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Metals Involvement in Alzheimer's Disease Pathogenesis

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1. Introduction

When Auguste Deter died in 1906, Alois Alzheimer, who had closely followed her mental degradation over the previous 5 years, obtained her brain and performed a post-mortem study. By using a silver-staining method just developed by Bielschowsky, Alzheimer identified aggregations of fibrils and what he called 'miliary foci'. Alterations of that nature had been already observed by others in post-mortem brains, but Alzheimer was the first to relate them to his patient's dementia. He described the case a year later and Auguste Deter remained in medical history as the first clinical case of what has been ever since referred to as Alzheimer's disease. For an English translation of Alzheimer's 1907 paper, see (Alzheimer et al., 1995).

1.1 General description

Alzheimer's disease (AD) is an irreversible neurodegenerative disorder, characterized by a gradual loss of the cognitive functions inexorably leading to dementia. From an early stage characterized by a mild loss of recent memory, the disease advances to more devastating symptoms such as faulty judgments and even personality changes, to terminate in a complete loss of reasoning ability and self sufficiency. Death normally ensues 8 to 10 years after diagnosis.

AD is often referred to as early- or late-onset depending on whether it appears before or after age 65, and is normally distinguished in familial, i.e., inherited, and sporadic, which is caused by a combination of genetic, lifestyle and environmental factors.

About 75% of AD patients have the sporadic form, which has normally a late-onset. The familial form, which affects the remaining 25%, can have instead either an early-onset (about 5% of all familial cases) or a late-onset.

The early-onset familial AD is attributed to mutations in one of three genes: amyloid precursor protein (APP), Presenilin1 or Presenilin2. The genetic causes of the late-onset familial AD are not fully assessed, although the inheritance of the $\epsilon 4$ allele of the apolipoprotein E (*ApoE4*) has been recognized as a risk factor for both familial and sporadic late-onset AD. Carriers of this allele have a 90% statistical risk of contracting AD if they are heterozygote and a virtual certainty if homozygote (Corder et al., 1993).

All forms share the same alterations that include neuron loss, synapse loss, amyloid plaques, neurofibrillary tangles and microgliosis. However, although it is well established that AD symptoms are due to a compromised neurotransmission originating from these alterations, there is not yet full agreement on what actually initiates the neurodegeneration.

1.2 Diagnosis

Since no single test or biochemical measurement can securely lead to a diagnosis of AD, intellectual deterioration is diagnosed as possible or probable AD on the basis of a careful analysis of the symptoms, the medical history of both patient and his/her relatives, a variety of neuropsychological tests and from the exclusion of alternative conditions, generally attained via neuroimaging. Almost universally physicians today follow the NINCDS-ADRDA Criteria for diagnosis of Alzheimer’s disease (McKhann et al., 1984), jointly published in 1984 by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association, which specify eight cognitive domains that are compromised in AD - memory, language, perceptual skills, attention, constructive abilities, orientation, problem solving and functional abilities - and give guidelines for impairment assessment and diagnosis. NINCDS-ADRDA criteria have proven reliable over the years and have been extensively updated in 2007 to account for recent advances in neuroimaging (Dubois et al., 2007).

1.3 Symptoms

Symptom progression of AD			
Function	Stage I (mild)	Stage II (moderate)	Stage III (severe)
Memory	Mild amnesia (recent memory)	Amnesia (recent and remote)	Not measurable
Language	Quasi-normal, anomia	Sensory aphasia	Severely impaired
Perceptual skills	Mildly impaired	Moderately to severely impaired	Not measurable
Attention	Reduced	Impaired	Not measurable
Constructive abilities	Reduced	Impaired	Not measurable
Orientation	Mildly impaired	Impaired	Not measurable
Problem solving	Mildly impaired	Impaired	Not measurable
Functional abilities	Almost normal, some mistakes	Unreliable	Not measurable
Behavior	Mild changes, signs of withdrawal	Absent	Agitated, delusional
Gait	Normal	Normal	Impaired
Posture	Normal	Normal or flexed	Bedridden
MMSE	25-21	20-11	10-0

Table 1. Symptom progression of AD; MMSE: Mini-mental state examination (Folstein et al., 1975).

1.4 AD pathology

1.4.1 Macroscopic pathology

Atrophy is the major macroscopic characteristic of the AD brain. Ventricles are dilated, gyri appear narrower and sulci wider than in a normal brain. The loss of tissue, which roughly correlates with the degree of cognitive decline, is so severe that an AD brain can weigh less than 1000g. Frontal, parietal and temporal lobes are all involved, the hippocampus in particular being severely affected. The primary sensory-motor cortex appears affected with some delay, whereas the occipital lobe is relatively spared.

1.4.2 Microscopic pathology

The 'miliary foci' and fibril aggregations that Alzheimer observed are today normally referred to as senile plaques (SPs) and neurofibrillary tangles (NFTs), and remain the hallmarks of AD.

SPs are clusters of protein fibrils that form in the extracellular space, made of an amyloid core, abnormal neurites and glial cells. The amyloid core is mainly an aggregation of Amyloid-beta ($A\beta$), a 4 kDa peptide 39 to 43 amino acids long, which is produced by the proteolytic cleavage of the APP, a transmembrane protein present in many cell types and highly concentrated on neuron synapses. Usually, APP is cleaved on the extracellular side of the neuronal membrane by the enzyme α -secretase, but occasionally it can be cleaved by two other enzymes (β - and γ -secretase) at different sites. The result is anyway the production of $A\beta$ segments that are released in the extracellular space and aggregate there in amyloid plaques. The gene for APP is located on the long arm of chromosome 21.

SPs are roughly spherical with a diameter reaching up to 200 μm , and are normally distinguished in 'diffuse' and 'compact': Diffuse SPs are amorphous deposits of non-fibrillar 'pre-amyloid' $A\beta$, which produce no alteration of the neuropil. Compact SPs, more often referred to as 'neuritic', show instead an amyloid core surrounded by dystrophic neuritis. The latter appear as clusters of radially oriented neuronal processes.

There are at least 3 subtypes of neuritic SPs: Primitive, which lack the amyloid core; Classical, in which a dense amyloid core is surrounded by dystrophic neuritis, which in turn appear interconnected by low density amyloid fibrils; Burned-out, which have only the dense amyloid core and at most a few neurites. This subdivision mirrors what researches believe is SPs' evolution: after being released, $A\beta$ segments forms diffuse plaques in the extracellular space until elements such as cytokines, ApoE, proteoglycans and others become embedded with the complex causing it to degenerate into neuritic plaques. In other words, SPs start as diffuse and then evolve into neuritic, first primitive, then classical, and finally burned out.

SPs appear to have also an anatomical hierarchy: higher-order association areas have the highest density of SPs, whereas the primary sensory-motor cortex the lowest. Striatum and cerebellum show a rather high density of SPs, but they are all of the diffuse type. The hippocampus appears relatively spared.

Only neuritic SPs are specific to AD, whereas diffuse SPs are found also in the brains of non-demented elderly individuals.

NFTs are aggregates of modified protein tau in the intracellular space. In normal conditions, the protein tau binds to microtubules, contributes to the progress of their formation and is a key element in their stability. In AD, tau undergoes hyper-phosphorylation which causes

the protein to aggregate. Moreover, upon hyper-phosphorylation, tau loses its binding capability so that the microtubules disintegrate. Each filament is made of two strands twisted around each other to form a helix, whose period is about 80 nm and diameter either 8 nm or 20 nm.

In AD, the areas most affected by NFTs are hippocampus, sibilum, amygdala, entorhinal and transentorhinal cortices. In the neocortex, higher-order association areas are more affected than the unimodal association areas, and the primary sensory-motor cortex is relatively spared. NFTs are also numerous in the nucleus basalis, limbic nuclei of the thalamus, locus ceruleus, substantia nigra, and the raphe nuclei of the brainstem.

NFTs' shapes seem to be influenced by the shape of the neuron in which they are. In fact, they look like a flame in pyramidal neurons, as for example in the hippocampus, whereas they appear more globular in rounded neurons, such as those of the nucleus basalis.

