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Metals Involvement in Alzheimer's Disease — A Patho-Genetic View

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1. Introduction

As Alois Alzheimer himself first observed, the brain of an individual affected by Alzheimer's disease (AD) shows aggregations of the peptide beta-amyloid ($A\beta$) and tau proteins, which form characteristic plaques and neurofibrillary tangles respectively [1].

Since $A\beta$ is known to participate in many normal body functions, its precipitation into plaques, often referred to as 'amyloid cascade', has been for a long time the only recognized, yet unexplained mechanism of AD pathogenesis [2]. In time, however, diverse phenomena, such as oxidative stress, aberrant inflammations, impaired energy metabolism and more have been gradually discovered to contribute to the cascade [3].

The observation that $A\beta$ aggregation in plaques is an age-dependent phenomenon, whereas $A\beta$ production is not, suggested that some other age-dependent mechanism must play a role in transforming $A\beta$ into a neurotoxic element. The fact that some metals, such as copper and zinc, are known to modulate glutamatergic neurotransmission [4] led researchers to hypothesize that late-age abnormalities in the homeostasis of one or more transition metals may play a role in the amyloid cascade.

Moreover, much evidence gathered on AD depicts an improperly functioning ceruloplasmin, an enzyme synthesized by the liver, which controls iron oxidization state. There is also evidence that a functional failure of systemic ceruloplasmin may be behind the iron-related redox processes that produce oxidative stress in the AD brain [5, 6]. Ceruloplasmin is the 'crosstalk' factor linking copper to iron metabolism, thus its failure is very likely to be a major actor in the dysfunctional metal metabolism affecting AD individuals. $A\beta$ may gain toxicity

upon some interaction with copper, in a process involving ceruloplasmin, even though the molecular mechanism still remains elusive.

This notion was further supported by the fact that the Amyloid precursor protein (APP) was discovered to possess selective copper binding sites, which mediate redox activities causing precipitation of A β even at low concentrations [7]. A β itself has been reported to possess selective high- and low-affinity metal-binding sites which, in normal conditions, bind equimolar amounts of either copper or zinc but, in conditions of acidosis, see zinc completely displaced by copper [8]. Thus, hyper-metallation was suggested to be the mechanism that gives A β its redox properties, triggering redox cycles through production of H₂O₂ that lead to self-oxidation of A β , formation of oligomers with diverse grades of complexity and finally to A β precipitation into plaques (Figure 1) [4, 9].

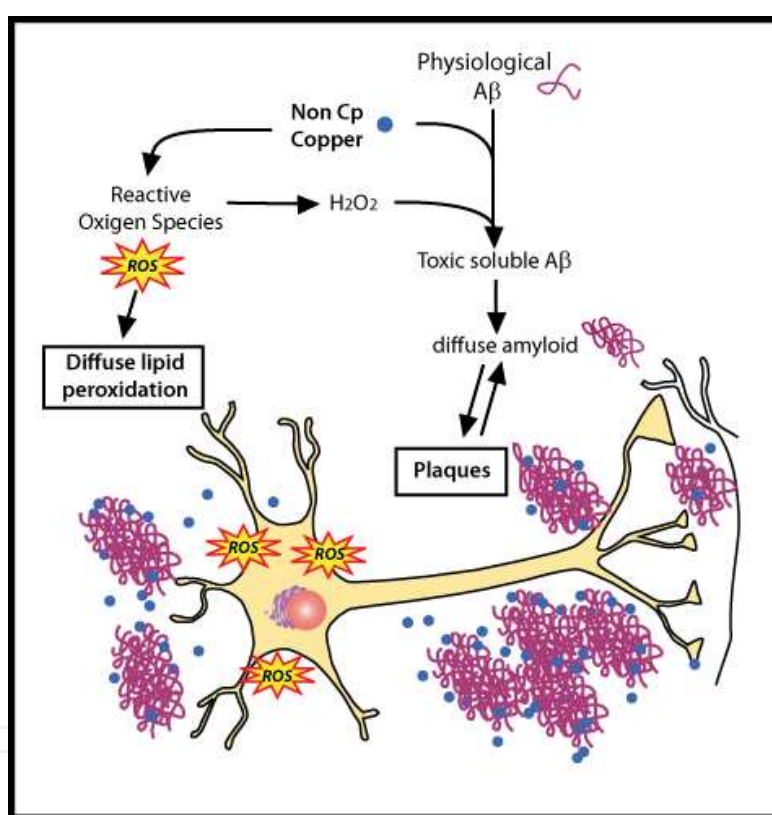


Figure 1. Systemic Non-Cp-copper in AD passes through the Blood Brain Barrier and causes an imbalance of copper in the brain. Here Non-Cp-copper can interact with physiological amyloid- β (A β), forming clusters of metal-toxic soluble A β that evolve in diffuse amyloid and, finally, in toxic plaques. Non-Cp-Copper can also interact with reactive oxygen species (ROS), which are responsible for lipid peroxidation in neuron membranes, protein oxidation, and cleavage of DNA and RNA molecules.

Diverse animal studies support the toxic role of copper in AD pathogenesis.

White et al. [10] showed that the copper contents of both liver and cerebral cortex in *APP*^{-/-} and amyloid precursor-like protein (*APLP2*^{-/-}) knockout mice were significantly increased, supporting the authors' hypothesis that the *APP* gene modulates hepatic and cortical copper

levels. However, the mechanism leading to increased brain copper content (due to *APP* gene knockout) remains known. Serum copper levels were not significantly more altered in *APP*^{-/-} and *APLP2*^{-/-} mice than in wild-type mice. It appears that a failure of copper excretion from the brain to the blood or, more likely, an imbalance of copper distribution between the Cp-bound and unbound fractions could be associated with the deposition of excessive copper levels in the brain in this knockout model. Besides demonstrating that the AβPP is an important regulator of brain copper homeostasis, White et al. also demonstrated that it potentiates the Aβ-mediated neurotoxicity by increasing oxidative stress.

