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Review

Copper-related diseases: From chemistry to molecular pathology

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ABSTRACT

The aim of this review is to give a general view on the current status of the scientific basis for the role of copper in human health and disease, outlining the roles of copper in human metabolism and bioenergetics, its coordination chemistry as well as its biological ligands involved in the multiple steps of metal assimilation and distribution. Copper bioavailability depends on four main factors: (1) the absorption of copper from the gastrointestinal tract; (2) copper transport in the portal blood; (3) the extraction of copper by hepatocytes from the portal blood supply; (4) copper uptake by peripheral tissues and by the central nervous system. The most important copper pumps, providing a permeation pathway for copper ions in man, such as hCTR1, hCTR2, DMT1, ATP7A, ATP7B, and MURR1, are extensively discussed. Different theories on the putative role of ceruloplasmin in copper transport in blood are presented. Data on interactions among other trace elements and copper, from the level of enterocyte to other systems in the body, are also shown. Finally, the function of different drugs, chelators and non-chelating agents, utilized in clinical practice in the therapy of Wilson's disease is treated, in particular the "reductive chelation" of penicillamine is discussed.

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1. Introduction

Wilson's disease (WD), also known as hepatolenticular degeneration, is a disorder of copper metabolism characterized by its accumulation in different organs, leading to liver cirrhosis, neurological disturbances and to a complexity of symptoms related to the disarrangement of copper transport and distribution in the whole body. Kinnier Wilson was the first who described this disease in 1912 [1] as "progressive lenticular degeneration", a definition which underlined the prevalent involvement of the central nervous system and in particular of the lenticular bodies. In his report Wilson also stressed the characteristic association, in this new disease of unknown origin, of neurological symptoms with liver cirrhosis. The low serum levels of ceruloplasmin, frequently found in patients affected by WD, have been considered for many years the main cause of the modifications in copper metabolism: ceruloplasmin was considered till 20 years ago the principal copper transporter in the human body, and its deficiency was considered the cause of the block of copper excretion and, accordingly, of its accumulation in different organs, mainly in liver and central nervous system (CNS) [2]. The discovery of a mutation in the ceruloplasmin gene in a Japanese family, resulting in ceruloplasmin absence in blood, was the black swan which frequently in science destroys the theory that "all swans are white". In fact, the autopsy performed in one of the family members died for decompensated cirrhosis, showed a massive accumulation of iron in the liver as well as in the central nervous system, copper levels being in the normal range [3]. Ceruloplasmin belongs to the family of multinuclear copper oxidases, which also include ascorbate oxidase and laccase, and has a ferroxidase activity with a relevant role in iron metabolism [4]: it is not only a copper transporter, but a copper enzyme which utilizes copper atoms as electron donator/acceptor in oxidative reactions. The ferroxidase activity of plasma ceruloplasmin can be considered as one of the most important mechanisms of its antioxidant properties, since clearing Fe(II) from plasma, the production of reactive oxygen species (ROS) generated in the non-catalyzed autoxidation of Fe(II) is avoided [5]. Ceruloplasmin plays an essential role in mobilization of iron stores, determining the rate of iron efflux from cells [6]. The debate on the pathogenesis of Wilson's disease received a strong acceleration in 1993, when the gene responsible for the disease was identified [7,8]. The gene, ATP7B, encodes a P-type ATPase which functions as a transmembrane transporter of copper atoms [9]. In liver, key organ of copper metabolism, ATP7B is mainly expressed in the Golgi apparatus of the hepatocytes, where it incorporates copper atoms in copper-enzymes. ATP7B is also localized at the biliary pole of hepatocytes, where it facilitates copper excretion into the bile [10]. A mutation in the WD gene may reduce the functionality of ATP7B, leading to a block in copper excretion into the bile and therefore to hepatic copper accumulation [11], chronic hepatitis [12], and cirrhosis [13]. As a consequence, copper serum levels may rise, leading to copper accumulation in the central nervous system [14], in the cornea [15], in the kidney [16,17], and in other organs [18,19]. The loss of functional ATP7B protein in the Golgi apparatus may cause failure in the incorporation of copper atoms in the apoceruloplasmin, resulting in the decreased ceruloplasmin blood levels found in the majority of WD patients. The low serum levels of ferroxidase ceruloplasmin may cause severe alterations in iron metabolism, leading to liver iron storage occasionally reported in WD carriers [20]. This paper aims to give a general view on the current status on the implication of copper in human health and disease, outlining its roles in human metabolism and bioenergetics, its coordination chemistry as well the biological ligands involved in the multiple steps of copper assimilation. In particular, we will focus our attention towards the interaction of copper status and liver function in health and in Wilson disease. Data on interactions between essential trace elements and copper will be presented, from the level of absorption in the gut to other systems in the body. The characteristics and the behaviour of different chelators utilized in clinical practice in the therapy of Wilson disease will be thoroughly discussed, going emphasis to the equilibria of complex formation and to the reductive action of penicillamine. It is worth of note that during the preparation of this manuscript two relevant articles appeared, concerning copper, copper homeostasis and its impact on neurodegenerative pathologies [21,22].