NFT develop first into early and then into fully grown tangles. After the death of the neuron some NFTs (often referred to as 'ghost' tangles) are visible also in the extracellular space.

It is important to remember, though, that NFTs are not AD specific and are frequent also in non-demented elderly individuals.

Neuropil threads are another form of neurofibrillary degeneration that in AD is found widely distributed throughout the gray matter, especially in distal dendrites and axons. They owe their name to their appearance which resembles short threads.

As mentioned above, the ultimate result of the presence of plaques and tangles in AD is a severe neuronal loss that can reach 60% in the hippocampus and 80% in the nucleus basalis and in some frontal and temporal areas.

2. Hypotheses on the causes of AD

2.1 Classic hypotheses

There are three major classic hypotheses on the origin of AD. The cholinergic hypothesis (Francis et al., 1999, Rossor 1983), which is the oldest and the one on which the majority of currently available drug therapies are based, proposes that AD is caused by a reduced synthesis of the neurotransmitter acetylcholine. The amyloid hypothesis (Hardy & Allsop, 1991) postulates instead that the fundamental cause of the disease is the deposition of A β , which is supported by the evidence that mutations in the gene for APP, which is known to cause A β aggregation, are linked to AD. The tau hypothesis, based on a study demonstrating that deposition of A β plaques does not correlate well with neuron loss (Schmitz et al., 2004), proposes that abnormalities in the tau protein initiate the disease cascade.

Safety and efficacy of more than 500 pharmaceutical treatments are being investigated in clinical trials worldwide on the basis of these three hypotheses. In 2008, two separate clinical trials showed positive results in modifying the course of the disease in mild to moderate AD: one with the metal complexing agent PBT2 (Lannfelt et al., 2008) and one with methylthioninium chloride (Wischik, 2008), a drug that inhibits tau aggregation, which unfortunately failed to confirm the positive results in its phase III.

2.2 The metal hypothesis

Recently, researchers have uncovered an important role played in AD neurodegeneration by transition metals via their properties to cause oxidative stress. In fact, copper and iron are known to participate in Fenton-type reactions that generate uncontrollable reactive oxygen

species (ROS) capable of damaging and destroying molecular and cellular compartments (Atwood et al., 2004).

Authors have widely reported enhanced metal concentrations in specific areas of AD patients' brains, in particular of iron, copper and zinc in cerebrospinal fluid (CSF) (Smith et al., 2010), of iron in the basal ganglia (Bartzokis et al., 2000, Bartzokis & Tishler, 2000), and of both iron and copper within SPs and NFTs (Good et al., 1992, Lovell et al., 1998). It has been also observed that APP possesses selective zinc and copper binding sites which mediate redox activity, causing precipitation of A β even at low concentrations (Bush et al., 1994). Also A β possesses selective high and low-affinity metal-binding sites. They can bind equimolar amounts of copper and zinc but, in conditions of acidosis, copper completely displaces zinc from A β (Atwood et al., 2000). A β reduces the metal ions by transferring electrons to O₂ and generating hydrogen peroxide in the process.

If on the one hand A β deposition in plaques is an age-dependent phenomenon, on the other hand A β production does not appear to increase with age. This seems to indicate that other age-dependent changes, as for example changes in metal homeostasis, may play a key role in A β transformation and neurotoxicity. Since copper and zinc are both modulators of the glutamatergic neurotransmission (Bush & Tanzi, 2008), abnormalities in metal homeostasis can have detrimental effects on synaptic processes, such as metal reuptake or storage in the synaptic cleft. All this evidence has eventually led to the proposal of a Metal Hypothesis of AD (Bush & Tanzi, 2008), which is based on the concept that it is the interaction of A β with specific metals, especially copper, that drives AD pathogenesis by promoting aggregation and neurotoxicity. By now, this view has become fully accepted and there is general agreement on the existence of a link between AD and oxidative stress phenomena triggered by transition metals. The Metal hypothesis of AD is now supported by the results of numerous clinical studies (Squitti et al., 2006, Squitti et al., 2002a, Squitti et al., 2003, Squitti et al., 2005).

3. Iron

3.1 Iron essentiality

Iron is essential for life. Of the 4-5 grams of iron normally present in a healthy body, about 2.5 g bind to hemoglobin and are used to promote respiration. Normally iron is absorbed from digested food or supplements. Average amounts are 1 mg/day for men, and 1.5-2 mg/day for women (Institute of Medicine Food and Nutrition Board, 2001). The majority is absorbed in the duodenum, where enterocytes of the duodenal lining reduce ferric Fe³⁺ to ferrous Fe²⁺, which is then transported across the enterocyte's cell membrane into the cell by the divalent metal transporter 1 (DMT1). After completing a number of functions, available Fe²⁺ is transported back out of the cell by ferroportin, which is distributed throughout the duodenum enterocytes. Hephaestin, a ferroxidase found mainly in the small intestine, oxidizes Fe²⁺ back to ferric Fe³⁺. At this point Fe³⁺ binds transferrin, a protein which takes it into circulation. Our body does not have a way to excrete iron in excess, so that the latter is stored inside another 450 kDa protein, ferritin, which can store up to 4500 atoms of iron per molecule (Fleming & Bacon, 2005).

3.2 Iron toxicity and the role of ceruloplasmin

Transported by transferrin, Fe³⁺ reaches the brain's capillaries and the Blood-Brain-Barrier (BBB), which is made of the brain capillary endothelial cells (BCECs) forming the wall of the

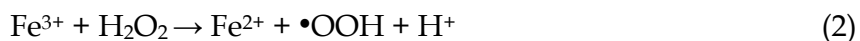
capillaries. The luminal side of the BCECs presents transferrin receptors that pick up the iron-loaded transferrin protein. An endocytosis is initiated and the receptor-transferrin complex is internalized into an endosome. Iron remains inside the endosome while the latter crosses through the BCEC and reaches the abluminal side. Here the endosome fuses with the external membrane, exposing and then releasing Fe^{3+} to the extracellular interstitial space. The apo-transferrin remains attached to the receptor 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 re-cycling.

In the extracellular space, two Fe^{3+} atoms bind to a passing transferrin molecule, which allows iron to reach the vicinity of a neuron. The neuron membrane displays transferrin receptors, an endocytosis is again initiated and the receptor-transferrin complex is engulfed in an endosome that sinks into the neuron. Differently from the BCECs, though, the neuron possesses DMT1s which allow Fe^{2+} to be released into the neuron's intracellular space, where iron can finally complete its nutritional function.

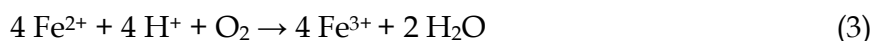
Unfortunately, a potentially dangerous outcome of this process is the reduction of iron back into Fe^{2+} . Iron is particularly dangerous in this oxidative state since it can easily enter Fenton reactions with H_2O_2 :



The hydroxyl radical $\bullet\text{OH}$ is the most reactive and vicious of all ROS species. Moreover, the above reaction easily proceeds as follows:



Fortunately, the neuron also displays ferroportin molecules on its membrane, which channel Fe^{2+} out into the extracellular space. At this point, a fundamental role in the neuron health is played by ceruloplasmin, a protein present on the astrocytic end-foot. In fact, ceruloplasmin catalyzes the oxidation of Fe^{2+} into Fe^{3+} , by way of the following chemical reaction:



Therefore, an unbalance of the ceruloplasmin content or efficiency induces iron-dependent oxidative damage to brain tissues (Patel et al., 2002).

Copper can enter exactly the same reactions, passing through Cu^{2+} to Cu^{1+} oxidative states. This confers to ceruloplasmin a special character as a 'crosstalk' factor linking copper to iron metabolism, which appears disarranged in AD.