Sparks and Schreurs [11] demonstrated that adding 0.12 ppm (0.12 mg/L) of copper to water given to a cholesterol-fed rabbit AD-model resulted in significantly enhanced cognitive waning and also exacerbated amyloid plaque deposition. This finding led to serious concerns in some Government Environmental Agencies about the content of copper in drinking water delivered to households via copper pipes. It must be kept in mind that cholesterol, though vital for neuronal transmission, synaptic plasticity and cell function, is also a well-established risk factor for atherosclerosis and AD [12]. Cholesterol to 7-hydroxy cholesterol oxidation, caused by Aβ, is extremely toxic for neurons [13]. Cp levels measured using o-dianisidine dihydrochloride as a substrate in the plasma of cholesterol-fed rabbit model after adding copper to drinking water, suggest an increase, although the change did not reach statistical significance. This suggests that a Non-Cp copper increase is a vehicle of copper within the brain.

In a different investigation [14], Sparks reconfirmed this earlier finding, reporting that other animal models, like spontaneously hyper-cholesterolemic Watanabe rabbits, cholesterol-fed beagles and rabbits, and *PS1* and *APP* transgenic mice showed considerably increased brain levels of Aβ when given copper-rich (0.12 ppm) drinking water. Notably, non-cholesterol fed *PS1* and *APP* transgenic mice models of AD demonstrated significantly enhanced levels of Aβ due to copper exposure via drinking water, demonstrating that the mouse model of AD exhibits vulnerability to copper even in the absence of cholesterol in the diet. This observation highlights the fact that both cholesterol and copper are separate causative factors whose interaction further enhances the formation of Aβ plaques.

In another study [15], Lu et al. confirmed the findings of Sparks and colleagues'. The authors demonstrated that Kunming strain mice fed with a high-cholesterol diet and distilled water containing 0.21 ppm copper exhibited significantly increased level of *APP* mRNA, coupled with the activation of caspase-3 in the brain, suggesting apoptosis mediated neurotoxicity. Strikingly, copper also increased cholesterol-induced learning and memory impairment in mice.

Moreover, it has been reported [16] that, in Sprague-Dawley rats, which underwent bilateral common carotid artery occlusion (2VO) and were administered with 250 ppm copper containing water for 3 months, chronic copper toxicity exacerbated memory impairment induced by 2VO coupled with an augmented expression of brain AβPP and β-site AβPP-cleaving enzyme 1 (BACE1) at both mRNA and protein levels. However, these copper-aggravated changes were ameliorated after copper was withdrawn from the drinking water.

As a whole, these experimental animal models demonstrated the toxicity mediated by copper in the AD cascade, showing that increased level of copper ingested with drinking water, or more generally through the diet, affects AD neuropathology.

All this evidence has eventually led to the proposal of the so called Metal Hypothesis of AD (Bush et al. 2008), which is based on the concept that it is the interaction of A β with specific metals, especially copper, that actually drives the amyloid cascade and AD pathogenesis.

One question remained: how does copper actually reach the brain? In fact, we normally ingest copper through the diet - via food, drinking water, beverage, supplements - and copper status in the body is regulated by the balance between duodenal absorption (intestine) and biliary excretion (liver). After crossing the intestinal lumen, copper is transported via portal circulation to the liver, where it is partly stored and partly redistributed to other organs. In the hepatocyte, copper is incorporated into ceruloplasmin, whose dimensions don't allow an easy crossing of the blood-brain-barrier (BBB).

An answer to the question came with the discovery that, although the vast majority of human copper circulates tightly bound to ceruloplasmin [17, 18], a faulty copper metabolism leads to the creation of a small pool of copper that goes into circulation loosely bound to and constantly exchanged among albumin, α 2 macroglobulin, peptides, amino acids and other low-molecular-weight compounds. Due to the loose character of the bindings, this portion is normally referred to as Non Ceruloplasmin copper (Non-Cp copper). The key difference between bound (to ceruloplasmin) and Non-Cp copper lies in the fact that the low-molecular-weight compounds can easily cross the BBB [18], thus carrying Non-Cp copper into the brain. There, copper can enter cycles of Haber-Weiss or Fenton reactions producing \cdot OH, against which our body has no defenses [19], and generate pleiotropic effects on the amyloid cascade [3].

The metal hypothesis has also gained support from consistent reports of enhanced concentrations of labile copper in areas of the brain that are considered critical for AD [20].

There is by now a solid body of literature reporting *in vitro* [8, 21, 22], experimental (reviewed in [23, 24]) and clinical evidence gathered over the last years which have shown that some systemic abnormalities in copper metabolism are shared between the AD and the Wilson's disease (WD). Wilson's disease is the paradigmatic disease of copper toxicosis or accumulation [18]. Although much less severe than in WD, it has been shown that the increases in Non-Cp copper correlate with some typical AD deficits, with the 'core' markers of AD in the cerebrospinal fluid [25], a poor prognosis of the disease [26], and the conversion from Mild Cognitive Impairment (MCI) to full dementia [27]. Meta-analyses have confirmed increased levels of total copper and Non-Cp copper in general circulation of AD patients compared with healthy control subjects [28-30]. A higher intake of copper in the diet was also associated with cognitive decline or with an increase in overall mortality. Specifically, in the 'Chicago Health and Aging Project' (3718 subjects followed from 1993 to 2002), a diet with a content of 2.75 mg / day of copper on average, along with a high saturated and trans fat intake, has been proved to be associated with cognitive decline, which was estimated to be equivalent to an extra nineteen years of aging [31]. In the 'Iowa Women's Health Study' (38772 older women followed from

1986 to 2008), the use of dietary supplements of copper has been shown to be associated with a 18% increase in total mortality [32]. Recently, it was shown that the increase of copper in the soil in 26 provinces and 3 municipal districts in China, between 1991 and 2000, is associated with an increased AD-related mortality. In geographic areas with higher concentrations of copper, the relative risk of AD-related death is 2.6 times higher than that in geographical areas with a lower content copper in the soil [33].

