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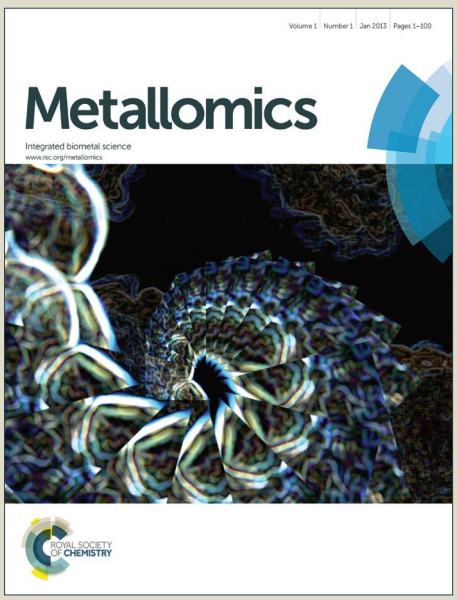

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Non-ceruloplasmin bound copper and *ATP7B* gene variants in Alzheimer's disease.

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Abstract

ATP7B, a protein mainly expressed in the hepatocytes, is a copper chaperone that loads the metal into the serum copper-protein ceruloplasmin during its synthesis and also escorts superfluous copper into bile, by a sophisticated trafficking mechanism. Impaired function of this ATPase is associated with a well-known inborn error of copper metabolism, Wilson's disease (WD). Several mutations of ATP7B are known, involving different regions of the protein, thus resulting in a plethora of phenotypes in WD patients. It is a consolidate notion that copper dysmetabolism occurs in Alzheimer's disease (AD) as well. Besides the molecular mechanisms relating copper to the protein hallmarks of this disease and neurodegeneration, more recently the observation that a free-copper in serum, not bound to ceruloplasmin (non-Cp-Cu), characterizes AD patients, prompted our research to identify possible genetic defects of the *ATP7B* gene in AD patients. Four specific single nucleotide polymorphisms and a WD rare mutation have a statistical association with AD. They contribute to characterize a copper subtype of AD. Additional facets of this AD phenotype, typified by higher levels of non-Cp-Cu, are presented and discussed in the framework of a copper failure as an accelerator risk factor of neurological disorders with different aetiology.

Copper handling in mammalian tissues.

Copper is an essential transition metal, co-factor for a number of vital enzymes in metabolism. Some of them are ubiquitous (like cytochrome c oxidase or superoxide dismutase 1 i.e. SOD1); others are more tissue specific (like lysyl oxidase, secreted by fibroblasts, or tyrosinase in skin melanocytes, dopamine β-monooxygenase in neurons, the plasma protein ceruloplasmin or the intestinal haephestin). They are involved in various metabolic pathways, ranging from oxidative phosphorylation, antioxidant defence, collagen or pigment synthesis, iron homeostasis, neurotransmitter synthesis [1-4].

Liver is the organ that controls copper body homeostasis, absorbing copper from blood (which in turn is enriched with copper by the intestine) and discarding unnecessary copper through the bile. On the basis of copper availability, two different routes allow cellular inward flux of copper: *via* Ctr1, a homotrimeric pore in the membrane, which is a high affinity passive copper transporter [5], or *via* DMT1, a Divalent Metal Trasporter 1 [6]. Specific copper chaperones are committed to donate copper in different subcellular paths [7]. Cytosolic enzymes (e.g. SOD1) receive copper from the small protein CCS (Copper Chaperone for Superoxide Dismutase), through a highly elegant mechanism of delivery, involving peculiar structure similarity between the chaperone and the monomer of the receiving protein [8]. Furthermore, cytosolic copper chaperone HAH1 (Human Atox1 Homologue) transfers copper to P-type ATPases, namely ATP7A and ATP7B, in the endoplasmic reticulum and *trans*-Golgi network, which in turn load the metal into cuproproteins to be secreted (e.g. ceruloplasmin, dopamine β-monooxygenase, peptidylglycine α-amidating monooxygenase) or, by trafficking to the cell membrane, eliminate excess copper from the cells. ATP7A generally traffics toward the basolateral membrane of polarized cells, while ATP7B to the apical side [9]. ATP7A and B show a high degree of homology and a peculiar pattern of expression. ATP7A is ubiquitous, but ATP7B is expressed mostly in the liver, and also in the brain. Increased