4. Copper

4.1 Copper essentiality

Copper is also an essential nutrient for man and is normally ingested via food, although it has been recently proposed that varying degrees of intake originate from drinking water piped through copper plumbing (Brewer, 2010). Copper status in the body is regulated by both duodenal absorption (intestine) and biliary excretion (liver). 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 and into low-molecular-weight compounds, and then routed into peripheral circulation or secreted into the bile for excretion. Absorption and

excretion interplay in such a way that an occasional over-ingestion in healthy adults normally results in a down-regulation of copper uptake in the duodenum and an up-regulation of biliary excretion.

4.2 Bound and free copper

About 85-95% of copper tightly binds to ceruloplasmin, whereas the remainder loosely binds to and is exchanged among albumin, α_2 macroglobulin, amino acids, peptides and several micronutrients. We will refer to the portion that binds to ceruloplasmin as 'bound' copper, and to the portion that binds to the loose compounds as 'free' copper (Linder et al., 1979), following a custom of Wilson's Disease clinical care.

A key difference between bound and free copper lies in the fact that the limited size of the low-molecular-weight compounds and the labile nature of their binding allow free copper to easily cross the BBB (Chutkow, 1978). A recent study (Choi & Zheng, 2009) has in fact shown that the bulk of copper transport into the brain is achieved by free copper ions travelling through the BBB, whereas ceruloplasmin-bound copper represents less than 1% of the brain bound copper. 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 $\mu\text{g/dL}$) (Jacobs, 2002).

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 (Brewer, 2007). In fact, ranges of plasma/serum copper values of healthy elderly individuals reported in the literature are very heterogeneous, adding complexity in the interpretation of their results (Squitti, 2011).

4.3 Copper toxicity

Although copper is an essential nutrient for man, it becomes extremely toxic when its levels loose balance. It is a transition metal and as such takes part in a variety of biological reduction and oxidation (redox) reactions, which make it an important cofactor of many redox enzymes. As mentioned above, copper can easily go into Haber-Weiss and Fenton reactions producing $\bullet\text{OH}$ (1), against which the body has no defenses (Gutteridge & Halliwell, 1990). An overload of this metal can easily lead to oxidative reactions resulting in cell damage and death.

Moreover, copper absorption and excretion is regulated by mechanisms controlled by genetic as well as environmental factors. Therefore, a copper toxicosis is generally the result of a failure of one or more of those mechanisms, although sometimes, but infrequently, a copper excess may be due to a clinical disease, such a liver cirrhosis. The genetic origin of copper toxicosis manifests itself most clearly in the failure to express a specific copper transporter, particularly in the liver (see below). Sheep, for example, are easily subject to copper toxicosis since they are not able to increase biliary copper excretion in response to an increased intake. Conversely, pigs are known to tolerate even severe increases of copper intake very well for the opposite reason (Bremner, 1979). However, toxicity per se ultimately stems from copper's ability to catalyze the production of compounds that generate oxidative stress, as expressed in reactions (1), (2), (3) detailed above.

The disruption 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 - of systemic copper, but both resulting in neurodegeneration. In these diseases, the genes coding for the two membrane copper transport proteins ATPase7A and ATPase7B are mutated. These two proteins are highly homologous, but with different patterns of tissue expression: ATPase7A (Menkes' protein) is mainly expressed in the intestine and in the BBB, while ATPase7B (Wilson's protein) is primarily found in the liver (Harris, 2000). The mutation impairs the function of the ATPase7A pump and prevents copper absorption at the intestinal level. This causes copper deficiency and reduces the levels of cuproenzymes (Harris, 2000). The location of ATPase7A at the choroid plexus makes this pump crucial for controlling the copper flux into the ventricles of the brain (Qian et al., 1998). Indeed, the copper deficiency in the brain of Menkes' patients is particularly severe because of a decrease in the activity of ATPase7A at the BBB. Defects of ATPase7B in Wilson's disease cause impairment of copper incorporation into ceruloplasmin, with consequent failure of copper release into the bile canalicula for excretion (Iyengar et al., 1988). 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 (Choi & Zheng, 2009, Hartter & Barnea, 1988, Iyengar et al., 1988). The holo-active form of ceruloplasmin depends on the ATPase7B activity, which mediates the incorporation of copper atoms into ceruloplasmin during its biosynthesis (Bielli & Calabrese, 2002). ATPase7B 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 (Bielli & Calabrese, 2002); 2) alteration of copper excretion through the bile via ceruloplasmin (Iyengar et al., 1988); 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 (Bielli & Calabrese, 2002). During aging, control over copper homeostasis can undergo progressive failure and even "normal" copper values could result in an altered copper burden in the aged brain (Deibel et al., 1996).

4.4 Copper chaperones

Besides the obvious function as means of transportation, chaperones are also the main defense system against copper excess. Copper import into the intestinal epithelial cells is mediated by the membrane protein Copper transporter protein 1 (Ctr1) (Figure 1) (Kim et al., 2008).

Based on its structural and biochemical properties, Ctr1 could be thought of as a Cu^{+1} -specific pore. In hepatocytes, intracellular copper is carried by specific chaperone proteins to Cu-dependent enzymes. So far, researchers have identified three copper chaperones, 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, ATPase7A and ATPase7B. 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. In a healthy physiological environment, virtually no copper remains unbound (Fig. 1) (Kim, et al., 2008). The chaperone system also includes metallothioneins, a family of proteins, whose expression is regulated by copper and other metals (Palmiter, 1994) 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 (Durand et al., 2010, Meloni et al., 2008, Yu et al., 2001). It was recently proven that this protein protects cultured neurons from toxicity generated by A β . A metal swap between Metallothionein-3 and soluble aggregated A β 1-40-Cu²⁺ avoids ROS production and consequent cellular toxicity. Finally, Cu,Zn SOD, 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 (Rossi et al., 1994).

