+} into Fe^{3+} . Two Fe^{3+} atoms bind to a passing transferrin molecule, which in turn is caught by a transferrin receptor on the neuron's surface. An endocytosis is initiated and the $\text{Rcpt}+\text{Tf}$ [Fe^{3+}] complex is engulfed in an endosome that sinks into the neuron (40).

5. ALZHEIMER'S DISEASE

AD is an irreversible, progressive neurodegenerative disorder, characterized by a gradual appearance of cognitive deficits accompanied by abnormal behavior 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.

5.1. Alzheimer's disease aetiology and neuropathology

AD can be schematically distinguished in a familial form with a mendelian inheritance (autosomal-dominant trait), which accounts for about 0.1% of the cases and usually have an onset before age 65, and a sporadic form, with a disease onset after 65 years of age, and an aetiology of complex disease, meaning that oligo-genes or poly-genes together with epigenetic or environmental factors contribute to the onset of the disease. Most of the familial AD cases can be attributed to mutations in one of three genes: APP, and presenilins 1 and 2. For the sporadic form, the best known genetic risk factor is the inheritance of the epsilon4 allele of the apolipoprotein E (APOE). The APOE4 allele increases the risk of the disease by three times in heterozygosis and by 15 times in homozygosis (41, 42). Both familial and sporadic forms share the same neuropathology, which is characterized by diverse pathological alterations that 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 cycles. In a schematic representation, five primary functional and anatomical features characterize the AD brain: (a) loss of neurons and synapses in the cerebral cortex and certain subcortical regions, which results in gross atrophy of the affected regions, including degeneration in the temporal lobe and parietal lobe, and parts of the frontal cortex and cingulate gyrus (41); (b) the density and distribution of extracellular amyloid plaques, composed mainly of aggregated, insoluble ABeta peptide in the entorhinal cortex, hippocampus and the nucleus basalis Meynert; (c) the presence of intracellular neurofibrillary tangles containing hyper-phosphorylated Tau; (d) increased oxidative damage of lipids, proteins and nucleic acids via ROS; (e) loss of biometal homeostasis (42). The deposition of aggregated ABeta and the hyper-phosphorylation of Tau have been shown to cause neuronal damage and contribute substantially to AD pathology (43).

5.2. Alzheimer's disease diagnosis

AD is usually diagnosed clinically from the patient history, collateral history from relatives, assessment of intellectual functioning including memory testing which can further characterize the state of the disease and the absence of alternative conditions, evaluated generally by neuroimaging techniques. The National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA, now known as the Alzheimer's Association) established the most commonly used NINCDS-ADRDA Alzheimer's Criteria for diagnosis in 1984 (44), extensively updated in 2007 (45). These criteria require that the presence of

cognitive impairment, and a suspected dementia syndrome, be confirmed by neuropsychological testing for a clinical diagnosis of possible or probable AD. Positive ABeta and Tau values in CSF as well as neuromaging evaluation are supportive markers of the diagnosis.

6. METALS AND OXIDATIVE STRESS IMPLICATION IN ALZHEIMER'S DISEASE

The main aim of the present section is to give an overview of the milestone *in vitro* studies which are now considered the 'core' of evidence at the basis of metal involvement in AD, which are described in paragraph 6.1, as well as to present some of our studies in living patients demonstrating that AD patients suffer for metal homeostasis abnormalities as summarized in paragraphs 6.2 to 6.5.

6.1. Copper involvement in alzheimer's disease

The metallochemistry of AD has gradually developed in the mid '90s, with the observation that the APP possesses selective zinc and copper binding sequences. These sites appear to mediate redox activity and cause precipitation of ABeta under mildly acidic condition even at very low concentrations (46). Such events might therefore be also occurring in the brain affected by AD. In addition, Aβ possesses selective high and low-affinity metal-binding sites, binding equimolar amounts of copper and zinc. In conditions of acidosis, copper completely displaces zinc from Aβ. This metal-induced precipitation of Aβ is completely reversed by chelation (47, 48) as observed in post mortem AD brain samples. Apart from metal dependent aggregation, it is metals such as copper and iron that confer the Aβ peptide its redox activity: in fact, Aβ reduces the metal ions, producing hydrogen peroxide by transferring electrons to O₂ (49, 50). This reduction reaction seems to mediate Aβ-induced oxidative stress and toxicity. Hydrogen peroxide is in fact a prooxidant molecule, triggering Fenton's like reactions and generating hydroxyl radicals (Table 2).

There are numerous additional processes in which copper may take part and generate toxicity. ABeta is generated from APP cleavage by beta- and gamma-secretases. Beta-secretase (BACE1) cleaves at the N terminus, while gamma-secretase cleaves at the C terminus of the ABeta sequence. BACE1 is an aspartic protease and may function as a dimer, whereas gamma-secretase is a complex of presenilin-1/2, Aph1, Pen2, and Nicastrin (51). It was reported that BACE1 modulates APP processing and the release of ABeta by interacting with CCS. The concept that copper content can modulate BACE1, i.e. the rate-limiting enzyme in the production of ABeta (52), is supported by the 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 (53). *In vitro* studies have demonstrated that different variants of ABeta (1-40, 1-42) have different affinity for copper, and that higher affinity generates higher toxicity (42). The results of *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-ABeta toxic effect (54).