levels of copper in the hepatocytes prompt ATP7B to traffic at the plasma membrane, thus extruding excess copper, into bile. In the liver, ATP7B loads copper into the ferroxidase ceruloplasmin, the most abundant serum copper protein (85-95% serum copper) which cannot release the metal [10]. Residual serum copper is bound to low molecular weight molecules (e.g. amino acids like histidine) or to albumin, which is a cysteine rich protein. This fraction represents an exchangeable pool of circulating copper, referred also as "free copper", and more correctly copper non-bound to ceruloplasmin (non-Cp-Cu), bioavailable for tissues and organs. Indeed, this copper pool can easily cross the blood brain barrier (BBB) and reach the brain, possibly accelerating neurodegenerative processes[11, 12]. It was demonstrated that brain is one of the organs containing the higher amount of copper in the human body. In fact, metal level reaches about 5 μg/g, which is similar to that of the liver. On the other hand, the cerebrospinal fluid (CSF) shows about tenfold lower copper concentration [12]. Copper, as free ions, enters the brain from the bloodstream. The expression of the copper protein transporters at both the blood/brain barrier (BBB) and at the blood/CSF barrier (BCB) indicates that also in the central nervous system (CNS), copper homeostasis is driven by the canonical mechanisms described for the other cell types. In fact, at the apical side of the brain endothelial cells of the BBB were identified Ctr1, which is likely responsible of the copper import from general circulation, whereas ATP7B might return excess copper to blood. Furthermore, ATP7B is also present at the basolateral side of epithelial cells of the choroid plexus, playing the same function. At the same time, ATP7A, located at the basolateral side, possibly releases Cu in brain parenchyma [12].

It is interesting to note that copper is not distributed homogeneously in the brain; its concentration at the synaptic cleft reaches 250 μM [13], a very high level. These findings, according to studies in synaptosomes and in hippocampal neurons, suggest that, besides the renowned roles of copper as enzyme cofactor, this metal may also affect neuronal transmission, long term potentiation, synaptic plasticity and excitotoxic cell death [12, 14] .

Mitochondria play a pivotal role in cell copper homeostasis. Delivery of copper to mitochondria is granted by a set of copper-transport proteins (Cox17, Sco1, Sco2, and others), which supply the metal to the active site of cytochrome c oxidase (COX) (complex IV of the electron transport chain). The function of the mitochondria copper chaperones strongly depends on the intracellular and mitochondrial redox status [3]. Besides canonical mitochondrial copper-chaperones, liver mitochondria contain a shorter isoform of ATP7B, possibly playing a role in the extrusion of copper from the organelle [15]. Mitochondria are also deeply involved in buffering copper in the cell; in yeast, they have been demonstrated to retain copper in the form of a non-proteinaceous, not yet identified store, which can be released on demand [16] and can also regulate copper entrance in the cell *via* Ctr1 [17]. Conversely, it has been demonstrated that cells overloaded with copper accumulate the metal in the mitochondria [16, 18]. Recently, it was also demonstrated that ATP7A activity impedes entry of excess copper in mitochondria in both human and mouse fibroblasts and that ATP7A dysfunction alters mitochondrial redox balance [19].

All copper chaperones show one or more copper binding domains, particularly rich in cysteine, histidine or methionine [8]. The presence of six copper binding domains in the regulatory N-terminal of copper ATPases, and their progressively occupancy by copper in dependence of copper concentration, are relevant to the signalling inducing ATPases trafficking toward and backward the plasma membrane in cytosolic vesicles [20, 21]. Furthermore, it was demonstrated that ATP7A trafficking in axons, also during the development of synapses, can be driven by calcium ions [22, 23].

Binding of copper to specific transporter proteins during trafficking and delivering is crucial, in order to avoid undesired transition of copper between oxidised and reduced state, which may affect intracellular redox status and radical-mediated toxicity. Furthermore, copper can displace other metals from metalloproteins [8]. The reduced form of glutathione and the metal-induced protein metallothionein both contribute to cytosolic copper buffering

potential [7], by virtue of their cysteine residues. Indeed, it has been found that the amount of free copper inside the cells in undetectable [5, 24].

Widespread literature supports the concept that neurodegeneration in the most common nervous system diseases (Alzheimer's disease, prion diseases, motor neuron diseases, Parkinson's disease) might be associated with disrupted copper homeostasis [11, 25-30].

Wilson's disease, a genetic impairment of copper homeostasis due to *ATP7B* mutations.

Wilson's disease (WD), also referred to as hepatolenticular degeneration, is a monogenic autosomal recessive disease, discovered in 1993 to be associated with mutations in the *ATP7B* gene (mapping to chromosome 13q14.3) as reviewed in [31]. WD has an estimated incidence of 1:30,000-50,000 [32, 33], although in Italian island Sardinia it reaches about 1:7,000 live births [34].

In Wilson's disease accumulation of copper in tissues and organs occurs: mutations in the gene *ATP7B* make the copper pump ATP7B to be non-functional, copper is not supplied to the Golgi apparatus, and is either not properly loaded into ceruloplasmin (decreased ceruloplasmin specific activity) or excreted through the bile. Copper is released by the liver in the blood as a non-Cp-Cu.

About 500 mutations and 800 single nucleotide polymorphisms (SNPs) have been identified in the *ATP7B* gene in WD patients worldwide (listed in a public database http://www.medgen.med.ualberta.ca/database). Most mutations are missense, but also non-sense, insertion, deletion, and splicing mutations have been described (for a review see [35]); most patients are compound heterozygotes. The wide range of mutations in the *ATP7B* gene may differently affect protein function and different pathways in the hepatocytes (pleiotropic effects) thus explaining the highly variable phenotypic clinical manifestation, which can range from a liver disease to a neurologic or psychiatric disease.