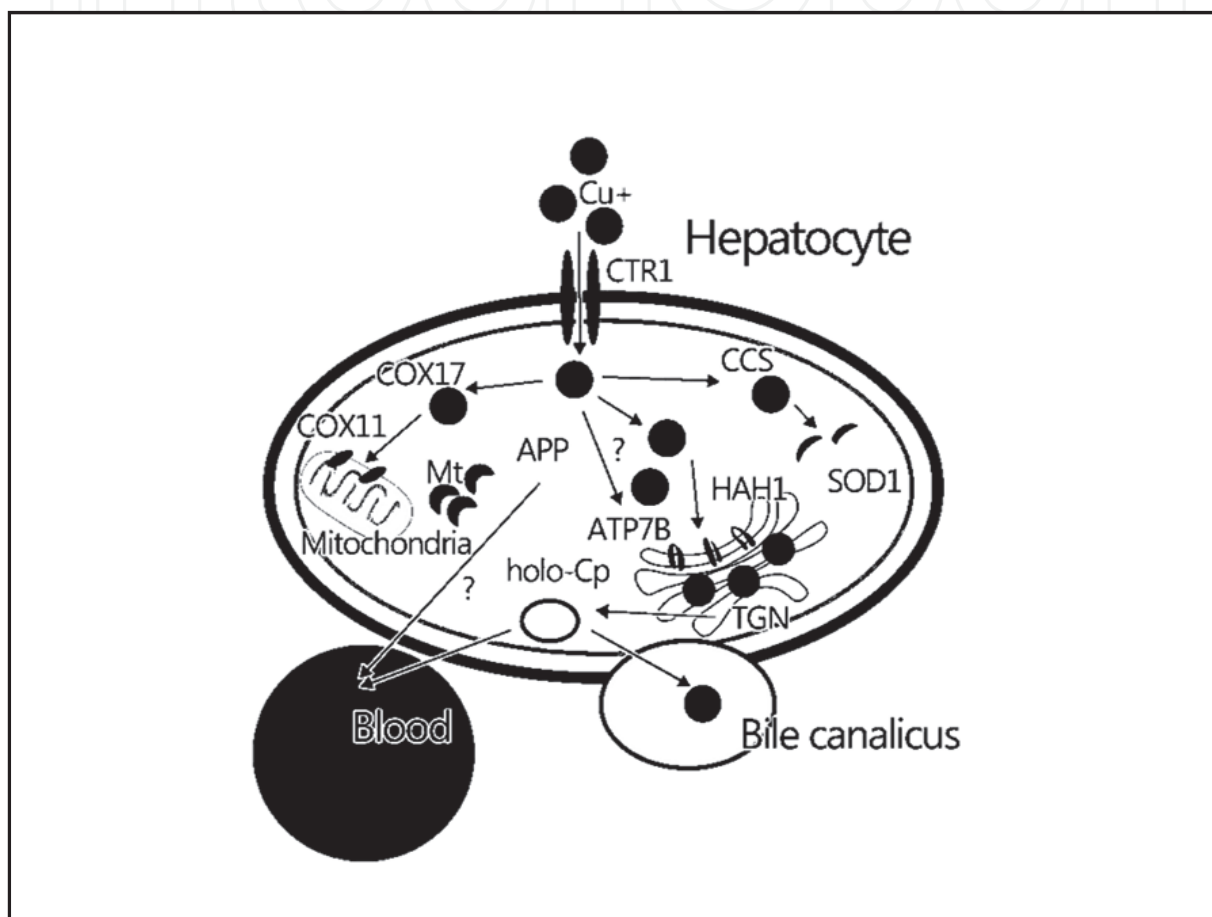


Fig. 1. Copper is metabolically finely regulated by the organism. Once copper crosses the intestinal lumen, it is transported into the liver via portal circulation. In the hepatocytes copper is incorporated into ceruloplasmin, other copper proteins and compounds and then routed into peripheral circulation 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.

4.5 Copper and Aβ

Neurodegenerative metallochemistry has developed on the observation that APP possesses selective zinc and copper binding sequences (Table 1). These sites mediate redox activity and can cause the precipitation of Aβ under mildly acidic conditions, even at very low concentrations (Atwood et al., 1998). Researchers believe that this is what happens in the AD brain (Atwood et al., 1998). Moreover, Aβ reduces the metal ions, producing hydrogen peroxide by transferring electrons to O₂ (Huang et al., 1999a, Huang et al., 1999b). This reduction is the key of Aβ-induced oxidative stress and toxicity, since hydrogen peroxide is a well known pro-oxidant molecule as it triggers Fenton's like reactions (1) (2) that generate hydroxyl radicals (see formulas in iron paragraph).

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 (Barnham et al., 2003b, Multhaup et al., 1996) Depletion of intracellular copper results in a reduction of APP gene expression (Bellingham et al., 2004b)
Zinc and copper interactions with Aβ	Zinc rapidly destabilized human Aβ40 solutions, inducing tinctorial amyloid formation (Bush et al., 1994) Aβ peptide with the sulfur atom of Met-35 oxidized to a sulfoxide is toxic to neuronal cells (Barnham et al., 2003a) Aβ peptide aggregation is induced by copper binding (Atwood et al., 2000) Aβ-Cu interaction generates ROS (Huang et al., 1999b, Multhaup et al., 1996) APP-Cu induced toxicity and oxidative stress in primary neuronal cultures, producing neuronal demise (Huang et al., 1999a, White et al., 1999a)
Solubilization of native Aβ from AD brain, transgenic mice and cell models by metal complexing agents	Solubilization of Aβ 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 (Cherny et al., 1999) bis(thiosemicarbazonato) 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 Aβ levels (Donnelly et al., 2008)
Cu-dependent catalytic conversion of dopamine, cholesterol, and biological reducing agents to neurotoxic H ₂ O ₂ <i>in vitro</i> and animal models	Aβ 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 catalyse copper-Aβ redox cycling (Opazo et al., 2002, Puglielli et al., 2005) Trace amounts of copper given to cholesterol-fed rabbits induced accumulation of Aβ in senile plaques and impaired the animals' learning capability (Sparks et al., 2006, Sparks & Schreurs, 2003)

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

APP binds copper in two domains, one located in the extracellular N-terminal region and the other in the C-terminal region within the A β peptide (review in Squitti & Zito, 2009). It has been proposed that APP reduces Cu²⁺ to Cu¹⁺ upon coordination, thus promoting the non-amyloidogenic cleavage pathway (Barnham et al., 2003b). The ablation of APP, which is normally expressed both in the brain and in a limited number of non-neural tissues as platelets, liver, kidney, and heart (Duce et al., 2010, White et al., 1999b) in knockout mice models produces metal dishomeostasis in these organs and tissues. APP-depleted knockout mice have shown a stunning 80% increase of copper concentrations in the liver and a 40% increase in the brain. Recently, Duce et al., (2010) reported that APP ablation causes an iron increase of 26% in the brain, 31% in the liver and 15% in the kidney.

As mentioned above, A β is generated from APP cleavage by α -, 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 (Edbauer et al., 2003). It was 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 (Angeletti et al., 2005), is supported by the recent evidence that copper (Cu²⁺) and manganese (Mn²⁺) potently 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 (Lin et al., 2008). *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 (Crouch et al., 2007). 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 (Crouch et al., 2005). Conversely, copper plays a role in the basal regulation of the APP gene. This has been shown in human fibroblasts over-expressing ATPase7A, a condition that causes copper intracellular depletion. Depletion of intracellular copper in these cells results in significant reduction of APP gene expression (Bellingham et al., 2004a, Bellingham et al., 2004b). 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 (Armendariz et al., 2004).

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 evidence that APP is involved in copper homeostasis as a copper detoxification/efflux protein.

Another line of evidence is the effectiveness of copper and iron chelators in hindering redox and toxic activities of A β and NTFs, whereas a replacement with those metals fully restores those activities (Sayre et al., 2000).

Regarding genetic risk factors, the *ApoE4*, which is the only well established risk factor for AD, has been demonstrated to interact with copper metabolism at different levels. It has been shown that ApoE protein possesses antioxidant properties which depend on 'bound' copper (Miyata & Smith, 1996, Moir et al., 1999, Sanan et al., 1994). ApoE4 isoform 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 . In fact, absolute and free copper concentrations are higher in carriers of the *ApoE4* allele than in non-carriers

(Gonzalez et al., 1999, Squitti et al., 2002a, Squitti et al., 2007). 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 (Babiloni et al., 2007b, Zappasodi et al., 2008).

The level of plasma homocysteine is a risk factor for AD (Babiloni et al., 2007a, Gorgone et al., 2009), and it's known that copper mediates Low-Density Lipoprotein (LDL) oxidation by homocysteine (Nakano et al., 2004).

A β binds copper and cholesterol, facilitating copper oxidation of cholesterol to 7-OH cholesterol and to 4-cholestenone (Puglielli et al., 2005), which are extremely toxic to neurons (Nelson & Alkon, 2005).

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 (Sparks & Schreurs, 2003). Thus, it appears that cholesterol increases A β formation and copper promotes A β aggregation and toxicity. Furthermore, cholesterol can catalyze copper-A β redox cycling (Opazo et al., 2002). The results from the cholesterol-fed rabbit model have been confirmed in transgenic mice (Sparks et al., 2006) 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 (Morris et al., 2006). As a result, serious concerns have been raised about a possible relationship between copper overexposure from vitamins or drinking water and cognitive disturbances (Brewer, 2010, Bush et al., 2003). The belief that copper toxicity is involved in the evolution of cognitive disturbances is also supported by recent investigations demonstrating an inverse correlation between cognitive performance and serum copper levels in cohorts of healthy aged individuals (Lam et al., 2008, Salustri et al., 2010a).

5. Metals in AD: A systemic view

The link between metals and AD has been traditionally investigated focusing on local metal accumulations in specific areas of the brain critical for AD. In this frame, authors have reported enhanced iron concentrations in AD brains, both in autopsy brain tissues and in CSF (Smith et al., 2010), in the basal ganglia (Bartzokis et al., 2000, Bartzokis & Tishler, 2000), and around SPs and NFTs (Good et al., 1992, Lovell, et al., 1998). Altered local concentrations of copper (Adlard & Bush, 2006), ceruloplasmin (Castellani et al., 1999, Loeffler et al., 1996), transferrin and ferritin (Connor et al., 1992) have been also reported.