Table 2. Milestones of the Evidence of Metal Implication in AD

Milestones	Key Findings
The amyloid precursor protein (APP) is a copper protein	APP has a copper binding domain which reduces Cu(II) to Cu(I) and produces oxidative stress (155, 156) Depletion of intracellular copper results in a reduction of APP gene expression (22)
Zinc and copper interactions with A β	Zinc rapidly destabilized human A β 40 solutions, inducing tinctorial amyloid formation (157) A β peptide with the sulfur atom of Met-35 oxidized to a sulfoxide is toxic to neuronal cells (158) A β peptide aggregation is induced by Cu binding (159) A β -Cu interaction generates ROS (50, 156) APP-copper induced toxicity and oxidative stress in primary neuronal cultures, producing neuronal demise (49, 160)
Solubilization of native A β from AD brain, transgenic mice and cell models by metal complexing agents	Solubilization of A β from post-mortem brain tissue was significantly increased by the presence of chelators, EGTA, N,N,N*,N*-tetrakis(2-pyridyl-methyl) ethylene diamine, and bathocuproine (48) bis(thiosemicarbazono) complexes - MII(btsc) examined in chinese hamster ovary cells overexpressing APP increased levels of bioavailable intracellular copper and zinc but also resulted in a dose-dependent reduction of ABeta levels (136)
Cu-dependent catalytic conversion of dopamine, cholesterol, and biological reducing agents to neurotoxic H ₂ O ₂ <i>in vitro</i> and animal models	ABeta binds copper and cholesterol, facilitating copper oxidation of cholesterol to 7-OH cholesterol and to 4-cholestenone, which are extremely toxic to neurons. Cholesterol can catalyze copper-ABeta redox cycling (69, 72) Trace amounts of copper given to cholesterol-fed rabbits induced accumulation of ABeta in senile plaques and impaired the animals' learning capability (71, 73)

Copper plays a role also in the basal regulation of the APP gene. This has been shown in human fibroblasts over-expressing ATP7A, a condition that causes copper intracellular depletion. Depletion of intracellular copper in these cells results in significant reduction of APP gene expression (21, 22). Moreover, a study of cDNA microarray technology demonstrated an up-regulation of APP and of the normal cellular prion protein (PrPc) 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 (55). Overall, this evidence shows that the APP gene harbors 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.

The Tau protein is a major component of the neurofibrillary tangles and also binds copper, which appears to promote aggregation (56). 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 (57).

Regarding genetic risk factors, the APOE4 was demonstrated to interact with copper metabolism at different levels. It has been shown that the ApoE protein possesses antioxidant properties which depend on bound copper (58-60). 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 ABeta (58-60). In fact, absolute and free copper concentrations are higher in carriers of the APOE4 allele than in non-carriers (61-63). 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 (64, 65).

The level of plasma homocysteine is a risk factor for AD (66, 67), and it's known that copper mediates LDL oxidation by homocysteine (68).

ABeta binds copper and cholesterol, facilitating copper oxidation of cholesterol to 7-OH cholesterol and to 4-cholestenone (69), which are extremely toxic to neurons (70). Trace amounts of copper, well below the levels considered safe for humans, given to cholesterol-fed rabbits have induced accumulation of ABeta in senile plaques and impaired the animals' learning capability (71). Thus, cholesterol increases ABeta formation and copper promotes ABeta aggregation and toxicity. Furthermore, cholesterol can catalyze copper-ABeta redox cycling (72). The results from the cholesterol-fed rabbit model have been confirmed in transgenic mice (73) and provided the rationale for the results of a large community prospective study showing a strong correlation between copper intake from a diet rich in saturated and trans fats and mental decline (74). As a result, serious concerns have been raised about a possible relationship between copper overexposure from food or drinking water and cognitive disturbances (75). 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 (76). 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 (35), is substantially more pronounced in the aged AD brain (37). Lovell *et al.* (37) 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