ATP7B is a large transmembrane protein; besides six copper-binding domains at the cytosolic N-terminus, it shows eight trans-membrane domains forming a channel for copper

translocation (the sixth containing a copper-binding motif, C-P-C), an ATP binding site, the phosphorylation domain (with a conserved aspartic acid residue) and the phosphatase domain. In ATP7B, trafficking induced by high copper level is concomitant with kinase-mediated phosphorylation and folding change of N-terminal domain; instead, dephosphorylation triggers the protein to recycle back to *trans*-Golgi. The protein shows several sites of phosphorylation [36], and different kinases are involved in the process [37]. However, the regulation of ATP7B trafficking seems to be more complex, because recent works indicate a complex interplay between phosphorylation and copper-dependent conformational changes affecting ATP7B intracellular location [36].

Therefore, each mutation may have a different impact on protein stability or its trafficking, and completely or only partially inactivate copper transport, thus explaining the wide range of phenotypes of the patients [35]. The prevalent mutation in Europe, including Italy, is H1069Q, located in the ATP-binding domain [38, 39], which makes the protein partially inactive, with a shorter half-life and an abnormal intracellular targeting [40, 41]. It allows limited synthesis of holo-ceruloplasmin (the holo-ceruloplasmin is the active form of the protein, distinguished from apo-ceruloplasmin, which is the inactive, labile apo-form), and is associated with late onset neurological symptoms [42].

Lutsenko[35] pointed out how diagnosis of WD can indeed be extremely difficult, due to wide variations in phenotypes in patients, and how important can be the interplay between genetic and lifestyle, including diet, and epigenetic factors. Indeed, monozygotic twins, carrying the same mutations in ATP7B, displayed different clinical symptoms [43, 44]. Genetic polymorphisms of *ATP7B* and copper levels in diet can also make the picture more complex. Indeed, *ATP7B* polymorphism affect cell location and trafficking of the protein [45], but this aberrant behaviour can be corrected by increasing copper level. Thus, in this case, copper deficiency in diet might make ATP7B less efficient in eliminating copper from cells.

Due to the primary expression of ATP7B in hepatic cells, copper excretion in bile is affected as well as the holo-ceruloplasmin synthesis. Thus in the liver overload of the metal occurs, as well as low ceruloplasmin levels in serum. Indeed, at the earliest stage of the disease, copper is 30-50 times higher than normal in the cytosol of hepatocytes. WD is progressive, sometimes undiagnosed for long time, and, if not treated, is lethal. It manifests symptomatically between 5 and 35 years of age, with highly variable degree of liver disease, including steatosis and inflammation, which may culminate in cirrhosis and/or with neurological and neuropsychiatric symptoms. The accumulation of copper in the cornea (Keiser-Fleischer rings), is a hallmark of this disease, and is mostly present in patients with the neurological manifestations [31, 33].

The mitochondrion constitutes an important and early target of copper toxicity in WD; indeed, changes in the morphology of liver mitochondria are pathognomonic of this disease. A study described copper accumulation in liver mitochondria in an animal model of this disease and copper-induced crosslinking of proteins within and between distinct mitochondrial membranes [46]. It is not known whether lack of function of mitochondrial ATP7B may contribute to copper accumulation in mitochondria. Recently, treatment of ATP7B-deficient rats with methanobactin, a high affinity copper chelator peptide produced by *Methylosinus trichosporium* and proposed as a new therapeuthical approach for WD, depleted mitochondrial copper and rescued mitochondrial damage [47], thus reinforcing the concept that mitochondrial damage plays a pivotal role in WD.

Many WD patients, about 40-50%, display neurological and/or psychiatric symptoms, which can be delayed with respect to clinically evident liver disease by many years but can also be the first clinical sign [32]. The copper content of brain is decreased [48] the labile component of copper is increased and deposition occurs particularly in the basal ganglia, and results in gliosis and neuronal loss. Widespread lesions in globus pallidus, head of the caudate nucleus and substantia nigra were found [49]. Cognitive, pyramidal and extrapyramidal

dysfunctions occur. Extrapyramidal signs and the imbalance of catecholamine metabolism include neurological WD in Parkinsonian syndromes [50]. Indeed, a nucleotide deletion at the 5'UTR region of a single allele of *ATP7B* gene was indicated as a risk factor for a late onset parkinsonism [51], supporting the hypothesis that Parkinson's disease may represent a heterozygote form of WD [52].