Also results of a recently completed Phase II clinical trial, based on using metal attenuating complexing compounds or Zinc therapy [34-37], appear to support the notion of a copper dysfunction in AD. The available evidence has now reached such a quantitative and qualitative level that the notion of a copper-related phenotype in AD has now started to be accepted [23]. This is a very important step, since many translational hypotheses may develop from this notion, in terms of both diagnostic and prognostic tools, with important repercussions in terms of preventive and therapeutic approaches. However, most of the literature dealing with the relationship between copper and AD focuses on local copper abnormal distribution, especially in those specific areas of the brain that are considered critical for the disease. Recently, this vision has started to appear limited. There is now a bulk of evidence suggesting that all modifications should be viewed in a wider framework of systemic, rather than local, metal dishomeostasis. This concept can be better understood looking at recent studies of the link between the status of serum ceruloplasmin and AD clinical signs and/or A β markers in the CSF [23]. Torsdottir et al. [38] reported a decrease in ceruloplasmin activity in AD patients. Lower levels of circulating ceruloplasmin in AD patients with different CSF markers of AD were reported by Brewer's [39], Arnal's [40] and Kessler's [41] groups. In 2008, our laboratory demonstrated a consistent and measurable increase of apo-ceruloplasmin (a defective form of ceruloplasmin, lacking copper and its ferroxidase activity) in the serum and CSF of AD patients [42, 43].

Since both WD and the early-onset form of AD are known to have a genetic origin determining the heritability of the disease, researchers have embarked in a wide range of studies in the attempt to find genes that cause the late-onset AD or at least contribute to it via damaging phenomena, such as oxidative stress, inflammation, apoptosis or an increased expression of A β . In order to encompass as much as possible of the huge genome world, researchers have also embarked in so-called large-scale genome-wide association studies (GWAS). These studies search for DNA sequence variations that appear more common in individuals with a certain disease than in individuals without that disease. GWAS typically analyze a multitude of single gene variations, generally called single-nucleotide polymorphisms (SNPs), and verify their association, if any, with the traits of a disease.

2. AD and APOE

So far, no specific gene has been found that can be reliably considered a cause of AD. Even genes, whose mutations have been found responsible for early-onset AD, appear to have a minor, or at least not a pivotal role in the late-onset form. However, numerous risk factors have

been identified in the last few years. Historically, the first one to be established is the inheritance of the $\epsilon 4$ allele of the apolipoprotein E (*APOE*), found on chromosome 19. *APOE* is the gene encoding the protein that carries cholesterol and other fats into circulation and manifests itself in a number of alleles, of which $\epsilon 2$, $\epsilon 3$ and $\epsilon 4$ are the most common. The allele $\epsilon 3$, the most common of the three, appears totally unrelated to the risk of developing AD. The slightly less common $\epsilon 4$, instead, has been definitely established to increase the risk of developing AD. The rare variant $\epsilon 2$ seems instead to provide some form of protection by delaying the onset age of the disease.

Individuals inherit two copies of the *APOE* gene, one from each parent, which can be different alleles. If one of the inherited alleles is $\epsilon 4$, the carrier has about a 3-fold increased risk of developing AD. Two copies of $\epsilon 4$ make this risk is much higher, reaching a 15-fold increase of the risk. In other words, it can be stated that *APOE* $\epsilon 4$ carriers have a 90% statistical risk of contracting AD if they are heterozygote and close to 100% if homozygote [44].

It must be emphasized, though, that we are dealing here with statistical risk: in fact, not all individuals who have one or two $\epsilon 4$ develop AD and AD occurs also in people who have no $\epsilon 4$. Thus, *APOE* $\epsilon 4$ is a 'susceptibility' gene, i.e., a gene that affects risk but is not the cause.

3. Other genes

The fact that *APOE* $\epsilon 4$ is neither necessary nor sufficient for the development of the disease has supported the quest for more genetic risk factors and GWAS have led to the identification of numerous genes now widely accepted as risk factors for AD. Major examples include:

CLU – The *CLU* gene on chromosome 8 encodes the protein clusterin, or apolipoprotein J, which is implicated in multiple biological processes, such as lipid transport, membrane recycling, cell adhesion and apoptosis. Two GWAS have independently found a statistical association between a SNP within *CLU* and the risk of having AD [45, 46]. Despite the fact that people who already have AD have more clusterin in their blood, and that clusterin blood levels correlate with faster cognitive decline in individuals with AD, there is no indication that levels of this protein can predict the onset of AD.

SORL1 – an *APOE* receptor in the neural system. Some variants on chromosome 11 have been related to AD [47], since a significant decrease in their expression has been found in AD patients and some authors have described a link between *SORL1* and APP regulation.

CR1 – encodes the protein C3b/C4b receptor, whose deficiency may contribute to chronic inflammation in the brain. Some specific variant of this gene have been identified as contributors to AD [46, 47].