We believe instead that the issue should be approached from the different perspective of systemic, rather than local, alterations. Results of many *in vivo* studies demonstrating correlations between abnormalities in metal homeostasis and specific deficits and markers of AD suggest that local accumulations should be viewed in a wider systemic alteration.

5.1 Iron

Besides of course its own levels in general circulation, typical markers of iron status are the levels of transferrin, ceruloplasmin, ferritin, transferrin saturation, together with the H63D and C282Y mutations of the HFE gene and transferrin's C2 polymorphism (TfC2). Since these gene variants are often associated with a distress of the liver, the classic liver function panel, i.e., albumin, transaminases (aspartate transaminase, AST; alanine transaminase, ALT) and prothrombin time, contribute to the picture.

In a study of 160 AD patients and 79 healthy elderly controls performed by our laboratory (Giambattistelli et al., 2011), we evaluated whether and how all the above listed markers of iron and liver status are interconnected. Our study revealed that AD patients have lower albumin, longer prothrombin time and higher AST/ALT values than controls, indicating a liver distress. Also, transferrin was lower and ferritin higher in AD patients. A multiple logistic regression backward analysis performed to evaluate the effects of these biochemical variables upon the probability to develop AD revealed that a simple one-unit decrease in serum transferrin 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. The role of genetic mutations is well described by the fact that while healthy controls carriers of the H63D mutation showed a normal iron status, AD patients carriers of the same mutation showed a panel resembling hemochromatosis, i.e., higher levels of iron, lower levels of transferrin and ceruloplasmin. This picture was not found in non-carrier AD patients. These results suggest that carrying the H63D 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 genetic mutation that appears to increase the probability of developing AD (Giambattistelli et al., 2011).

In another study, we measured serum levels of iron, ceruloplasmin and transferrin, calculated the transferrin saturation and evaluated the activation of the ceruloplasmin-transferrin (Cp-Tf) system, expressed by the Cp/Tf ratio, in relation to the main cognitive and anatomical deficit of AD, namely MMSE and medial temporal lobe atrophy (Squitti et al., 2010c). Results demonstrated that the values of ceruloplasmin, peroxides and Cp/Tf, besides being elevated, inversely correlated with MMSE scores, while medial temporal lobe atrophy positively correlated with Cp/Tf and negatively with serum iron levels. All these findings demonstrate that the alterations of iron metabolism that accompany the disease are systemic rather than local, and indicate that the role attributed by existing literature to local metal accumulations in brain areas critical for AD should be rather viewed in the frame of a wider systemic alteration, which could be approached taking into account both circulating biochemical markers variations and their genetic makeup. In this line, we recently explored the hypothesis that polymorphisms of ATP7B, the gene encoding the protein that controls the levels of free copper in the body and whose defects cause Wilson's disease, have higher frequencies in AD than in healthy individuals. Two groups of 190 AD patients and 164 controls were studied in order to compare the frequency of alleles for these two polymorphisms using a 'case-control' design. Two polymorphisms previously associated with Wilson's disease - GG genotype in the SNP 2495 A>G (Lys832Arg) (exon 10) and GG in c.1216T>G (Ser406Ala) (exon 2) - showed an association with susceptibility to AD (Bucossi et al., 2010). These polymorphisms also show a link to non-ceruloplasmin copper levels.

5.2 Copper

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 (Arnal et al., 2010, Bocca et al., 2005, Gonzalez et al., 1999, Smorgon et al., 2004, Squitti et al., 2006, Squitti et al., 2009, Squitti et al., 2002a, Squitti, et al., 2003, Squitti et al., 2007, Zappasodi, et al., 2008), many others no variation (Basun et al., 1991, Baum et al., 2010, Gerhardsson et al., 2008, Jeandel et al., 1989, Kapaki et al., 1989, Molina et al., 1998, Ozcankaya & Delibas, 2002, Snaedal et al., 1998), and two very recent studies even a decrease of copper in plasma (Vural et al., 2010) and serum (Brewer et al., 2010) of AD patients. Moreover, the latter

two studies are in line with two older studies showing that low plasma copper concentrations correlate with clinical worsening in AD, one reporting a copper plasma-decrease in more severe vs. less severe patients (Kessler et al., 2006), and the other a direct correlation between low plasma copper concentrations and cognitive decline (Pajonk et al., 2005).

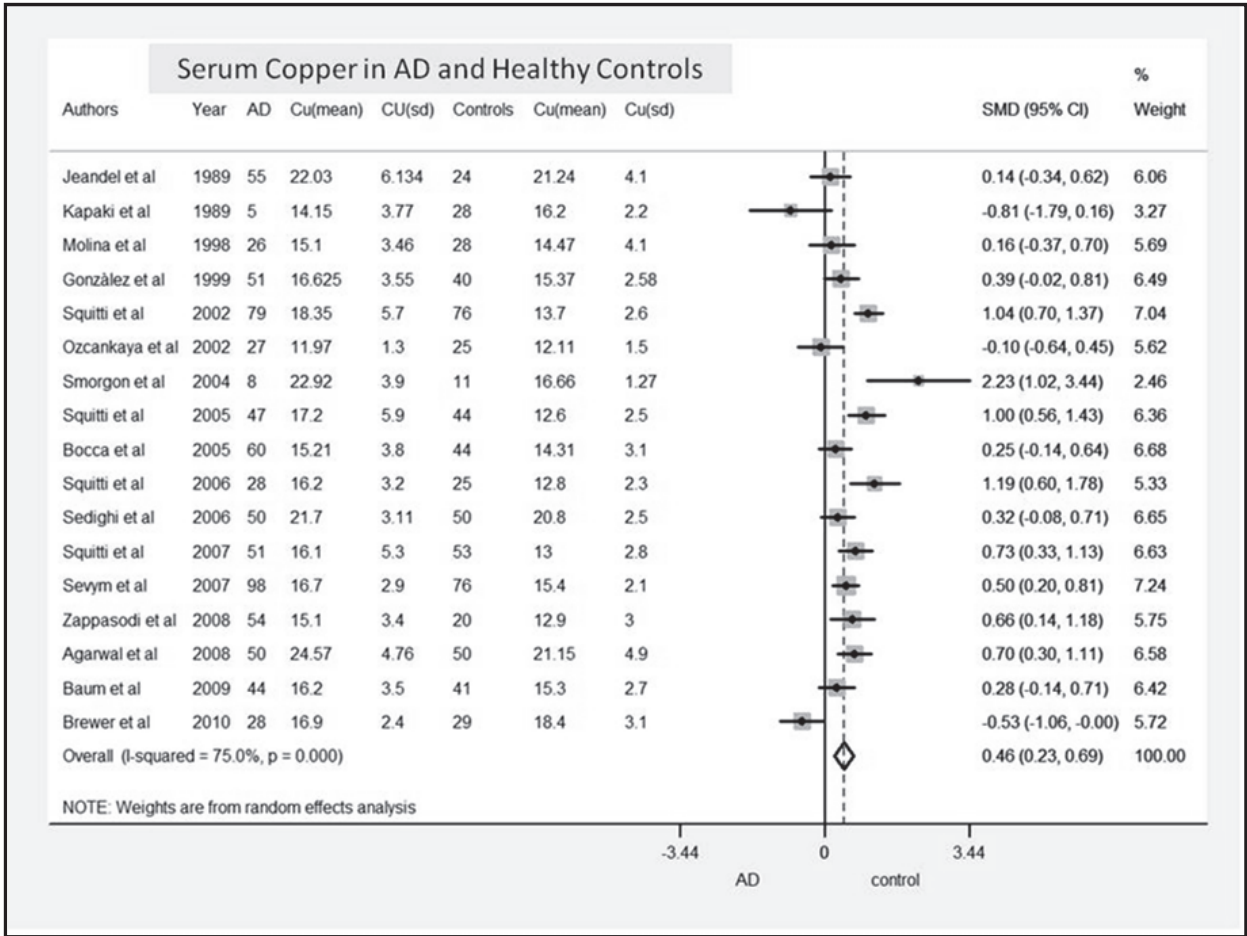


Fig. 2. Standardized mean difference (SMD) in copper serum level. The square represents the SMD between patients and controls. The size of the squares is proportional to the sample size of the study, the whiskers represents the 95% confidence interval. The diamond represents the pooled estimate based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

Recently, we have evaluated these heterogeneous results in a meta-analysis, which has analyzed data from all the serum, plasma and CSF studies published since 1983 on AD patients, to gain an objective evaluation of whether systemic copper variation are associated or not with AD. 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. After an initial selection based on statistical criteria, data from 21 studies on serum copper and 5 studies on plasma copper were merged for a pooled total of 966 AD patients and 831 controls (Bucossi et al., 2010). The analysis concluded that AD patients have actually higher levels of serum copper than healthy controls (Fig. 2). Even though moderate, the assessed copper increase is sufficient to unambiguously distinguish AD patients from healthy controls.