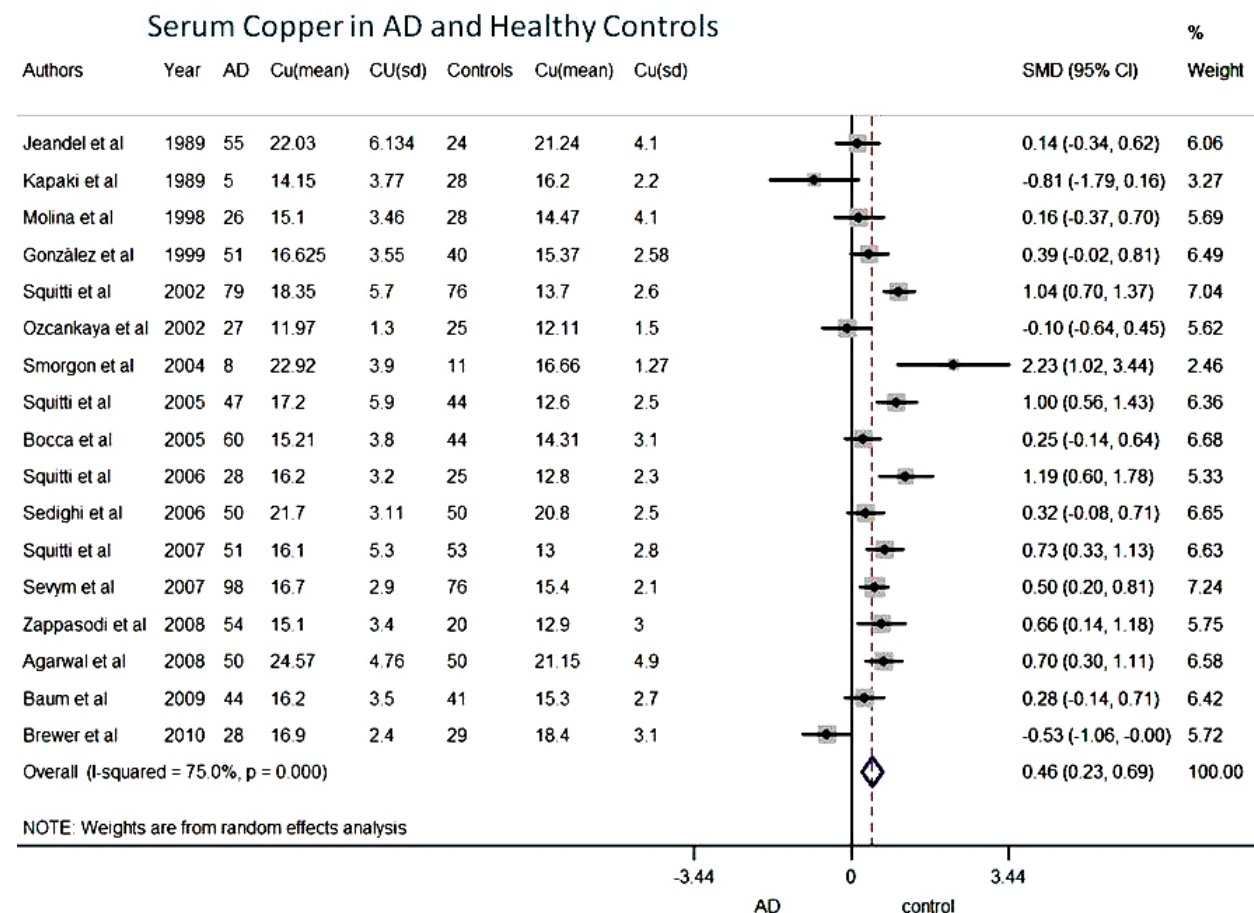


Figure 2. Standardized mean difference (SMD) in Cu serum level. The square represents the SMD between patients and controls. The size of the squares is proportional to the sample size of the study, the whiskers represents the 95% confidence interval. The diamond represents the pooled estimate based on random effects model, with the centre representing the point estimate and the width the associated 95% CI.

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 (77).

6.2. Copper in Alzheimer's disease: studies on living patients

Our research group has contributed to this issue by collecting evidence of copper involvement in AD, primarily via clinical studies. We 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 circulating and CSF copper levels (6, 7, 33). The existence of systemic copper dysfunctions in AD has been a controversial issue for many years. In fact, many studies have reported an increase of circulating copper in AD patients with respect to healthy controls (61-63, 65, 78-85), many others no variation (26, 86-94), and two very

recent studies even a decrease of plasma (27) and serum (95) copper in AD patients. Moreover, the latter studies are in line with two older studies showing that low plasma copper concentrations correlated with clinical worsening in AD, one reporting a copper plasma-decrease in more severe vs. less severe patients (96), and the other a direct correlation between low plasma copper concentrations and cognitive decline (97). Recently, we have evaluated these heterogeneous results in a meta-analysis, which has analyzed data from all the studies published since 1983 on AD patients and healthy controls. Data from 21 studies on serum/plasma copper have been merged for a pooled total of 966 AD patients and 831 controls (Figure 2) (25). Meta-analysis is a quantitative method that combines the results of independent reports to distinguish between small effects and no effects, random variations, variations in sample used or in different analytical approaches. We used this approach to gain an objective evaluation to the question whether systemic copper variations are associated to AD or not. The analysis demonstrated that AD patients have higher levels of serum Cu than healthy controls (Figure 2). Even though moderate, the assessed copper increase was sufficient to unambiguously distinguish AD patients from

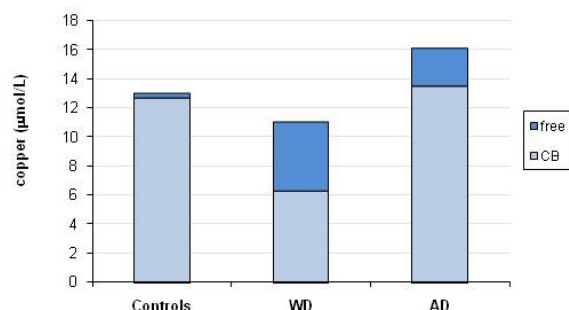


Figure 3. Free copper in Alzheimer's disease (AD). The figure shows copper distribution its 2 serum pool - copper bound to ceruloplasmin (CB) and free copper - in healthy controls, WD and AD patients.

healthy controls. Plasma data did not allow conclusions, due to their high heterogeneity, but the meta-analysis of the combined serum and plasma studies confirmed higher Cu levels in AD (Figure 2). The analysis of CSF data, instead, revealed no difference between AD patients and controls. These results confirm our previous interpretations of copper serum-increases in AD than in healthy controls. In particular, when we measured copper, iron, total hydroperoxides, transferrin, ceruloplasmin and copper-enzymes Cu/Zn SOD and TRAP levels in the sera of diverse AD patients cohorts (62, 63, 83, 98-100) we found that copper, peroxides and Cu/Zn SOD activity (98) were higher, TRAP was lower and ceruloplasmin and iron did not differ between AD patients and healthy controls. Moreover, these changes appeared to be specifically referred to the AD patients since they were not present in vascular dementia (VAD) patients, with the exception of TRAP that was lower in VAD patients in comparison to healthy controls (85). Diverse explanations can be advocated to account for systemic copper abnormalities in AD. Inflammation can be one reason, whose role in AD is well established (101). In fact, ceruloplasmin, which accounts for 85-95% of circulating copper, is an acute phase reactant, whose levels increase during the inflammatory response. In a previous study (100), we investigated whether markers of inflammation in general circulation were abnormal in AD, and showed that levels of ceruloplasmin were higher in AD than in healthy controls, though close to the significance threshold ($p=0.05$). However, that ceruloplasmin increase could not account for the pronounced rise of serum copper estimated in the patient sample analyzed in that study. In fact, when we performed a deeper analysis by distinguishing ceruloplasmin bound from the free copper pool (102), results revealed that the copper increase in our AD patients was attributable to this latter fraction (83, 84, 100). This is why we started studying free copper in AD. However, free copper abnormalities can be advocated as an additional rather than alternative explanatory variable of copper disturbances in AD, as also other research groups recently confirmed (79, 95, 103, 104). Interestingly, Boll *et al* (105) found an increment of free copper in the CSF of AD patients, but the reduced number of samples precluded conclusive results. Also previous studies of ours pointed in this direction and have characterized the increase of free

copper in AD patients and its correlation with the main deficit of the disease as explained in the following paragraph.