PICALM - located on chromosome 11, encodes phosphatidylinositol-binding clathrin assembly protein, which is linked to the process by which brain nerve cells (neurons) communicate with each other. GWAS have identified several functional SNPs in the *PICALM* gene [45-50]

In AD, GWAS have consistently shown that the effect size and the strength of association of *APOE* variants are greater than the best of *APOE*-unrelated associations. This stresses the

relevance of *APOE* as risk factor for AD, although also other explanations should be considered: firstly, most GWAS do not study the variants' true susceptibility but are rather based on the identification of their tagging markers. The latter generally show much greater heterogeneity than the former in terms of both alleles and extent of linkage. Consequently, it is possible that some variants have in reality bigger effect sizes than the ones seen by GWAS. Secondly, current GWAS platforms are often insufficient to detect rare variants. Thus, the existence of some variants that have a big effect size but happen to be rare can remain undetected. Moreover, the linear modeling framework often used in GWASs considers only one SNP at a time, thus ignoring the genomic and environmental context of each SNP [51]. This is an important limitation since the genotype-phenotype relationship is most likely characterized by significant genetic heterogeneity and complex gene-gene and gene-environment interactions [52]. Recently, there has been a shift away from the 'one SNP at a time' method toward a more holistic approach that includes the gene's environmental context.

Another approach is selecting a candidate gene on the basis of hypotheses regarding the disease and then analyze all sequence changes of that gene. Direct sequencing has proven an effective way to discover rare variants with large effect sizes. Recently, studies using next-generation sequencing have led to the identification of rare frequency coding variants in *PLD3* [53] and *TREM2* genes [54, 55] associated with the risk of AD.

3.1. *ATP7A* and *ATP7B*

Two serious disorders are today recognized to be due to a dyshomeostasis in copper metabolism: Menkes disease (MD) and Wilson's disease (WD). Both are caused by a mutation of one gene: *ATP7A* in MD and *ATP7B* in WD, which generate a dysfunction of the proteins they encode: ATP7A and ATP7B, respectively [56]. These two proteins are copper pump proteins or transporting ATPase and, although they have somewhat different distributions in diverse cell types, both are key in regulating copper levels in the body.

The *ATP7A* gene is located on the long (q) arm of the X chromosome. The protein it encodes, ATP7A, is found virtually everywhere in the body except in the liver and delivers copper wherever it is needed within tissue cells. In the small intestine, it contributes to control the absorption of copper from food. Mutations of the *ATP7A* gene lead to synthesis of dysfunctional proteins, of which some engage in disorderly copper transport while others are even unable to bind the metal. More than 100 mutations have been identified as causes of MD.

ATP7B is a gene of chromosome 13, expressed mainly in the liver, although it has also been detected in the brain, heart, kidney, lung, mammary gland and placenta [57]. The protein it encodes, ATP7B, maintains the body copper homeostasis. In the hepatocyte, ATP7B receives copper from ATOX1 and it moves across the trans Golgi network where it incorporates this metal into apo-ceruloplasmin generating a holo-active form. When intracellular copper levels exceed cell needs, ATP7B moves toward the bile canaliculi and carries out the excretion of copper [58].

Technically, mutations of the two genes have somewhat opposite effects: in MD, the dysfunctional *ATP7A* results in an unbalanced distribution of copper throughout the body. Copper

accumulates in some tissues while it remains insufficient in others, where the decreased supply reduces the activity of enzymes that are necessary for the health of numerous body parts, such as bone, skin, blood vessels, and the nervous system. In WD, instead, the dysfunctional *ATP7B* causes: (i) a failure in the incorporation of copper into the holo-ceruloplasmin, resulting in increased levels of Non-Cp copper being released into the blood stream and in low circulating levels of that protein; (ii) decreased levels of copper released into the bile canaliculi, causing cell over-feeding and intracellular copper deposits, which accelerate apoptotic cell death [59].

We know that in WD huge amounts of Non-Cp copper enter the brain through the BBB, where labile copper accumulates and leads to neurodegeneration. WD is considered the hallmark of copper toxicosis.

Since increased copper levels characterizing WD are shared in a smaller scale by AD patients, the *ATP7B* gene and its variations have of course become the focus of much of our research focusing on copper. Some authors have expressed doubts about this research direction by pointing out that neurodegeneration in WD leads to movement disorders with little or no effect on cognition, while AD neurodegeneration affects chiefly cognition. Moreover, onset ages are very different in the two pathologies as WD appears typically in childhood, whereas late-onset AD by definition after 60-65y. In other words, even accepting the fact that the two pathologies share copper toxicity, manifestations are so different that it seems unreasonable to claim that the same gene causes both of them. In reply to this valid concern, other authors have argued that *ATP7B* has a high variability. Thus, the possibility that this gene is a causative gene for WD when in homozygosis and a susceptibility gene for AD (interacting with environmental factors and other risk genes) cannot be ruled out. Other genes have shown unexpected diverse effects. *ATP7A*, for example, which is causative of MD, has been recently recognized to be associated with a mild form of occipital horn syndrome [60]. Moreover, recent evidence has demonstrated that certain missense mutations of this gene can cause a syndrome restricted to progressive distal motor neuropathy even without signs of copper deficiency [61].

Our laboratory has pursued the link between copper and AD pathogenesis on the assumption that the excessive Non-Cp copper production in the body is actually due to a faulty *ATP7B* causing a flaw in the incorporation of copper into nascent ceruloplasmin in the liver [56]. On this basis, we have embarked in an extensive study of the *ATP7B* gene and of the protein *ATP7B*, which is the only one known to catalyze that incorporation.

Unfortunately, analysis of the *ATP7B* gene is not an easy task, due to its huge variability. The 1000 Genomes project has identified 1,358 variations of *ATP7B* in human populations. At least 500 of them have been recognized as disease-causing [62]. Worldwide detection of *ATP7B* mutations is actually difficult [63] since most mutations are rare, reported only within single families and often prevalent in specific ethnic groups [32]. As a result, the database regarding both the gene's properties and the possible dysfunctions of the proteins they encode is still largely insufficient [64].