Plasma data did not allow conclusions because of extremely high heterogeneity, but the meta-analysis of the combined serum and plasma studies confirmed higher copper levels in AD. The analysis of CSF data, instead, revealed no difference between AD patients and controls. Previous results from our laboratory are in line with the meta-analysis outcome. When we measured copper, iron, transferrin and copper-enzymes as ceruloplasmin and Cu,Zn SOD, together with total hydro-peroxides and total radical-trapping antioxidant potential (TRAP) levels in the sera of diverse AD patients cohorts (Rossi et al., 2002, Squitti et al., 2006, Squitti et al., 2004, Squitti et al., 2002a, Squitti et al., 2005, Squitti et al., 2007), we found that copper, peroxides and Cu,Zn SOD activity (Rossi et al., 2002) were higher, TRAP was lower and ceruloplasmin and iron did not differ between AD patients and healthy controls. 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 (Squitti et al., 2003).

Diverse explanations can be advocated to account for the systemic copper abnormalities in AD. Inflammation can be one reason. 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 (Squitti et al., 2005), our laboratory 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. However, it was clear that the level of 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 between bound and free copper, results revealed that the copper increase in our AD patients was attributable to this latter fraction (Squitti et al., 2006, Squitti et al., 2009, Squitti et al., 2005). It must be remembered, though, that free copper abnormalities should be considered as an additional rather than alternative explanatory variable of copper disturbances in AD (Althaus, 2008, Arnal et al., 2010, Brewer et al., 2010, Hoogenraad, 2007).

5.3 The role of free copper

The considerations above support the notion of a fundamental distinction between the biochemical properties of bound and free copper.

The levels of free copper are higher in AD patients than in healthy individuals. One ultrafiltration study, aimed at finding filterable free copper in AD sera, has also revealed concentrations of free copper 3.7 times higher in AD patients than in controls (Squitti et al., 2006). Free copper levels correlate with AD main cognitive deficits (Squitti et al., 2006, Squitti et al., 2009, Squitti et al., 2004, Squitti et al., 2002a, Squitti et al., 2003, Squitti et al., 2005), with neuro-anatomical (Squitti et al., 2002a) and electrophysiological changes (Babiloni et al., 2007b, Zappasodi et al., 2008), with accepted AD markers (Squitti et al., 2006) and with known genetic risk factors, such as *ApoE4* (Squitti et al., 2002a, Zappasodi et al., 2008). Copper systemic abnormalities in AD resemble those observed in Wilson's disease, where the free fraction transported by micronutrients plays a fundamental role, while ceruloplasmin appears fragmented. Ceruloplasmin fragmentation, revealed by fragments <50 KDa, has been reported in AD patients showing higher-than-normal levels of free copper (Squitti et al., 2008). This suggests impairment in the incorporation of copper into the protein during the biosynthesis (see the "copper chaperones" section). It must be noted, though, that in Wilson's disease the free copper percentage is much higher than the one estimated in AD (Siotto et al., 2010) (Fig. 3).

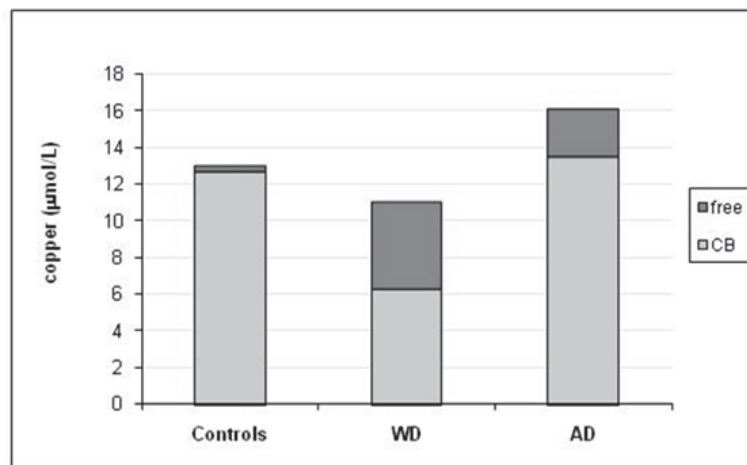


Fig. 3. Free copper in Alzheimer's disease (AD). The figure shows copper distribution its two serum pool - copper bound to ceruloplasmin (CB) and free copper - in healthy controls, Wilson's disease (WD) and AD patients.

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 clinical study by our laboratory 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 (Squitti et al., 2007). Very recently, we reproduced the same evidence in a bigger cohort of AD associated with iron abnormalities (Giambattistelli et al., 2011). 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 (White et al., 1999b). 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 (White et al., 1999b). 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 is related to CSF copper increase in AD patients, possibly interacting with A β (Squitti et al., 2006). 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 (Choi & Zheng, 2009). 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 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 that we evaluated in clinical studies on AD patients (Squitti & Salustri, 2009).

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 (Squitti et al., 2006), is sustained by the negative correlation between copper and A β in the CSF found by other authors (Strozyk et al., 2009). This evidence is also supported by the fact that copper

detectable in the CSF is not ceruloplasmin-bound, but is bound to a not yet identified ligand (Que et al., 2008).

The occurrence of copper increase or decrease in the brain is a debated issue. Some authors suggested that there is a decrease in bulk tissue levels in AD brains, and particularly in the neocortex (Adlard & Bush, 2006). Loeffler and coworkers (Loeffler et al., 1996) reported instead that ceruloplasmin and copper are actually increased in AD. Deibel and coworkers (Deibel et al., 1996) and Platin and coworkers (Platin et al., 1987) reported lower levels of copper in the amygdala and in the hippocampus of AD brains than controls, however the authors determined nanograms of copper on micrograms of weight tissue, instead of nanograms of copper on micrograms of proteins present in the sample, and it is known that the AD brain tissue has plenty of plaques and not of proteins, so their evaluation at least arises some concerns. Religa's and Squitti's groups (Religa et al. 2006, Squitti et al., 2007) did not find a copper decrease in AD. Also our recent meta-analysis of copper-related literature (Bucossi et al., 2010) revealed no difference in CSF copper between AD patients and healthy controls. So caution is needed when facing this issue. Seminal studies indicate that copper dyshomeostasis in the brain, present in normal aging (Maynard et al., 2002), is substantially more pronounced in the aged AD brain (Lovell et al., 1998). In fact, copper levels in the plaque-free neuropil of an AD brain are approximately 4 times higher than in the neuropil of a healthy brain, and approximately 30% higher in the A β plaques than in plaque-free regions.

Our laboratory has started investigating metal involvement in AD as revealed by systemic markers detectable in general circulation, and clinical indices of prognosis or AD conversion. In particular, in order to evaluate whether information on iron or copper abnormalities can help in the AD prognosis, we assessed levels of copper, iron, zinc, transferrin, ceruloplasmin, peroxides, TRAP, free copper and *ApoE4* genotype in 81 mild or moderate AD patients (Squitti et al., 2009). We studied the association of these wide set of parameters with the patients' scores in the MMSE (primary outcome), in the Activities of Daily Living (ADL) and Instrumental of Daily Living (IADL) tests (secondary outcomes), performed at study entry and after 1 year. 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%). 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 identify those patients at higher risk for a more severe decline (Squitti et al., 2009).