The bulk of the studies on WD focuses on liver pathogenesis and damage and is of general notion that liver toxicity may be ascribed to copper-induced radical-mediated damage, while the molecular mechanisms underlying WD neurodegeneration are not well understood. Liver symptoms in WD can be treated by lifelong administration of copper-chelators (D-penicillamine, triethylene tetramine, best known as Trientine, or tetrathiomolybdate) to promote copper excretion from the body, or of zinc or both and also with a low-copper diet [33]. Zinc ingestion reduces the body's capacity to absorb copper by potentiating mucosal block in the intestine, through a 25-fold increase of the expression of methallothioneins, which tightly bind atoms of copper and trap them into enterocytes. Copper is eliminated through exfoliation with stools, thus preventing copper transfer into the blood [53]. However, neurologic deficits may persist despite treatment. Furthermore, it has been observed that chelation therapy may even worsen neurological symptoms. This is known as the 'paradoxical effect': copper mobilization by chelators lead to a burst of toxic non-Cp-Cu pool [31, 54]. Approximately 5% of WD patients require liver transplantation; however, liver transplantation cannot recover irreversible basal ganglia damage and neurological rehabilitation. Of note, in two homozygotic twins liver transplantation resulted in different neurological outcome [55].

Copper as a risk factor for Alzheimer's disease

As per the World Alzheimer report 2015 (http://www.alz.co.uk/research/world-report-2015), 9.9 million new cases of dementia each year worldwide have been estimated, implying one new case

every 3.2 seconds. The regional distribution of new dementia cases is 4.9 million (49% of the total) in Asia, 2.5 million (25%) in Europe, 1.7 million (18%) in the Americas, and 0.8 million (8%) in Africa. The global costs of dementia reached US$ 818 billion in 2015.

Based on evidence of the monogenic familial forms of Alzheimer's disease (AD), the key mediator of AD pathology has been settled in the brain amyloid-β peptides that forms dimers and oligomers, leading to aggregation and extracellular plaques, responsible for altered function of neural cells and neurodegeneration, which are accompanied by intracellular aggregates of phosphorylated tau protein forming neurofibrillary tangles. However, AD exists primarily as a sporadic complex disease, and the aetiology of this form has to take into account polygenic and multifactorial effects, along with environmental risk factors. Besides age and familiarity, modifiable factors include diabetes mellitus, midlife hypertension, midlife obesity, physical inactivity, and depression, lifetime prevalence of major depressive disorder, smoking, and low educational attainment [56 209].

Amongst causative risk factors, the excess of serum copper has been proven as a fact in late onset AD. Meta-analyses along with large population studies have demonstrated the association of copper imbalance with cognitive decline. More precisely, meta-analyses demonstrated copper imbalance in AD, consisting in increased serum copper [57], increased non-Cp-Cu [58] and decreased brain copper [59] .

These are some clues that indicate a role for copper in the aetiology and progression of AD. It has been also suggested, in fact, an intriguing interplay between copper and the proteins involved in AD. Amyloid-β peptides bind Cu (2+) with very high affinity and this explains copper accumulation found in AD plaques [60, 61]. In turn, copper-binding triggers amyloid-β peptides aggregation and copper redox activity, which are both involved in AD pathogenesis [26, 62-64].

Amyloid cascade hypothesis in AD suggested that the toxic form of amyloid-β peptides consisted in soluble oligomers, which once aggregated, became less toxic, and even protective.

However, recent reports indicate that, in the presence of bound redox copper, the aggregates maintain their redox activity and produce hydroxyl radical from hydrogen peroxide [63].

On the other hand, the plasma-membrane amyloid precursor protein (APP) (which produces β-amyloid following aberrant proteolysis by γ-secretase) might be physiologically involved in copper brain homeostasis [65, 66]. More recently, reports demonstrate that copper can be involved in APP-dependent synaptogenesis and modulate APP trafficking [67, 68].

Large population studies sustain that an excess of copper has a detrimental effect on cognition. The Chicago Health and Aging project (about 3,700 subjects in a 9-year longitudinal study) demonstrated that people with a higher intake of copper and of saturated and trans-fats had a 3-fold faster rate of cognitive decline [69]. The Rancho Bernardo study (San Diego, Southern California) evaluated about 1,500 subjects and demonstrated that people who had lower copper in their blood were mentally clearer compared with those with excessive copper, who had problems with short term and long term memory [70]. Along the same line, the Iowa Women's Health Study (including 40,000 elder women), demonstrated that use of copper supplements increased the rate of mortality by 18% [71]. Most of multi-vitamin/mineral dietary supplements contain approximately 2 mg of inorganic copper/pill [29], which further increases the copper pool in the body. Thus, the intake of copper with dietary supplements usually exceeds the copper Recommended Dietary Allowance [72]. A study on metal concentration in the soil of mainland China collected data from 26 provinces, 3 districts within 1991-2000 [73], demonstrated that the increase of copper in the soil was associated with an increased rate of mortality for AD, and that in the geographic areas with higher copper concentrations, the relative risk of AD was 2.6 times higher than in the areas with low copper. As a whole, the data reported clearly demonstrate that copper represents a risk factor for AD.