The first significant information on the structure of the *ATP7B* protein was gained from a homology modeling study [65]. Some more light was later shed by nuclear magnetic resonance (NMR) spectroscopy studies [66-70]. Recently, significant progress in the comprehension of

ATP7B structural organization has come from the solution of the crystal structure of the bacterial copper ATPase LCopA [71]. The LCopA protein model has been employed as a template to analyze ATP7B core domain on the basis of its sequence homology to build interpretations of WD mutations [64].

We know that mutations leading to a complete abolition of ATP7B function, chiefly early stop mutations and mutations in regions of the gene that have a high functional importance, lead to an early and predominantly hepatic dysfunction. Conversely, point mutations in regions that are functionally less important are associated with a later onset and predominantly neurological or psychiatric dysfunctions [17].

An effective strategy to characterize genetic compositions, which has become popular in recent years, is the so-called *in-silico* analysis, i.e., performed via computer simulations. Several computational procedures have been developed to analyze the effects of genetic variants on the protein function. The advantage of this type of analysis is that it allows analysis of huge amounts of data, delivered by high-throughput sequencing technologies, in relatively short time. These procedures take into account different factors associated with the protein properties, such as chemistry constraints, three dimensional structure, and amino acid sequences of homologous and orthologous proteins [72].

Our laboratory has used a new *in-silico* approach based on amino acid sequence, utilizing four among the most used bioinformatics tools (i.e. Polyphen- 2, SIFT, Panther, and PhD-SNPs). We have applied this approach to non-synonymous SNP (nsSNPs) detected in the *ATP7B* gene to profile WD-causing and WD-non-causing mutations, while obtaining at the same time useful information about the gene's domains that could potentially harbor loci of susceptibility for other disorders related to copper metabolism [73].

3.2. rs7323774 and rs2147363

In pursuit of the identification of regions in the *ATP7B* gene linked to copper dishomeostasis, our laboratory has embarked in an investigation of the association between copper-related biochemical markers in serum (bound copper, Non-Cp copper and ceruloplasmin) and variants of the *ATP7B* gene.

In one study of 399 AD patients and 303 healthy elderly controls, we focused our attention on a set of four SNPs that had been reported to be informative of the *ATP7B* gene structure [74]: rs1801243 (missense substitution: Ser406Ala), rs2147363 (intronic variant: c.1544-53A>C), rs1061472 (missense substitution: Lys832Arg) and rs732774 (missense substitution: Arg952Lys). We had already found in a previous study that those SNPs among the four that lay in the transmembrane domains appear to have an association with AD [75].

We first stratified the AD and control groups into three 'classes' according to their Non-Cp copper levels: 'low Non-Cp copper' (<1 µmol/L), 'medium Non-Cp copper' (≥1, <1.6 µmol/L) and 'high Non-Cp copper' (≥1.6 µmol/L) (Figure 2). Results showed antithetic distributions for patients and controls: the 'low Non-Cp copper' class accounted for 27% of AD cases vs. 61% of controls; the 'medium Non-Cp copper' for 11% of AD vs. 10% of controls and the 'high Non-Cp copper' for 62% of AD versus 29% of controls. The serum copper profile was then

analyzed in relation to the selected gene variants in the sole 'high Non-Cp copper' class (patients with AD, n = 109; controls, n = 53).

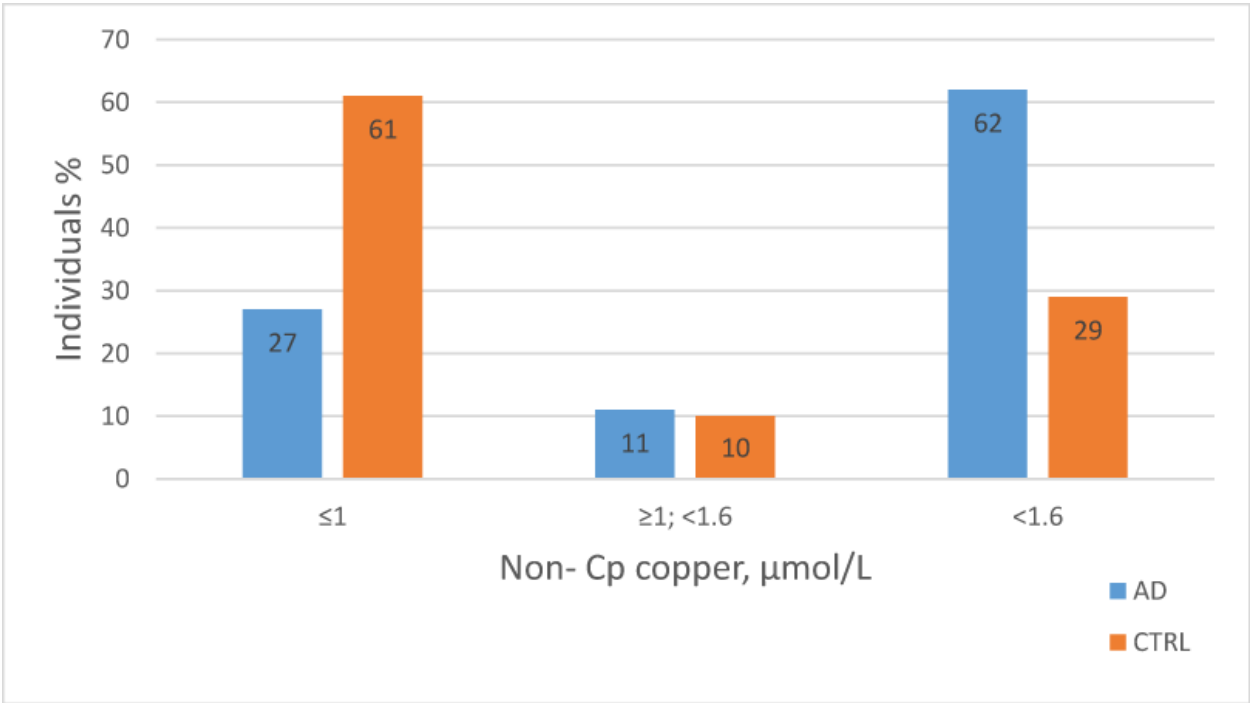


Figure 2. Stratification of the AD and control groups (CTRL) into three 'classes' according to their Non-Cp copper levels: 'low Non-Cp copper' ($<1 \mu\text{mol/L}$), 'medium Non-Cp copper' ($\geq 1, <1.6 \mu\text{mol/L}$) and 'high Non-Cp copper' ($\geq 1.6 \mu\text{mol/L}$). The bar graph reports the percentage of AD (first bar from left) and CTRL (second bar from left) for each class.