6.3. Free Copper involvement in alzheimer's disease: studies on living patients

The comparison with values obtained from normal subjects indicated that free copper levels are higher in AD patients than in healthy elderly controls and correlate with the main cognitive (62, 83-85, 99, 100, 106), neuro-anatomical (62), electrophysiological deficits (64, 65), with accepted AD markers, namely CSF Aβ and Tau proteins (83), and with known risk factors, such as APOE4 allele, of the disease (62, 65). 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 (83).

Copper systemic abnormalities in the AD resemble those observed in Wilson's disease, where a micronutrient-associated copper fraction exists, independent of ceruloplasmin, which, instead, appears fragmented. It must be noted, though, that in WD this fraction is much higher than the one estimated in AD (107) (Figure 3).

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 (29). We have also reported fragmentation of ceruloplasmin, revealed by the presence of low-molecular-weight fragments (<50 KDa) of ceruloplasmin in AD serum samples from selected patients with higher-than-normal levels of free copper (108), 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, is a copper systemic abnormality that 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 – PT – and lower albumin levels than controls matched for age, sex and risk factors for cardiovascular diseases and medication intake (63). Very recently, we reproduced the same evidence in a bigger cohort of AD associated with iron abnormalities (109). The hypothesis that copper, as well as iron (see below) 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 (19). 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 (19). 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 ABeta (58). This evidence fits very well with previous results in a mouse model of brain uptake of radiocopper [$^{67}\text{Cu}(\text{II})$], as also confirmed by data of a recent study (7). In the [$^{67}\text{Cu}(\text{II})$] mouse model, a net brain copper uptake occurs and parallels the free copper increase in the injectate, starting from a concentration of $\text{Cu}(\text{II})$ of 3.2 ng/ml, corresponding to 0.05 $\mu\text{mol/L}$, much lower than the 2.5 $\mu\text{mol/L}$ value that we evaluated in clinical studies on AD living patients (110). The belief that there is a direct interaction between copper and ABeta, based on the strong negative correlation between free copper in serum and ABeta in the CSF (83), is also sustained by the negative correlation between copper and ABeta in the CSF found by other authors (111).

In a recent study, we assessed levels of copper, iron, zinc, transferrin, ceruloplasmin, peroxides, TRAP, free copper and APOE genotype in 81 mild to moderate AD patients, age mean 74.4, SD = 7.4 years, clinically followed up for 1 year to evaluate whether these markers could give information about the prognosis of the disease. We studied their association with scores in the MiniMental State Examination (MMSE) (112) (primary outcome), Activities of Daily Living (ADL) and Instrumental of Daily Living (IADL) (secondary outcomes) performed at study entry and after 1 year that were analyzed by multiple regression analysis. Our study revealed that free copper can predict the annual change in MMSE, adjusted for the baseline MMSE: it raised the explained variance from 2.4% (with only sex, age and education) to 8.5% ($p = 0.026$). When the annual change in MMSE was divided into <3 or ≥ 3 points, free copper was the only predictor of a more severe decline (predicted probability of MMSE worsening 23%: (OR= 1.23; 95% CI=1.03-1.47; $p=0.022$). In other words, this study showed an association between systemic copper deregulation and unfavorable evolution of cognitive function in AD patients, demonstrating that free copper identifies those patients at higher risk for a more severe decline (84).

In a very recent study we tested the hypothesis that free copper was increased significantly enough to distinguish also individuals affected by mild cognitive impairment (MCI) from healthy controls (106). To verify this hypothesis a sample of 83 MCI subjects were compared with 100 elderly controls in terms of levels of serum copper, free copper, ceruloplasmin, APOE4, iron, transferrin, and TRAP. The groups were compared also in terms of demographic and cardiovascular risk factors. The comparison with an additional group of 105 mild to moderate AD patients was also evaluated. The multinomial logistic regression analysis demonstrated that APOE4 and free copper differentiated MCI from healthy control subjects. Chances to acquire MCI increased about 24% for each free copper unit ($\mu\text{mol/L}$) increment. APOE4 and free copper differentiated the MCI group also from the AD group. APOE4 and free copper appeared associated to MMSE worsening, as did age and sex (106). A follow-up study is in progress on MCI subjects, which will test

whether free copper can provide information about who, within the MCI subjects, will progress to AD.

In another study we have investigated normal women over age 50 (mean age 60.9); we studied their free and ceruloplasmin bound copper, and measured MMSE and several other measures of memory and cognition (19 neuropsychological test battery) (113). We found that free (but not ceruloplasmin bound) copper levels correlated inversely with MMSE and certain other measures of memory, that is, the higher the free copper, the poorer the performance on these measures of cognition (114).

Subsequently, we investigated the relationship between depression which was advocated as a risk factor of AD (115), markers of oxidative stress and neurotransmission, as expressed by sensory cortex excitability. Serum levels of oxidative stress markers and somatosensory magnetic fields, evoked by external galvanic stimulation, were measured in 13 depressed patients and 13 controls (116). Depressed had higher levels of total and free copper than controls and lower levels of transferrin. They also showed lower sensory cortex excitability, which correlated with copper levels in controls, but not in depressed patients. Transferrin correlated with sensory cortex excitability in both patients and controls, although in opposite ways. Copper level results associated with the patients' clinical status. Pro-oxidant agents appear to affect neuronal excitability and clinical state of depressed patients, as free copper excess alters their cortical glutamatergic neurotransmission (113).