Non-Ceruloplasmin-copper in serum in Alzheimer's disease.

The late onset form of AD exists primarily as a sporadic complex disease, in which polygenic and multifactorial effects, incomplete penetrance of associated genes and of non-genetic risk factors contribute to the disease onset. Recently, the scientific community has devoted attention to non-Cp-Cu, the fraction of circulating copper not bound to ceruloplasmin, which has been found increased in some neurological disorders [31]. The copper imbalance described in literature so far is mainly represented by an increased size of the pool of serum non-Cp-Cu [53, 74, 75]. Because of its looser binding to albumin and micronutrient pool in the blood, non-Cp-Cu is available to meet tissue needs in the body. If non-Cp-Cu pool increases, it can become toxic to the brain, since it can cross the BBB. Many studies highlight the strict connection between excess of non-Cp-Cu (higher than normal reference values) and AD onset and progression. These studies have been carried out comparing non-Cp-Cu serum levels in AD patients or MCI with healthy controls or patients with vascular disease or Parkinson's disease [76-78]. The probability of having AD associated to increased values of non-Cp-Cu in the blood varies from 1.22 to 3.3 (**Table 1**)., the probability of having AD increases about threefold when AD is compared to healthy subjects [odd ratio (OR) 3.21; 95% Confidential Interval (CI) 1.53-6.71; $p<0.002$] [79]. In the MCI condition, which is considered a prodromal stage of AD, the OR of the conversion from MCI to full AD is 3.3 (95%CI 1.21—9.24; $p= 0.02$ [80]). Meta-analyses and systemic reviews stressed the imbalance of this specific copper fraction in serum as a risk factor for AD and Mild Cognitive Impairment (MCI) subjects [80]

Worsening of cognitive function in AD patients has been associated with copper metabolism abnormalities [80, 81]. Several studies carried so far on independent cohorts or AD populations reported information on the relative risk of AD onset (odds Ration, Relative risk, Hazard ratio) associated with copper imbalance (**Table 1**). In particular, it has been demonstrated that high serum levels of non-Cp-Cu are associated with MCI condition [76, 77] and with an increased rate of conversion from MCI to full AD in a 6-year longitudinal study [80]

Moreover, recently it has been demonstrated an association between serum ceruloplasmin specific activity and risk of AD [82], highlighting an imbalance between holo- and apo-form of ceruloplasmin as a mirror of a copper systemic imbalance. The specific activity of the protein which indicates the amount of active enzyme in circulation, is represented by the ratio between the enzymatic activity of Cp and the concentration of Cp in serum (eCp/iCp: enzymatic, eCp/ immunoturbidimetric, iCp). The analysis showed that beside an increase of the risk of having AD for non-Cp–Cu ($p=0.008$) there is a decrease of the risk for ceruloplasmin specific activity ($p=0.001$). Furthermore, an estimated model including non-Cp-Cu, eCp/iCp, age and *APOE*-epsilon 4 allele (the only established risk gene for AD), showed a good power in discriminating AD patients from healthy controls, with sensitivity of 66% and a specificity of 93%.

ATP7B genetic variants and Alzheimer's disease

Two recent genome wide association studies (GWAS) identified loci affecting metal metabolism [83, 84]. These studies identified two loci on chromosome 1, which were found to have significant association with copper concentration in erythrocytes. A deeper analysis revealed that the genes in these regions in chromosome 1, do not code for proteins with known functions, which have been associated to disease involving copper metabolism alterations.

Among the disease-associated loci discovered by GWAS, CLU, the gene that encodes clusterin resulted the most associated to an increased risk sporadic AD occurrence [85].CLU is a molecular chaperone involved in apoptosis, in protein folding, and in Aβ clearance. It has been reported that CLU binds both ATP7A and ATP7B, facilitating their degradation *in vitro* and modulating their copper-export function [86]. As expected, analyzing the chromosomal region where is located ATP7B gene, GWAS did not find any significant SNPs association with AD. This negative outcome regarding ATP7B can be explained either by the GWAS paradigm "common disease – common

gene variants", or by the complex structure of *ATP7B* gene. In fact, GWAS hardly detect rare variants, which instead are supposed to account for the missing hereditability of complex diseases, especially if referred to the *ATP7B* which is a high polymorphic gene [87] .With a different perspective, using a hypothesis driven approach[88], we hypothesized that the mechanism underlying the increase of non-Cp-Cu in AD could be related to genetic defects related to (or which interfere with) the ATP7B function, causing an altered copper loading into nascent ceruloplasmin. To pursue this issue, we started an extensive hypothesis-driven candidate gene association study of *ATP7B* gene in AD patients, which is still in progress. The nature of *ATP7B* gene make the analyses very complex. The 1000 Genomes project has identified more than 1,300 variations of *ATP7B* in human populations [88]. Worldwide detection of *ATP7B* mutations is actually difficult since most mutations are rare, reported only within single families and often prevalent in specific ethnic groups. As a result, the database regarding both the gene's properties and the possible dysfunctions of the proteins they encode appears still unsatisfactory [89].