The main result of this study was that individuals who are GG homozygous for *ATP7B* rs7323774 SNP have higher levels of Non-Cp copper in their serum. This was true for the entire sample of patients and controls but was definitely more pronounced in AD individuals. Non-Cp copper distributions of AD patients turned out antithetic to those of controls and results showed that this is due to those *ATP7B* variants that are located in regions encoding *ATP7B* transmembrane domains, i.e., variants that we know to be associated with copper dyshomeostasis in AD. It must be noted, however, that we selected the variants on the basis of the information they could deliver on the *ATP7B* gene structure, not for their impact on gene function. Consequently, it is obvious that rs1801243, rs2147363, rs1061472 and rs732774, even though significantly associated with AD [74], could not be the loci responsible for the effects of the *ATP7B* gene on AD, either in terms of Non-Cp copper derangement or in terms of an increased risk of the AD [76].

In another study of 286 AD patients and 283 controls we focused on the rs2147363 and rs7334118 variants of the *ATP7B* gene. All genotype frequencies in our controls were within the ranges reported previously in the European origin populations of the HapMap project (available on <http://hapmap.ncbi.nlm.nih.gov>).

We analyzed the genetic association between SNPs and AD risk. Our study revealed a significant association between rs2147363 and AD. When data were adjusted for confounding variables (i.e., age, gender, and *APOE* genotype), significant results were obtained for the recessive model (OR: 1.63, 95% CI: 1.03–2.57; $p = 0.035$) and Log-additive model (OR: 1.51, 95% CI: 1.05–2.16; $p = 0.025$).

In order to verify whether rs2147363 has any functional variants in LD, we also analyzed the SNPs that showed a complete LD ($D' = 1$) with this *ATP7B* variant in Tuscans in Italy (TSI), which is the HapMap population most genetically related to our sample. We identified ten SNPs in complete LD with the rs2147363. We prioritized these variants using FastSNP. This analysis highlighted that one variant is a non-synonymous SNP (medium-high risk), four are intronic enhancers (very low-low risk), four are intronic with no known function (no effect), and one is a downstream variant with no known function (no effect) (Table 1).

SNP ID (rs)	Possible Functional Effects	Region	SIFT	PolyPhen2
rs7334118	Missense (non-conservative); Splicing regulation	coding	Damaging	Possibly Damaging
rs4943053	Intronic enhancer	intronic	-	-
rs9535803	Intronic enhancer	intronic	-	-
rs9535809	Intronic enhancer	intronic	-	-
rs2147362	Intronic enhancer	intronic	-	-
rs11839458	Intronic with unknown function	intronic	-	-
rs9526820	Intronic with unknown function	intronic	-	-
rs9535827	Intronic with unknown function	intronic	-	-
rs9535794	Downstream with unknown function	3-UTR	-	-
rs9535806	Intronic with unknown function	intronic	-	-

Table 1. Prioritization and functional analysis of *ATP7B* SNPs in LD with rs2147363.

To predict the functional impact of the non-synonymous SNP (rs7334118), we used two different bioinformatics tools: SIFT and Polyphen2. The application of both the SIFT algorithm using orthologous sequences and the Polyphen2 tool highlighted that this coding variant may have an adverse effect on the *ATP7B* protein.

To verify the hypothesis that genetic association of rs2147363 is due to LD with the rs7334118 SNP, 176 AD subjects and 169 healthy controls among the study population were genotyped for the SNP rs7334118. Two AD patients were carriers of the rs7334118G allele, whereas no healthy individual with this *ATP7B* mutation was identified. Even though this coding variant was identified only in AD patients, its allele frequency in the AD group is in line with the minor allele frequency observed in a general population of European origin (information available at dbSNP). Thus, this result has to be confirmed in a bigger healthy control sample.