At present, we are exploring the hypothesis that single nucleotide polymorphisms (SNPs) of ATP7B - the gene which encodes the protein that tightly controls the levels of free copper in the body and whose defects cause WD - have higher frequencies in AD than in healthy individuals, to identify risk factors among those genes that control systemic copper. This study is part of a larger project aimed at identifying the molecular processes at the basis of copper dysmetabolism in AD. In this first step we have studied a cohort of 178 AD patients and a 150 healthy subjects and analyzed the results concerning 4 SNPs. GG genotype in the SNP 2495 A>G (Lys832Arg) (exon 10) and GG in c.1216T>G (Ser406Ala) (exon 2) resulted significantly associated with a higher probability of having AD (in progress).

6.4. Iron involvement in alzheimer's disease

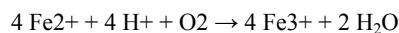
There is also total agreement on the evidence of a link between AD and oxidative stress phenomena triggered by iron whose disarrangement has been generally viewed as originating independently of systemic influences. In this frame, authors have reported enhanced iron concentrations in AD brains, both in autopsy brain tissues and in CSF (117) especially in the basal ganglia (118, 119), and around the senile plaques of ABeta or the neurofibrillary tangles commonly found in AD brains (37, 120). Authors have also reported altered local (within the brain) concentrations of specific proteins regulating iron levels, such as ceruloplasmin (121, 122), transferrin and ferritin (123).

6.5. Iron involvement in Alzheimer's disease: studies on living patients

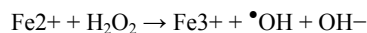
On a different line, we were interested in verifying whether iron homeostasis abnormalities were associated with AD. To do so we started analyzing typical markers of iron status in general circulation (iron, transferrin, transferrin saturation, ferritin, ceruloplasmin) together with mutations in HFE (H63D and C282Y) and in Transferrin C2 (TfC2), which were reported to associate with AD. Since these gene variants are often accompanied by liver distress, we decided to analyze also the typical panel for liver function (albumin, transaminases, PT) in the cohorts under investigation (160 AD patients and 79 healthy elderly controls (109). In particular, we were interested in evaluating whether and how markers of iron and liver status were interconnected and contributed to AD onset. This study revealed that albumin was lower, PT longer and AST/ALT values higher in AD patients than in controls, indicating a liver distress confirming previous observations. Also transferrin was lower and ferritin higher in AD. Multiple logistic regression backward analyses performed to evaluate the effects of these biochemical variables upon the probability to develop AD revealed that a one-unit transferrin serum-decrease increases the probability of AD by 80%. A one-unit albumin serum-decrease reduces the AD probability by 20%, while a one-unit increase of AST/ALT ratios generates a fourfold probability increase. While healthy controls carriers of HFE mutation were normal for iron status, AD patients carriers of the HFE H63D mutation showed a panel resembling hemochromatosis, i.e., higher levels of iron, lower levels of transferrin and ceruloplasmin and activation of the ceruloplasmin-transferrin (Cp-Tf) system (as revealed by calculating the ceruloplasmin to transferrin ratio - Cp/Tf -). This picture was found neither in H63D non-carrier AD patients, nor in healthy controls. We concluded that carrying the HFE mutation is not itself sufficient to increase the risk of AD. Rather, it is a synergy between iron increase, a condition of liver dysfunction and the H63D mutation that appears to increase the probability of developing AD (109).

In another study, we measured serum levels of iron, ceruloplasmin and transferrin, and calculated the transferrin saturation and the activation of the Cp-Tf system (124) in relation with the main cognitive and anatomical deficit of AD, namely MMSE and medial temporal lobe atrophy (125). The results of this study demonstrated that ceruloplasmin, Cp/Tf, transferrin saturation and peroxides levels were abnormally higher in AD. Elevated values of ceruloplasmin, peroxides and Cp/Tf inversely correlated with MMSE scores, while isolated medial temporal lobe atrophy positively correlated with Cp/Tf and negatively with serum iron. This finding demonstrates that systemic alterations of iron metabolism accompany the disease and indicate that the role attributed by existing literature to local iron accumulations in brain areas critical for AD should be rather viewed in the frame of a wider systemic alteration. In this scenario, antioxidant systems play of course a role. Overall, the Cp-Tf antioxidant system has been advocated as the main plasma antioxidant system (124). Our recent data refresh previous literature on this

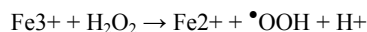
matter, mainly studied in the early '80. We were particularly interested in this antioxidant system since it appears as the crosstalk system between iron and copper metabolism which are both damaged in AD. Coherently with these findings, the Cp-Tf system was activated in AD. In the Cp-Tf system, ceruloplasmin catalyzes the oxidation of ferrous iron (Fe²⁺) into ferric iron (Fe³⁺), by way of the following chemical reaction:



Ceruloplasmin-catalyzed iron oxidation and removal from circulation by transferrin is a very important process since it reduces the amount of Fe²⁺ available for participation in Fenton's reactions (see the 'Iron Homeostasis' section), whose byproducts are instead extremely vicious. In fact, ferrous iron can also convert hydrogen peroxide into the highly reactive radicals OH[•] and OOH[•]:



which, in turn, proceeds as follows:



Copper can enter exactly the same reactions, passing through Cu²⁺ to Cu¹⁺ oxidative states. Ceruloplasmin can also loosely bind additional copper atoms to its 6 structural ones (8), sequestering potential noxious metals. The Cu-Tf system, in fact, represents the serum capacity to sequester exchangeable metals (copper, iron) (124), thus decreasing from general circulation the labile metal pool prone to partake in reactions generating oxidative damage. In other words, if they were not oxidized and removed by the coordinated ceruloplasmin and transferrin actions, iron and copper would cyclically produce highly reactive radicals, which the circulating blood would then distribute all over the organism. Nonetheless, it is important to keep in mind that if, on the one hand, this mechanism avoids or reduces production of lethal oxidative species in general circulation, on the other hand, when transition metals move into the cells, as it could be the case for iron in neurons but also copper – see the WD condition -, they can cause even a worse brain damage, if a concomitant neuronal metals defective efflux does occur (18, 19). This the case of WD - the utmost example of copper toxicosis - in which large amounts of free copper enter the brain and cause abnormal glial cells and degenerated ganglion cells in cerebral cortex, putamen and dentate nucleuses (126). Evidence of a defective iron or copper efflux from neurons, linked to APP, was also reported in several studies on cell cultures and APP knock-out mice (APP^{-/-}), in which APP expression is experimentally suppressed (18, 19). As reported above, the ablation of APP produces metal dysbalance in several organs and tissues (18, 19). In particular, in a previous section we have discussed the increase of copper concentrations in the liver (80%) and in the brain (40%) in APP^{-/-} mice. Recently, some authors (18) demonstrated that APP ablation causes an iron increase of 26% in the brain, 31% in the liver and 15% in the kidney as well. They

also showed that, like ceruloplasmin, APP catalytically oxidizes Fe²⁺ (18). All together, these studies demonstrate that APP plays a role in copper and iron mobilization, indicating that its abnormalities in AD have either a systemic effect on those metals' homeostasis or a local effect on neuronal metal efflux. Our recent results of increased brain atrophy in AD patients are in support to this concept, as medial temporal lobe scores associated with either decreasing levels of serum iron or increased values of Cp/Tf ratios (125), and also with copper serum-increases (62).

7. TREATMENT OF ALZHEIMER'S DISEASE WITH METAL COMPLEXING AGENTS

Since late '80s, there has been wide interest upon the idea that properly tuning the redistribution of metals (aluminum, iron, selenium, zinc, copper) via metal complexing or ligand agents may positively affect the natural history of AD. Numerous recent reviews reporting detailed information about the studies in which metal complexing agents have been thoroughly tested as potential therapeutics for AD (127-129), or which discuss the pros and cons of the basal choices at the basis of the use of such compounds (20, 110), or even the challenges associated with these sort of treatment (130) have been published so far. A summary of the most intriguing molecules with metal redistribution properties invented so far, together with their potential or proved applications in ameliorating the clinical history of AD is reported herein.

7.1. *In vitro* studies and animal models on treatment of alzheimer's disease with metal complexing agents

Early *in vitro* studies showed the effect of metal chelators in solubilizing ABeta deposits. Experiments showed that zinc-induced aggregation of ABeta40 could be reversed by the divalent metal ion chelator ethylenediamine tetra acetic acid (EDTA) (131). Solubilisation of AD brain-derived ABeta was achieved with N,N,N',N'-tetrakis(2-pyridylmethyl)ethylene diamine (TPEN) and 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline (bathocuproine) (48). Moreover, bathocuproine, bathophenanthroline, diethylenetriamine penta-acetic acid (DTPA) halted Aβ redox activity complexing copper (132). Another metal ligand molecule, [1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-bis(2-octadecyloxyethyl)ester,N,N'-disodiumsalt] DP-109 (133), was found to reduce the burden of amyloid plaques and of cerebral amyloid angiopathy in Tg2576 mice. There also exists a lipophilic amyloid-targeting metal chelator - XH1 -, designed with amyloid-binding and metal chelating moieties, which reduced APP protein expression in human cells and ABeta pathology in APP transgenic mice (134). Similar results were achieved with the lipophilic metal chelator DP-109, which markedly decreased amyloid plaques in APP transgenic mice (133).

Ammonium Tetrathiomolybdate forms a stable tripartite complex with copper and proteins (110). Given with food, tetrathiomolybdate can complex 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, tetrathiomolybdate 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 (110), it has no known biological activity, and is largely cleared in the bile. A recent study tested the ability of tetrathiomolybdate to reduce Aβ pathology and spatial memory impairment in both a prevention and a treatment paradigm in Tg2576 mice. The study demonstrated that tetrathiomolybdate lowered brain copper concentrations and reduced Aβ levels in a prevention paradigm, but not in a treatment paradigm, suggesting that lowering systemic copper may achieve anti-amyloid effects if initiated early in the disease process (135).

Metal bis(thiosemicarbazonato) complexes - MII(btsc), where M stands for either Copper (II) or Zinc (II) - can affect extracellular levels of ABeta. Treating Chinese hamster ovary cells overexpressing APP with engineered MII(btsc) increased levels of bioavailable intracellular copper and zinc but also resulted in a dose-dependent reduction of ABeta levels (136). Iron-regulated APP in ABeta peptide in cell culture were decreased by (-)-epigallocatechin-3-gallate, the main polyphenol constituent of green tea (137). It was demonstrated that this molecule has metal-chelating and radical-scavenging properties that have an effect on iron metabolism in AD (137).

Nanoparticles conjugated to chelators were shown to easily cross the BBB, chelate metals, and exit through the BBB with their corresponding complexed metal ions (128, 138). Early studies from Liu's group (128, 138) showed that these nanochelators can effectively remove Fe from tissue sections of AD brain and also from ferritin.

Some studies (53, 139) recently demonstrated the potential application of curcumin, a commonly used spice extracted by turmeric, as an anti-inflammatory (140) and anti-oxidant (141) molecule with neuroprotective properties. Some authors (142) demonstrated its metal chelator and neuroprotectant (143) effects. In particular, Lin *et al.* demonstrated in PC12 cells (53) that this compound has strong effects on APP and BACE1 transcription up-regulation mediated by copper. The authors suggest that curcumin might have a combined effect on suppressing APP and BACE1 transcriptions, blocking the effect of copper. Due to its multi-functional effect new derivatives have been created to improve curcumin brain bioavailability (144). Clinical studies are currently in progress to verify whether the use of this natural, non-toxic, neuroprotective compound with brain access could offer potential therapeutic benefits against neuronal damage.