Our hypothesis driven approach was inspired by the study by Gupta et al. [90], which presented a set of SNP markers. These SNPs were highly heterozygous across most world populations and could be used in combination with analysis of prevalent mutations as a comprehensive strategy for determining pre-symptomatic and carrier sibs of WD patients. We decided to take advantage of this knowledge and started our studies in AD from those alleles, which were informative of the *ATP7B* gene structure [91] (Fig 1 modified from Squitti et al. [92]). We focused our attention on a set of four SNPs, namely: rs1801243 (missense substitution: Ser406Ala), rs2147363 (intronic variant: c.1544-53A>C), rs1061472 (missense substitution: Lys832Arg) and rs732774 (missense substitution: Arg952Lys). We found high frequencies of the minor allele in two SNPs causing non synonymous substitutions: the rs1801243 (c.1216T>G) associated with amino acid change Serine to Alanine in position 406 and the rs1061472 (c.2495A>G) that causes the amino acid substitution of Lysine to Arginine in position 832. Subsequently, we studied another non-synonymous change Arginine in Lysine in position 952 (rs732774).

In a larger study population, we demonstrated a significant association of rs1061472 genotypes and AD risk and revealed an association for the rs772774 [93].

Linkage disequilibrium (LD) analysis pointed out that the four original SNPs and their LD SNPs covered 96% of the ATP7B gene sequence, distinguishing two ‘‘strong LD’’ blocks. Genetic association analysis indicated that one of them, the LD block containing the gene region encoding for transmembrane domains (rs732774-rs1061472 [94, 95]) had a stronger association with AD than the other. Unknown spots potentially associated with AD lying within rs732774-rs1061472 region could be rare loci.

In an independent sample of 287 AD patients we have assessed non-Cp-Cu serum concentrations, rs1801243, rs1061472, rs732774 *ATP7B* genetic variants and the *APOE4* genotype [94, 95]. Patients were distributed into two groups on the basis of a non-Cp-Cu cut-off (1.9 μM). The study revealed that the two AD subgroups did not differ regarding age, sex, MMSE score, or *APOE4* frequency allele, but they did differ regarding allele, genotype and haplotype frequencies of rs1061472 A>G and rs732774 C>T after multiple testing corrections, so demonstrating genetic heterogeneity between the two AD groups

High copper-rich food consumption has little or no direct effect on body copper content under normal physiological conditions[96]. Genetic make-up, including copper modifier genes can significantly change this scenario since copper intake and genetics are closely connected in terms of copper toxicosis: a high, or even normal, copper dietary intake in individuals with a genetic susceptibility to copper exposure causes metal toxicosis. On this basis, we have developed and suggested a diet at a copper content in line with the RDA for those subjects who are bad copper metabolizers as revealed by copper markers testing [88].

Conclusions

In the first decade of the 2000s the involvement of some *ATP7B* gene variants as a risk factor for Parkinson's disease was suggested [51]. Studies carried out in the second decade of the 2000s, starting from completely different hypothesis, proved that the *ATP7B* genetic variants represent a risk factor also for sporadic AD. If on the one hand, it may be surprising that clinically different neurological disorders such as Parkinson's disease and AD can share a more or less pronounced copper imbalance, on the other hand it is grounded that *ATP7B* mutations have pleiotropic effects. In facts, they cause the wide spectrum of symptoms characterizing the WD, ranging from a typical liver disease in the earlier stage of the disease, to the neurological symptoms like movement disorders or psychiatric disorders, including Parkinsonian syndromes. These new concepts support copper failure as a causative accelerator risk factor in neurodegenerative disorders, and primarily in AD.

This several evidences discussed in our work pave the way to biomarker science applied to copper in AD. Biomarker science sets the stage for developing drugs that can be used to take control of disease-causing molecular pathways before the manifestation of clinical symptoms, as demonstrated for MCI conversion to full AD [80].

Acknowledgments

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Figure legends

Figure 1. Human ATP7B depicted on the basis of homology-modelled structure. The table reports the *ATP7B* gene SNPs studied and the specific nucleotide and amino acid substitutions, located in a specific ATP7B domain, and the relative Odd Ratio (OR) associated with AD.