To test whether the intronic region, in which rs2147363 is located, plays a role in the regulation of the *ATP7B* gene function, the GERP++ and is-rSNP algorithms were used. rs2147363 nucleotide position resulted non-conserved, whereas, in the intronic region around this SNP, 7 nucleotide positions on the 16 analyzed (43%) achieve the GERP threshold [77]. Table 2 describes the findings of the is-rSNP algorithm. This analysis predicted the presence of binding sites of 8 transcription factors (TF) binding sites, and, in two cases (i.e., Zfp423 and PLAG1), the prediction results were significant (adjusted $p < 0.05$). The main result of this detailed genetic analysis is that the significant association between rs2147363 SNP and AD can be explained on the basis of the presence of TFs binding sites in the region in which this variant is located. Thereby, rs2147363 may be associated with a cis-regulatory function. Specifically, we observed two of the genetic models of rs2147363 SNP associated with AD: a recessive model that suggested a 1.63-fold increased, and a log-additive model that suggested a 1.51-fold increase in the risk of having AD. The Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) analyses highlighted that log-additive model achieved the most reliable outcome. This suggests that C allele of rs2147363 SNP is additive: its presence in one gene copy will lead to an increase in AD risk; its presence in both gene copies will lead to a further increase. Additionally, we tested the reliability of our coding or noncoding hypotheses to explain this intronic association. When we considered the coding hypothesis, we analyzed the SNPs in complete LD ($D' = 1$) with rs2147363 in TSI. The functional prediction analysis showed that the SNP rs7334118 has a complete LD ($D' = 1$) with rs2147363. Moreover, rs7334118 has been recorded in the Wilson's Disease Mutation database (available at <http://www.wilson-disease.med.ualberta.ca>) and in Human Genome Mutation Database (available at <http://www.hgmd.cf.ac.uk/>) as a Wilson's disease-causing variant, it corresponds to H1207R, located into the P-domain, the domain in which phosphorylation of ATP7B occurs; in particular the residue lies in the ATP-hinge which connects the nucleotide-binding domain (N-domain) and the P-domain (Figure 3). Even though the genotype analysis in our case-control population revealed the presence of two carriers of the rs7334118G allele in AD patients and none in healthy controls, this *ATP7B* mutation could not alone explain the genetic association observed for rs2147363. Specifically, two AD patients heterozygous for the rs7334118G mutation were also carriers of the C allele in rs2147363 (1%). On the contrary, the other 166 AD patients, carriers of the C allele in rs2147363, were negative for the rs7334118 WD mutation (99%). However, this result does not exclude additional rare variants with large functional effects may explain this intronic association.

We used the HapMap database to identify functional coding variants, in which only few rare variants are present. Using other genomic databases, such as the database of 1,000 Genomes Project, may deliver new candidate variants that could be tested to explain our intronic association. Conversely, in-silico analyses on rs2147363 revealed that this intronic SNP can have a regulatory role on ATP7B function. In particular, the GERP analysis highlighted that the intronic region around rs2147363 resulted significantly conserved, and the is-rSNP algorithm significantly predicted the presence of two TF-binding sites in the rs2147363 region. These independent findings suggested that this genomic region has a regulatory function, and consequently rs2147363 can be associated with clinical phenotypes related to ATP7B dysfunction.

Position	Nucleotide	GERP++ score
chr13: 52542783	C	2.17
chr13: 52542784	A	2.37
chr13: 52542785	G	-7.84
chr13: 52542786	G	4.09
chr13: 52542787	G	0.889
chr13: 52542788	C	-1.02
chr13: 52542789	C	1.93
chr13: 52542790	T	-0.596
chr13: 52542791	C	4.11
chr13: 52542792	T	-3.02
chr13: 52542793	A	2.98
chr13: 52542794	G	-3.44
chr13: 52542795	G	2.55
chr13: 52542796	T	-7.19
chr13: 52542797	T	3.25
chr13: 52542798	G	4.87

Table 2. GERP analysis of the genomic region around rs2147363. Conserved positions are highlighted in bold.

Specifically, Zfp423 and PLAG1 have been predicted to have a binding-site in the genetic region of rs2147363. Both these TFs are involved in complex metabolic processes. For example, Zfp423 has been reported to regulate neural and adipocyte development [78], whereas *PLAG1* is a proto-oncogene that activates genes involved in uncontrolled cell proliferation (e.g., *IGFII*, *CRLF1*, *CRABP2*, *CRIP2*, and *PIGF*) [79]. In this framework, our data on Zfp423 support the hypothesis that *ATP7B* can be involved in molecular pathways linked to brain activity regulation, suggesting new perspectives in the interpretation of neurologic signs of WD and AD. This conclusion fits well with our original hypothesis that *ATP7B* can harbor variants that may account for some of the missing heritability of AD [80]. In particular, the present data furnished a new insight into the copper hypothesis: non-coding regions can play a role in the *ATP7B* function, and, thereby, genetic variation in *ATP7B* cis-regulatory elements within non-coding regions may be associated with AD risk. Furthermore, genetic data adjusted for age confounding effect confirmed the association between rs2147363 and AD risk. However, although in-silico analyses support our hypothesis, further functional studies are necessary to confirm the role of non-coding variants in the *ATP7B* gene function and in AD risk.

3.3. K832R (rs1061472) and R952K (rs732774)

In a recent study, we focused our attention on two functional changes in the *ATP7B* gene in particular: K832R (rs1061472) and R952K (rs732774).

K832R corresponds to a non synonymous amino acid substitution and the corresponding AAG>AGG mutation in exon 10 specifies this amino acid change in the A-domain, within the ATP binding domain region of the protein. R952K (AGA>AAA) corresponds to a non-synonymous substitution in the loop between Tm 5-Tm6 of the protein (Figure 3). Our aim was to verify whether and in what way these amino acid changes have a disturbing effect on the function of the ATP7B protein in terms of metal binding properties or ATP hydrolysis, which can eventually result in copper dyshomeostasis.

We recruited 251 AD patients and 201 controls. As reported in the original article [76], for the K832R substitution, the minor allele frequency (MAF) resulted 40% in Italian Tuscans, 45% in Utah residents with Northern and Western European ancestry, while 42% in our controls. For the R952K substitution, MAF was 39% in Italian Tuscans, 44% in Utah residents and 43% in controls. The LD analysis revealed an association between K832R and R952K substitutions in both AD patients ($D' = 0.79$) and controls ($D' = 0.81$). A high LD between K832R and R952K was confirmed also in all HapMap populations.

Allele frequency distributions of both *ATP7B* SNPs differed between AD and controls: K832R substitution genotype frequencies were K832/K832 10%, K832/R832 47%, and R832/R832 43% (χ^2 test, $p = 0.022$) in AD patients, while K832/K832 15%, K832/R832 54%, and R832/R832 31% in our controls. R832/R832 genotype was more frequent in AD and this frequency variation was maintained also when taking into account the Bonferroni correction ($\alpha = 0.025$) as well as in the logistic regression analysis, which took into account age and gender as confounding factors. Our analysis revealed that patients with the *ATP7B* homozygous R832 genotype had a 1.71-fold higher risk of developing AD than controls [adjusted OR= 1.71 (1.12–2.60); $p = 0.012$]. Genotype frequencies of the R952K substitution were R952/R952 15%, R952/K952 41%, and K952/K952 45% in AD patients, and R952/R952 16%, R952/K952 54%, and K952/K952 30% in our controls.