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 (Squitti et al., 2011). 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. A multinomial logistic regression analysis demonstrated that *ApoE4* and free copper differentiated MCI from healthy 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 (Squitti et al., 2011). 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 (Salustri et al., 2010a). We studied their free and ceruloplasmin bound copper, and measured MMSE and several other measures of memory and cognition (19 neuropsychological test battery). 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.

Subsequently, we investigated the relationship between depression, which is advocated as a risk factor of AD, markers of oxidative stress and neurotransmission, as expressed by sensory cortex excitability (Dal Forno et al., 2005, Salustri et al., 2010b). Serum levels of oxidative stress markers and somatosensory magnetic fields, evoked by external galvanic stimulation, were measured in 13 depressed patients and 13 controls. Depressives 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 compounds appear to affect neuronal excitability and clinical state of depressed patients, as free copper excess alters their cortical glutamatergic neurotransmission (Salustri et al., 2010a).

6. Treatment of AD with metal complexing agents

Since the '90s, researchers have cultivated the idea that a tuned redistribution of metals (aluminum, iron, selenium, zinc, copper) via metal complexing or ligand agents may positively affect the natural progression of AD. Numerous studies have been published reporting on tests with metal complexing agents as potential therapeutics for AD (Duce & Bush, 2010, Liu et al., 2010, Price et al., 2007), and their pros and cons, as well as the challenges associated with their usage, are under debate (Hegde et al., 2009, Squitti & Salustri, 2009, Squitti & Zito, 2009). The following is a summary of the most promising molecules with metal redistribution properties developed so far, together with a description of their potential or proven applications.

Zinc-induced aggregation of A β can be reversed by the divalent metal ion chelator ethilendiamine tetra acetic acid (EDTA)(Huang et al., 1999a). Solubilisation of A β derived from AD brains was achieved with N,N,N',N'-tetrakis (2-pyridylmethyl) ethylene diamine (TPEN) and 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline (bathocuproine) (Cherny et al., 1999). Bathocuproine, bathophenanthroline and diethylenetriamine penta-acetic acid (DTPA) halted A β redox activity complexing copper (Atwood et al., 2004).

Another metal ligand molecule [1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-bis (2-octadecyloxyethyl) ester,N,N'-disodiumsalt] DP-109 (Lee et al., 2004), was found to reduce amyloid plaques and cerebral amyloid angiopathy in Tg2576 mice.

XH1, a lipophilic amyloid-targeting metal chelator designed with amyloid-binding and metal chelating moieties, reduced APP protein expression in human cells and A β pathology in APP transgenic mice (Dedeoglu et al., 2004). Similar results were achieved with the lipophilic metal chelator DP-109, which markedly decreased A β plaques in APP transgenic mice (Lee et al., 2004).

Ammonium tetrathiomolybdate forms a stable tripartite complex with copper and proteins (Squitti & Salustri, 2009). 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, it has no known biological activity, and is largely cleared in the bile (Squitti & Salustri, 2009). A recent study tested the ability of tetrathiomolybdate to reduce A β 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 A β levels in the prevention paradigm, but not in the treatment paradigm, suggesting that lowering systemic copper may achieve anti-amyloid effects if initiated early in the disease process (Quinn et al., 2010).

Metal bis(thiosemicarbazonato) complexes - MII(btsc), where M stands for either Copper (II) or Zinc (II) - can affect extracellular levels of A β . 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 A β levels (Donnelly et al., 2008).

Iron-regulated APP in A β peptide in cell cultures were decreased by (-)-epigallocatechin-3-gallate, the main polyphenol constituent of green tea (Reznichenko et al., 2006). It was demonstrated that this molecule has metal-chelating and radical-scavenging properties that have an effect on iron metabolism in AD.

Nanoparticles conjugated to chelators were shown to easily cross the BBB, chelate metals, and exit through the BBB with their corresponding complexed metal ions (Liu et al., 2010). Early studies from this group (Liu et al., 2005) showed that these nanochelators can effectively remove iron from tissue of AD brain and also from ferritin.

Some studies (Lim et al., 2001, Lin et al., 2008) recently demonstrated the potential application of curcumin, a commonly used spice extracted by turmeric, as an anti-inflammatory and anti-oxidant molecule with neuroprotective properties. Some authors (Cole et al., 2007) demonstrated its metal chelating and neuroprotectant (Jiao et al., 2006) effects. In particular, Lin and coworkers (Lin et al., 2008) demonstrated in PC12 cells 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. 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 found to reverse the aggregation and redox activity of A β (49%) by its low affinity for copper in Tg2576 mice (Cherny et al., 2001).

6.1 Desferrioxamine

Desferrioxamine is a hexadentate chelator that does not cross the BBB. This chelator was tested for AD efficacy in a single-blinded study and it was reported to slow the clinical

progression of dementia (Crapper McLachlan et al., 1991). The target metal of the study was aluminum. 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 observed which, together with a decoppering effect, might have been at the basis of the positive clinical outcome.

Rationale	Agent	Type of study	Outcome
Induction of a negative metal balance throughout the whole body, as it happens with D-penicillamine or Zinc compounds in the treatment of Wilson’s disease.	Desferrioxamine	One single-blinded study (Crapper McLachlan et al., 1991).	The clinical progression of dementia was reported to slow down.
	D-penicillamine	Prospective, randomized, double blind, parallel, placebo controlled phase II clinical trial (Squitti et al., 2002b).	H ₂ O ₂ levels decreased in a group of AD patients with respect to both their own levels before the treatment and the levels of the AD patients who had taken the placebo
Studies based on the hypothesis that solubilization of A β plaques could be achieved by stripping them of their metal content or by facilitating the delivery of metals into the cell	Clioquinol	Prospective, randomized, double blind, parallel, placebo controlled phase II clinical trial (125-375 mg/BID over 36 weeks) (Ritchie et al., 2003).	Slowing of the rate of clinical decline in a subset of AD patients with moderate dementia
	PBT2	Prospective, randomized, double blind, parallel, placebo controlled phase II clinical trial (12-week treatment) (Lannfelt et al., 2008).	Dose-dependent reduction of A β concentrations in the CSF and positive impact on two executive functions.
Studies based on the hypothesis that copper supplementation can slow AD progression	Copper	Prospective, randomized, double-blind, placebo-controlled phase II clinical trial. Sixty-eight AD patients were given oral copper supplementation [Cu-(II)-orotate-dihydrate; 8 mg Cu daily, for 12 months (Kessler et al., 2008).	Cu supplementation did not change the rate of clinical decline.
Studies based on the hypothesis that aluminium could cause AD	Zinc aspartate	Preliminary trial with zinc aspartate administrated <i>per os</i> and i.v. for three to twelve months to 10 AD patients (Constantinidis, 1992).	Improved memory, understanding and communication in 8 of the 10 patients treated. The 2 patients who did not respond to the treatment had been given zinc aspartate only <i>per os</i> .

Table 3. Milestones in the Treatment of AD Patients with Metals or Metal Complexing Agents

The fact that desferrioxamine showed such an effect without crossing the BBB could be explained by the induction of a negative metal balance throughout the whole body, as it happens with D-penicillamine, Zinc compounds or ammonium tetrathiomolybdate in the treatment of Wilson's disease.

6.2 D-penicillamine

D-penicillamine has been widely used for treatment of Wilson's disease. It functions as a copper chelator, controlling both the reactivity and bioavailability of that metal, ultimately facilitating its disposal through the urine. However, adverse events have been reported which have drastically limited its use.

A study of D-penicillamine tolerability and efficacy in the treatment of AD had to be interrupted before finishing recruitment, due to a number of serious adverse events (Squitti et al., 2002b). Some conclusions were drawn from the patients who had completed the treatment at the moment of interruption (18 patients out of 34). Serum and urine copper were measured together with red blood cells Cu,Zn SOD (Squitti et al., 2002b, Rossi et al., 2002). While serum copper had remained stable, urine copper levels were drastically elevated, indicating that large amounts of copper were indeed being removed from the tissues. Cu,Zn SOD activity was drastically reduced, revealing that this enzyme could be used as an indicator of general depletion of bioavailable copper. A significant reduction was also observed in the hydroperoxide levels.