Gene SNPs	Amino acid substitution	exon	Genotype	Risk (OR)	ATP7B domain	Haplotype
ATP7B_rs1061472 A\G	Lys832Arg	10	GG	1.71	A-domain	G832/A952
ATP7B_rs 732774 G\A	Arg952Lys	12	AA	1.82	Luminal loop between TM5-TM6	
ATP7B_rs1801243 T\G	Ser406Ala	2	GG	1.8	Metal binding unit 4	
ATP7B_rs2147363 A\C	intronic	3/4	CC	1.63		
ATP7B_rs7334118A\G	His1207Arg	17	GG		P-domain:ATP hinge	

Linkage Disequilibrium (D'=1)

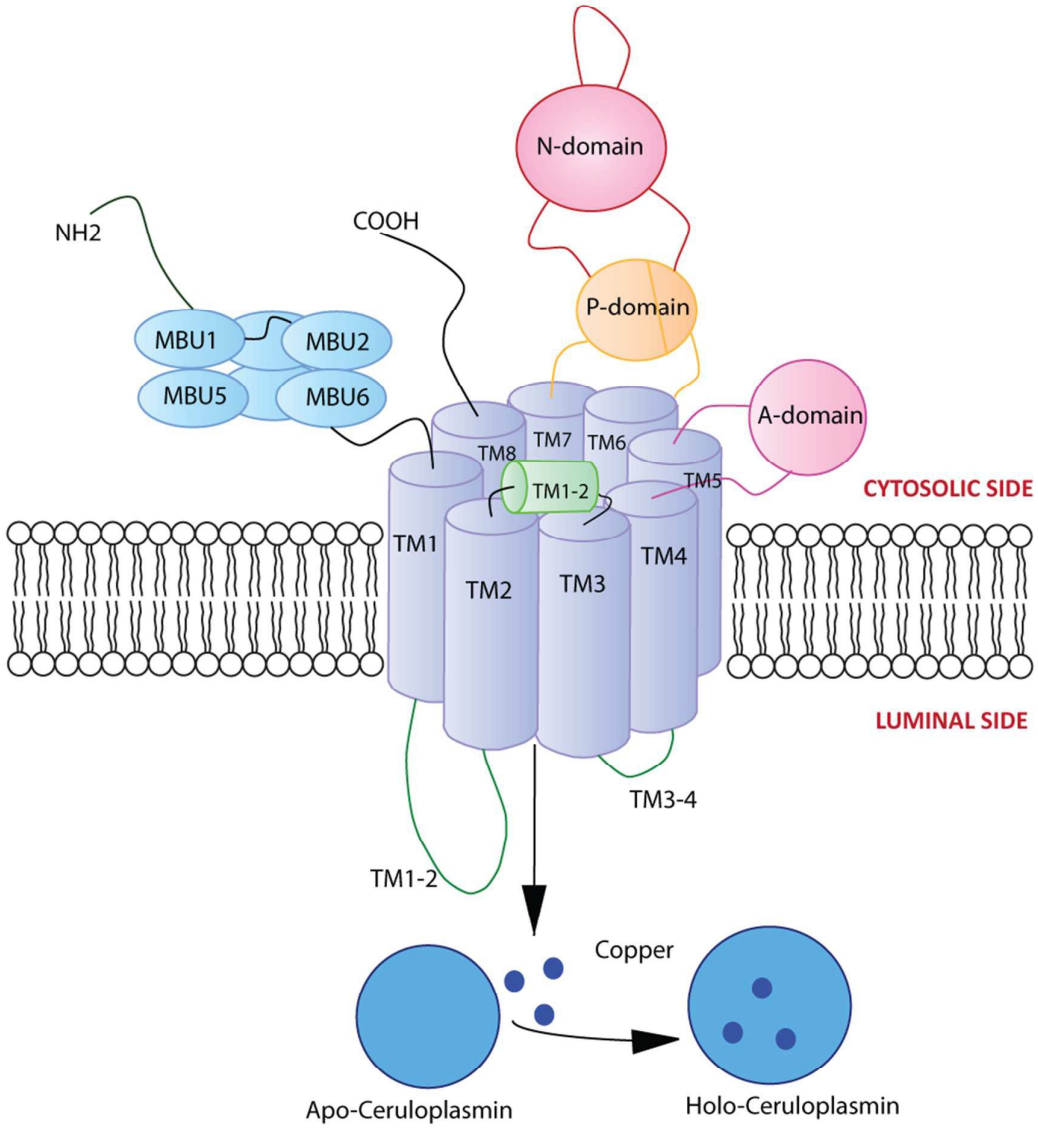

Table 1. From 2002 to 2014 eleven studies demonstrated that copper failure increases the risk of Alzheimer's disease (AD) in terms of Odd Ratio (OR), Relative Risk (RR) and Hazard Ration (HR) of Alzheimer's disease