Clioquinol (see also below) has been proven to be able to reverse the aggregation and redox activity of ABeta (49%) by its low affinity for copper in Tg2576 mice (47).

7.2. Clinical trials on the treatment of alzheimer's disease patients with metal complexing agents

7.2.1. Desferrioxamine

Desferrioxamine is a hexadentate chelator that does not cross the blood brain barrier BBB. This orthodox chelator was tested for AD efficacy in a small ($n = 48$), single-blinded study in which placebo-treated and untreated patients were pooled. In addition, this study did not report any measure of the cognitive performance of the patients. Given to AD patients for two years it slowed the clinical progression of dementia (145). The target metal of the study 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. A generalized decrease of metal concentration was seen. This effect and a decoppering effect in particular – since copper has not been replaced during desferrioxamine administration – might have been at the basis of the clinical outcome. The fact that desferrioxamine showed such an effect without crossing the BBB could be due to the induction of a negative metal balance throughout the whole body as it is extensively documented for the treatment of WD with D-penicillamine or Zinc compounds.

7.2.2. D-penicillamine

D-penicillamine has been used for treatment of WD; it functions as a copper chelator, controlling both the reactivity and bioavailability of that metal, ultimately facilitating its disposal through the urine. Nowadays, conspicuous adverse events have been reported and this limited its use in WD. In fact, a study on the tolerability and efficacy of D-penicillamine for the treatment of AD had to be interrupted for ethical reasons before finishing recruitment, due to adverse events, including the death from cardiac arrest of one patient under treatment (146). This forced the conclusions of the study to be drawn only from those patients who had completed the treatment at the moment of interruption (18 patients out of 34, i.e. 53%). Both serum and urine copper and red blood cells Cu,Zn SOD were measured (98, 146). While serum copper remained stable, its urine levels were drastically elevated by therapy, indicating that large amounts of copper were indeed being removed from the tissues. The activity of Cu,Zn SOD was drastically reduced, below control levels, revealing that this enzyme could be used as a good indicator of general depletion of the bioavailable copper (98). We noted a significant reduction in hydroperoxide levels in the treated AD patients at difference from serum copper levels which did not change during the treatment period, as expected. In fact, it has to be kept in mind that copper, when is complexed with a chelating agent, as for example D-penicillamine, is still detectable in the blood sample even though it is not bioavailable (as demonstrated by the drastic decrease of Cu,Zn SOD activity in our case) (98, 146). Even though we could reach the positive result of decreasing the bioavailable copper and hydroperoxides content in patients *in vivo*, the clinical relevance of our data could not be fully assessed, because the 24 week observation period of the study was unfortunately insufficient to detect differences in cognitive decline compared to the placebo control group. As a matter of the

fact, Crapper McLachlan and colleagues (145) have shown a positive effect of chelation therapy in AD by treating patients for a 24-month period and Ritchie *et al.* (147) reported some positive results with clioquinol (see below) after 36 week. In our study with D-penicillamine, surprisingly, in spite of the abundant literature existing in the field, the follow-up period of 24 weeks was not sufficient to detect any cognitive decline in the placebo group and a comparison with treated patients was therefore precluded. It must be noted, however that in our D-penicillamine pilot study we had also observed an important antioxidant change in the serum of both treated and placebo patients, probably because both groups were taking vitamin B6 to prevent the expected D-penicillamine induced deficit of this vitamin (146).

7.2.3. Iodochlorhydroxyquin (clioquinol) drug class

In early 2000s clioquinol was tested in man (148) revealing that a treatment of 21 days resulted in a significant improvement of patients' cognitive performance. This study was followed either by a case report on 2 AD patients showing that clioquinol treatment could ameliorate focal cerebral glucose metabolism and clinical deterioration (149) or by a double blind placebo-controlled clinical trial carried out by Ritchie and colleagues in which 36 patients were treated with 125 mg/day twice daily (bis in die -BID) for the first 12 week, then 250 mg BID between week 13-24 and finally 375 mg BID in the latest period of week 25-36. The trial revealed that clioquinol treatment slowed the rate of clinical decline in a subset of AD patients having more severe dementia (147). However, in the subgroup of patients with moderate dementia (ADAS-Cog > 25 at baseline) the difference in ADAS-Cog between clioquinol and placebo did not reach statistical significance at week 36 ($p = 0.07$) (147). In addition, in this subgroup there were no significant effects of the drug on the CSF ABeta42 levels. As stated by the authors (147), the results of the clioquinol study supported a proof of concept in humans that a drug targeting ABeta-metal interaction can have a significant effect on slowing the progression of AD. Clioquinol is capable of crossing the BBB, and it was believed that it solubilized ABeta plaques by stripping them of their metal content. However, from subsequent *in vitro* investigations, it was unclear whether the positive results gained by this phase II clinical trial were related to an attenuated ABeta-metal ion interaction or to some other mechanisms. Moreover, the study was concerned with caveats generally referred to small scale trials. On these bases, the relevance of the clioquinol conclusions have been questioned, as extensively reported in a recent review (130). Argumentations against the chelating effectiveness of clioquinol were referred mostly to the evidence coming from a study by Treiber and co-workers (150). In this study, by using a yeast model system, the authors (150) demonstrated that the addition of clioquinol to the culture medium drastically increased copper concentration within the yeast cells. Thus, they refer the positive effects of clioquinol on AD to an increase of copper uptake into the neural cell, instead to an attenuated ABeta-metal ion interaction outside the cell. However, these authors do not report information about the bioavailability of the