Despite the positive effect in decreasing the bioavailable copper and the content of hydroperoxides, the clinical relevance of those results could not be fully assessed because of the short duration (24 weeks) of the observation, during which patients in the placebo arm did not worsen, precluding the detection of cognitive differences between the treated and the placebo groups. Crapper McLachlan and colleagues (Crapper McLachlan, et al., 1991) have shown a positive effect of chelation therapy in AD by treating patients for a 24-month period, and Ritchie and coworkers (Ritchie et al., 2003) reported some positive results with clioquinol (see below) after 36 weeks.

6.3 Iodochlorhydroxyquin (clioquinol) drug class

In the early 2000s, clioquinol was tested in man revealing that a treatment of just 21 days resulted in a significant improvement of patients' cognitive performance (Regland et al., 2001). This study was followed by a case report on two AD patients showing that clioquinol could ameliorate focal cerebral glucose metabolism and halt clinical deterioration (Ibach et al., 2005), and by a double-blind placebo-controlled clinical trial, in which 36 patients were treated with 125 mg/day for the first 12 week, then 250 mg/day between week 13 and 24 and finally 375 mg/day in the latest period of weeks 25-36 (Ritchie et al., 2003). The trial revealed that clioquinol slowed the rate of clinical decline in a subset of AD patients having more severe dementia. However, in the subgroup of patients with moderate dementia the difference between clioquinol and placebo did not reach statistical significance. In addition, in this subgroup the drug had no significant effects on the plasma A β levels.

As stated by the authors, the results of the clioquinol study supported a proof of concept in humans that a drug targeting A β -metal interaction can have a significant effect on slowing the progression of AD.

Clioquinol is capable of crossing the BBB, and it is believed that it solubilizes A β plaques by stripping them of their metal content. However, after *in vitro* investigations, it is unclear

whether the positive results shown by the clinical trial were related to an attenuated A β -metal ion interaction or to some other mechanisms.

In the end, the relevance of the clioquinol conclusions has been severely questioned (Hegde et al., 2009). Arguments against the chelating effectiveness of clioquinol referred mostly to the evidence coming from a study by Treiber and co-workers (Treiber et al., 2004), who, by using a yeast model system, demonstrated that the addition of clioquinol to the culture drastically increased copper concentration within the yeast cells. Thus, they attributed the positive effects of clioquinol on AD patients to an increase of copper uptake into the neural cell, instead of an attenuated A β -metal ion interaction outside the cell. However, the authors (Treiber et al., 2004) did not report information about the bioavailability of the intracellular copper-clioquinol complex.

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 (White et al., 2006). Clioquinol, by 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. This is why molecules of the clioquinol drug class have been defined as metal protein attenuating compounds (MPAC), and have been attributed both chelating and ionophoretic properties.

Establishing the exact localization of clioquinol within the cell, or the copper bioavailability of the putative clioquinol derivatives-copper-complex, could be of help to define the molecular mechanisms either 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. In the end, clioquinol was withdrawn from human experimentation.

New compounds were then developed, such as PBT-2, a second-generation compound of the clioquinol drug class, that lacks iodine. PBT-2 has been recently tested on AD patients in a phase II, double-blind, randomized, placebo-controlled 12 week trial. 74 patients completed the trial and well tolerated the 250 mg of PBT-2 daily dosage. This study revealed that PBT2 induced a significant dose-dependent reduction of A β concentrations in the CSF, even though no drug effects on either A β 40 or A β 42 plasma levels were detected. Moreover, PBT-2 was found to preserve two executive functions, as evaluated via category fluency and trail making tests (Adlard et al., 2008, Lannfelt et al., 2008). This promising MPAC is now been tested in a phase III clinical trial.

6.4 Zinc therapy

Some agents used in anti-copper therapies are specifically aimed at maintaining a state of copper malabsorption (Squitti & Zito, 2009). Zinc acetate and other zinc salts, such as zinc carbonate, zinc sulfate, zinc gluconate, zinc oxide, zinc chloride and zinc stearate, are currently used in the treatment of Wilson's disease (Squitti & Zito, 2009). Zinc antagonizes the absorption of copper in the gut by increasing metallothioneins concentrations in the mucosa up to 25 times. The liver is the main human storage place for copper and metallothioneins are the major copper binding proteins in this organ. 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 within 2 weeks when zinc sulphate is administered in a dose of 600 mg/day. The safety and positive effects shown by zinc compounds, such as zinc sulphate or aspartate, in treating Wilson's disease

strongly recommend the testing of these compounds in AD. Encouraging results in this direction were obtained in a preliminary study by Constantinidis (Constantinidis, 1992), who reported improvements in memory, understanding, communication, and social contact in eight out of ten AD patients treated with zinc compounds.

6.5 Side effects of anti-copper complexing agents

The side effects of anti-copper complexing agents and MPAC are 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 shows a correlation with dose strength and frequency of drug assumption. For ammonium tetrathiomolybdate, a relatively new copper-lowering agent, these events have been associated with the reduction of serum ceruloplasmin levels, which is assumed to be a marker of copper defective bioavailability. For example, at ceruloplasmin concentrations of 5 mg/dL or less, bone marrow suppression is common; it is somewhat frequent at concentrations between 5 and 10 mg/dL, it is only occasional between 10-18 mg/dL, and very rare above 18.

Clioquinol usage in humans has been halted as it has been related with subacute myelo-optic neuropathy.

Zinc compounds have only minor adverse events, limited to gastrointestinal troubles.

7. Conclusion

The results of the studies described in this chapter should be regarded as single pieces of a complex mosaic of systemic disarrangements which result in severe neurodegeneration via a variety of biochemical phenomena, among which oxidative stress plays a leading role. In this view, systemic metal abnormalities, even if mild but over a long period of time, have disrupting effects on central processes controlling the defenses against metal-induced damage. Glutamatergic synapses definitely represent focal sites where the metal-A β toxic action can be promoted resulting in A β precipitation (Bush et al., 2003, Bush & Tanzi, 2008). The identification in AD of a free copper serum-level increase, as well as of disturbances of iron homeostasis and the activation of the antioxidant Cp-Tf system, appear coherent with this picture. Not only can free copper cross the BBB and enter reactions promoting A β toxicity, but it can systemically activate the Cp-Tf system, promoting iron subtraction from general circulation and its consequent internalization into neural cells. Both pathways converge towards neurodegeneration and neuronal death. 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, may possibly have a positive effect on the natural progression of AD.

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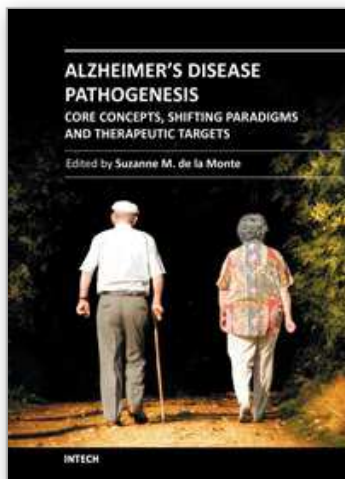
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Alzheimer's Disease Pathogenesis: Core Concepts, Shifting Paradigms, and Therapeutic Targets, delivers the concepts embodied within its title. This exciting book presents the full array of theories about the causes of Alzheimer's, including fresh concepts that have gained ground among both professionals and the lay public. Acknowledged experts provide highly informative yet critical reviews of the factors that most likely contribute to Alzheimer's, including genetics, metabolic deficiencies, oxidative stress, and possibly environmental exposures. Evidence that Alzheimer's resembles a brain form of diabetes is discussed from different perspectives, ranging from disease mechanisms to therapeutics. This book is further energized by discussions of how neurotransmitter deficits, neuro-inflammation, and oxidative stress impair neuronal plasticity and contribute to Alzheimer's neurodegeneration. The diversity of topics presented in just the right depth will interest clinicians and researchers alike. This book inspires confidence that effective treatments could be developed based upon the expanding list of potential therapeutic targets.

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