The χ^2 test indicated that two distributions differed ($p = 0.006$) and the association between the risk allele K952 and AD was maintained after checking for age and gender as possible confounders in a logistic regression model. In summary, patients with the *ATP7B* homozygous K952 genotype had a 1.82-fold increased risk of AD compared with controls [adjusted OR= 1.82 (1.19–2.80); $p = 0.006$].

We also performed a haplotype association analysis for the two SNPs in order to investigate their combined effect on AD risk. The most common haplotype was R832/K952, which contained a risk allele at each SNP locus. It was distributed as follows: 60.2% in AD patients and 53.2% in controls ($X^2 = 4.85$; $p = 0.028$).

The second more frequent haplotype was K832/R952, which contained no risk alleles and its frequencies differed between the two cohorts, being 28.8% in patients and 37.3% in controls ($X^2 = 7.21$; $p = 0.007$).

A logistic regression model was used to check these associations when taking into account age as a possible confounder. The model confirmed the association ($p = 0.018$) and revealed that the haplotype K832/R952 confers some protection against AD [adjusted OR= 0.68 (0.49–0.93)]. Thus, the haplotype association analysis revealed that the presence of alleles with normal function, i.e., K832 and R952, is protective, even though the haplotype lies in a gene with significant disease-risk.

It is important to notice that the SNPs of *APOE*ε4 and *ATP7B* (both K832R and R952K) are independent AD risk factors, as there was no difference in the frequency of the *ATP7B* alleles between carriers and non-carriers of the *APOE*ε4 variant (consistently $p > 0.2$), even when the analysis was restricted to assessment of only the AD population (consistently $p > 0.2$). In summary, the RR genotype in K832R raises the relative risk of developing AD by 71%, while KK in R952K by 82%. The haplotype R832/K952, instead, appears to confer protection against the disease by reducing the relative risk by 32%. These results seem in conflict with the fact that GWASs carried out so far have never found an association between AD and the 13q14.3 chromosomal region where the *ATP7B* gene lies. However, we already described above how GWAS, which are not well equipped to detect rare variants and fail to take into account the genomic and environmental context of the investigated diseases, may underestimate *ATP7B* haplotypes that are instead significantly associated with high risk of AD in individuals exposed to inorganic copper [80].

4. Conclusions

The form of AD that has been the subject of this chapter accounts for about 95% of all AD cases and appears rather late in life, normally after 60. For this reason it is called late-onset Alzheimer's disease (LOAD), a term that well differentiates it from the inherited form, familial AD, which is called early-onset AD because it develops much before 60 and accounts for the remainder 5%. In opposition to familial AD, LOAD was initially called "sporadic" because early researchers saw no link between the disease and hereditary factors and assumed that the appearance of the disease was occasional and totally casual.

As we have seen above, this appeared untrue later, when researchers discovered the role of several polymorphisms of the *APOE* gene in the disease pathogenesis. The term sporadic continued to be widely used but we now know that LOAD is strongly influenced by both genetic and environmental factors and appears to have a complex pattern of inheritance. It remains true, however, that so far no gene has been found to be fully causative of LOAD, a fact that is often referred to as *missing heritability*.

Due to the lack of a secure culprit, the role of genetic mutations in LOAD is often understated. In an attempt to compensate, this chapter has described some of the most meaningful evidence constituting the genetic background believed to provide a combined contribution to susceptibility (to become sick), which remains a statistical entity. It must be kept in mind that not only genetic heterogeneity contributes to susceptibility but also other factors, such as *reduced penetration*, a term describing that a predisposing genotype might be present without the

pathology necessarily appearing, or even *phenocopy*, in which there are no predisposing genotypes and yet the pathology develops due to environmental factors. The distinction between *genetic inheritance* and *genetic risk* is fundamental and has helped understand how the disease incidence can be decreased by changes in life style.

The chapter has also described GWAS, which have linked a substantial number of genes to the pathogenesis of AD. It is important to notice that each gene, when taken alone, accounts only for a small percentage of the disease incidence and often lacks clinical significance: the above mentioned *CLU* gene, for example, has a proven correlation with AD-related cognitive decline but its odds ratio tells us that it increases the risk of developing LOAD just 0.1-0.15 times. Moreover, this gene has no clinical relevance since it does not predict the disease onset.

However, all the genetic evidence presented here should be regarded as the framework in which systemic metal unbalances develop. For this reason, we have described in a consequential but also factual fashion the role of systemic copper in the toxic processes leading to AD, which we believe to be among the most important phenomena in AD pathogenesis.

We conclude with the introduction of an important, and somewhat innovative, notion concerning copper in AD. We have shown how multiple variants of the *APOE* gene have an actual effect on the statistical risk to develop AD and how they are also linked to level variations of the portion of copper that does not bind to ceruloplasmin. These variants are also in linkage disequilibrium with rare mutations, which can have a relevant effect on the risk of the disease. We are now starting to explore this field by searching for multiple rare variants in the *ATP7B* gene, which may account for portions of the odds of developing AD, defining a new gene for AD susceptibility. Looking at all these associations as a whole, genetics appears as the factor guiding the association between copper abnormalities and the clinical picture of the disease. In other words, the fact that *ATP7B* multiple rare variants in practice modify the copper homeostasis of an individual and have an effect on his/her risk of developing AD is an expression of the causative character of this gene on the susceptibility of the disease.

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