intracellular copper-clioquinol complex, disparaging the relevance of their observation. Some information to this regard could be found in a subsequent study demonstrating a decreased intracellular metal bioavailability linked to the accumulation of soluble A β outside the cell (151): clioquinol drug class, facilitating the delivery of metals (copper and zinc but not iron) into the cell, seemed to activate the phosphoinositol 3-kinase mediated protein kinase pathways, ultimately leading to an increase in the secretion of matrix metalloproteinases, which can degrade A β outside the cell (151). This is why clioquinol drug class molecules have been defined as metal protein attenuating compounds (MPAC), and both chelating and ionophore (i.e. a lipid-soluble molecule usually that transports ions across the lipid bilayer of the cell membrane) properties have been attributed to them. Establishing the exact localization of clioquinol within the cell (cell membrane, cytoplasm) or copper bioavailability of the putative clioquinol derivatives-copper-complex could be of help to define the molecular mechanisms triggered or prevented by this class of molecules. Further clioquinol phase II/III studies were stalled by difficulties in preventing di-iodo 8-hydroxy quinoline contamination upon large-scale chemical synthesis as well as its cytotoxicity profile which caused clioquinol withdrawn from human experimentation. New compounds were then developed. Among these, PBT-2 - a second-generation compound of the clioquinol drug class - that lacks iodine - has replaced clioquinol. It has been recently tested for treatment on AD patients in a phase II, double-blind, randomised, placebo-controlled 12 week trial. 74 patients completed the trial and well tolerated the 250 mg of PBT-2 daily dosage. This salient study revealed that PBT2 induced a significant, dose-dependent reduction of CSF ABeta concentrations even though no drug effects on either Ab40 or Ab42 plasma levels were detected. Moreover, PBT-2 was found to preserve two executive functions as evaluated via category fluency test and trail making part B (152, 153). This MPAC, which promises effectiveness in counteracting AD progression, is now under phase III clinical trial investigation.

7.2.4. Zinc therapy

Agents commonly used for chronic or maintenance of an anti-copper therapy include those that maintain a state of copper malabsorption, such as zinc acetate (reviewed in (20)). Zinc acetate and other zinc salts (zinc carbonate, zinc sulfate, zinc gluconate, zinc oxide, zinc chloride and zinc stearate) are currently used for the comprehensive treatment of WD including initial treatment (reviewed in (20)). Liver represents the main human storage for copper and metallothioneins the major copper binding proteins in this organ (see paragraph 3.3). Zinc antagonizes the absorption of copper in the gut by increasing metallothioneins concentrations in the mucosa up to 25-fold. Diffusible free copper present in the blood is sequestered in a non-toxic form in the mucosal cells lining the intestine by metallothioneins. In this form copper is then excreted with the stools. The copper body balance becomes negative in 2 weeks when zinc sulphate is administered in a dose of 600 mg/die. Very safe compounds such as zinc sulphate or aspartate are recommended to be tested in AD. Encouraging results in

this direction can be found in a preliminary study by Constantinidis (154), reporting improvements in memory, understanding, communication, and social contact in eight out of ten AD patients treated using zinc compounds (reviewed in (20)).

7.2.5 Side effects of anti-copper complexing agents

The side effects of anti-copper complexing agents and MPAC has been primarily limited to anemia and leucopenia, due to bone marrow depletion of copper, and occasionally to liver toxicity. The frequency of occurrence of these effects correlates to dose strength and frequency of the drug assumption. For a relatively new copper-lowering agent, which is ammonium tetrathiomolybdate these events have been correlated with the reduction of serum ceruloplasmin levels, assumed to be a surrogate marker of copper bioavailability. For example, at a ceruloplasmin concentration of 5 mg/dL or below, bone marrow suppression is common; between 5 and 10 mg/dL it is frequent, between 10-18 mg/dL is only occasional, and above 18, is very rare. Recovery, on suspension of therapy (drug holiday) or reduction of the dose for 1-2 days, follows quickly. Clioquinol's use has been halted for human use essentially because it has been related with subacute myelo-optic neuropathy. Zinc compounds have solely minor adverse events limited to gastrointestinal troubles.

8. PERSPECTIVE

Data of the studies presented can be interpreted as single pieces of a scenario of a complex disarrangement of transition metals that, via oxidative stress, affects patients with AD. In this view, a systemic metal dysfunction, even though of a mild nature, but over a long time course, may have a disturbing effect on central processes which control brain protection against metals-mediated damage, eventually leading to oxidative damage in defined areas or sites of the brain critical for AD. Glutamatergic synapses can well represent the focal sites where the ABeta-metal toxic action could be promoted and result in ABeta precipitation, downstream AD pathology (1, 2). The identification of a serum-level increase in free copper, as well as disturbances of iron homeostasis and activation of the main antioxidant system of the body, the Cp-Tf system, in AD, appears coherent with this picture. Moreover, if on one hand free copper can itself cross the BBB and enter possible reaction of ABeta toxicity, on the other hand it can systemically activate the Cp-Tf system, promoting iron subtraction from general circulation and its internalization into neural cells with a defective iron efflux linked to APP, eventually leading to neurodegeneration. Both pathways converge towards neuronal death via generation of ROS. In this framework, properly tuning the redistribution of metals via molecules which induce or maintain a state of copper malabsorption, such as zinc compounds, or metal complexing agents (146), may positively affect the natural history of AD (20, 110). Despite the high affinity of some metal-complexing agents towards their specific target metal, a more generalized effect in sequestering diverse metals seems likely, thus improving their chances of success in slowing the

progression of AD. PBT-2 from clioquinol drug class seems very close to gain this important result (153).

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