Clinical studies analyzing copper link to Alzheimer's risk	**Copper increases the risk of Alzheimer's with relative risk or odds ratio ranging from 1.22 to 5.16**			
Serum copper and risk for Alzheimer's disease	**Subjects**	**risk (OR, RR, HR)**	**CI 95 %**	**p value**
Squitti et al., Neurol 2002 [97]	AD vs healthy	**1.8**	1.36 -2.43	p<0.05
Squitti et al., Neurol 2003 [98]	AD vs VAD	**2.06**	1.28 - 3.31	p<0.003
Squitti et al., Neurol 2009 [81]	1 year longitudinal study in AD	**1.23**	1.03-1.47	p<0.022
Squitti et al., J Alzh Dis 2011 [76]	MCI vs healthy	**1.22**	1.05-1.41	p<0.01
Squitti et al., Curr Alzh Res 2013 [79]	AD vs healthy	**3.21**	1.53-6.71	p<0.002
Squitti et al., Ann Neurol 2014 [80]	6 years longitudinal study in MCI	**3.3**	1.21-9.24	p=0.02
ATP7B gene variants and risk for Alzheimer's disease				
Squitti et al., Rejuvenation Res 2013 [93]	AD vs healthy	**2.3**	1.41-3,77	p<0.001
Bucossi et al J Alzh Dis 2013 [91]	AD vs healthy	**1.3**	1.06-1.69	p=0.015

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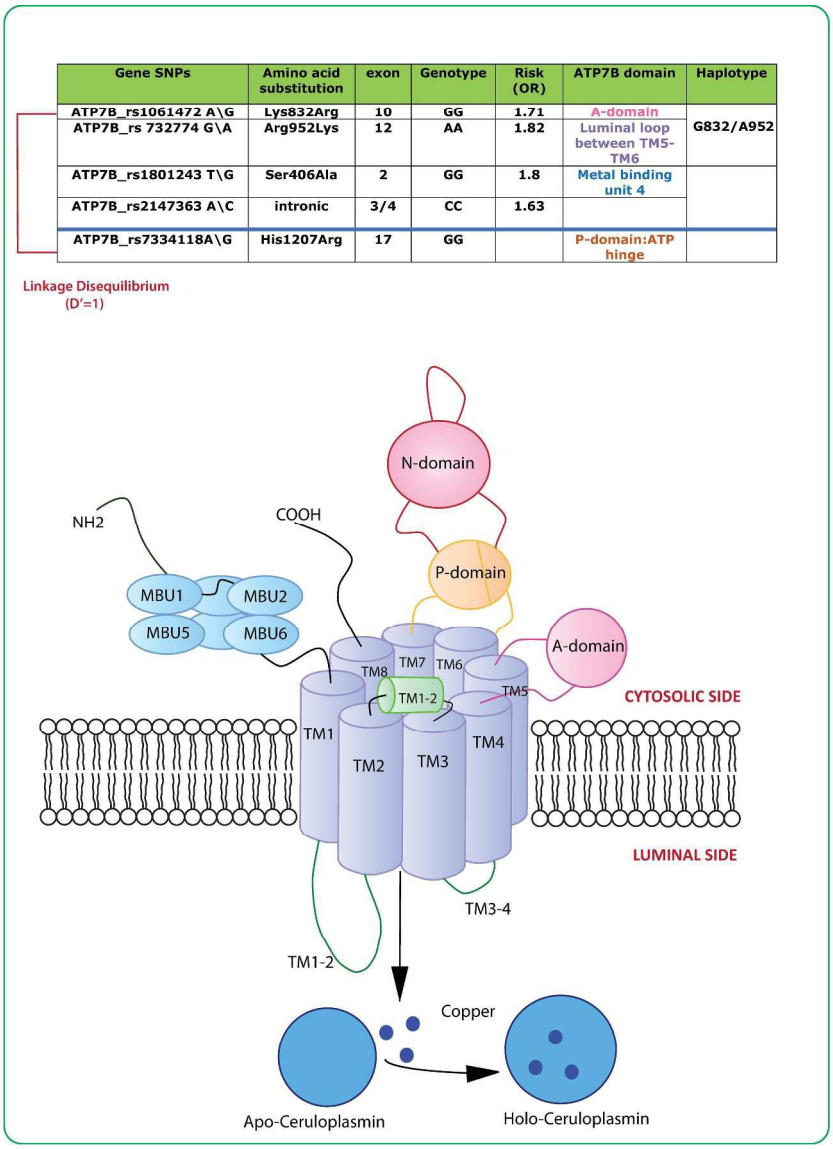

Human ATP7B depicted on the basis of homology-modelled structure. The table reports the ATP7B gene SNPs studied and the specific nucleotide and amino acid substitutions, located in a specific ATP7B domain, and the relative Odd Ratio (OR) associated with AD.

275x313mm (300 x 300 DPI)

Significance to metallomics

This minireview is aimed at summarizing the most relevant findings in the field of copper link to neurodegeneration. The molecular mechanisms of metal-dependent physiological processes in humans are taken into account. Abnormal values of a specific fraction of copper in serum is addressed with the aim of explain the molecular basis of the breakdown of copper homeostasis in some neurological disorders, such as Wilson's disease, Alzheimer's disease, Parkinson's disease. The minireview spans from an overview of the ATP7B copper-pump function in the hepatocyte to clinical studies and gentic studies showing the connection between the altered release of non-ceruloplasmin copper in serum generated by a defective functioning of this ATPase and variants of *ATP7B* gene associated with an increased risk of having Alzheimer's disease.