2. Copper in humans

2.1. Copper as a pro-oxidant

ROS formation during acute exposure of liver cells to copper overload is generally considered the crucial event leading to cell death [23]. Copper is a transition metal characterized by a low redox potential between Cu(II) and Cu(I) 0.158 V in water that accounts for its capacity to exchange electrons with other chemical compounds [24]. The biological utility of copper mainly derives from this aptitude to cycle between its oxidized and reduced forms. The effects of metals characterized by variable oxidation states, as copper and iron, depend markedly on the predominant form of the metal, which in turns depends on redox potential and coordinating environment [25]. For this reason copper is utilized by a large number of enzymes, probably more than 300 in humans, involved in oxidative reactions. The copper redox state is one of the critical variables that affect ion transport pathways. Copper uptake and its intracellular oxidative effects are mainly caused by the cupric ion, whereas the effects on membrane transport are largely due to cuprous ion, likely formed at the membrane surface, where sulfydryl groups act as reducing agents [26]. Copper ion redox state is critical for methaemoglobin formation in trout erythrocytes [27]. Cu(I) is supposed to be found more often into the cell membrane and inside the cell, where the local redox potential is considerably lower than in blood [28]. Studies on copper speciation in humans have been scarcely developed: Cu(I) has a great affinity for thiol group (–SH), its most important coordinating group in biologic environment [27]. The cellular and biochemical mechanisms by which copper storage may induce cell damage and cell necrosis is still debated [30]. It has been hypothesized that excess copper could trigger peroxidative cell damage by ROS production and consequent oxidative injury [31]. In fact, oxidative stress is often cited as a possible cause of damage in cells exposed to high copper concentrations, even if no supporting quantitative data are presented. In hepatocytes incubated with high concentrations of Cu(II) lysosomes were identified as the major site of endogenous cytotoxic ROS formation, and in another study by confocal laser scanning microscopy the origin of ROS generated in hepatocytes was identified in mitochondria, suggesting an important role for mitochondria in copper-induced apoptosis [3]. In the same study, co-incubation of Cu-treated hepatocytes with the iron chelator deferoxamine significantly inhibited ROS production preventing hepatocytic cell death. Copper exposure was found to activate extracellular signal-regulated kinase (ERK) in trout hepatocytes, stimulating its phosphorylation to pERK; activation of ERK, as well as of p38, were responsible for enhanced ROS formation, and for apoptotic cell death [33]. Hepatocyte necrosis induced by Cu(II) has been also partly attributed to membrane lipid peroxidation, and to the increase of intracellular calcium levels observed when the hepatocytes were incubated with high Cu(II) levels [34]. Exposure of HepG2 cells, an in vitro system typical of liver parenchymal cells, to high copper dosages, was constantly associated with the induction of heat shock protein 70 (hsp70) [35] and of metallothioneins [36], providing a protective mechanism against copper toxicity. In patients affected by Wilson disease and in Bedlington terriers, an animal model of WD [30], oxidative

injury to hepatic mitochondria has been reported [29]. These observations confirm previous reports [37], indicating mitochondria as the main target of copper-related peroxidative damage in Wilson disease. A severe mitochondrial involvement could be at the basis of the development of a cellular energy deficit and of increased intracellular free calcium levels, both leading to apoptotic cell death [32].

2.2. Copper physiology

Copper is an essential trace element for living organisms, taking part in all aspects of metabolism, including mitochondrial oxidative phosphorylation, free radical detoxification, neurotransmitter synthesis and denaturation, pigment formation, connective tissue synthesis and iron metabolism [38,39]. In human body copper is found in relatively high amounts: a healthy 70 kg adult contains about 110 mg of copper, the major part (46 mg) in skeleton and bone marrow, 26 mg in skeletal muscles, 10 mg in liver, 8.8 mg in brain and 6 mg in blood [40]. The great part of copper in the human body, in physiological conditions, is probably functional, being copper atoms involved as cofactor of different redox enzymes. The best known among these are: ceruloplasmin [4]; cytochrome *c* oxidase, the terminal enzyme of electron transport and oxidative phosphorylation; superoxide dismutase, an antioxidant enzyme able to remove superoxide radicals from tissues; lysyl oxidase, essential for cross-linking of collagen and elastic fibers; monophenol mono-oxygenase, involved in the synthesis of melanin; dopamine-beta-mono-oxygenase, required for the synthesis of dopamine; and peptide-alpha-amidating mono-oxygenase, essential for the synthesis of pituitary hormones [40]. Copper plays further additional non-enzymatic functions in angiogenesis [41], nerve myelination and activity of endorphin [42]; it also plays and an essential role in brain development, shown by the presence of demyelination and by neurodegeneration in patients affected by Menkes disease, the best documented congenital copper deficiency in humans [43,44]. Copper is essential for reproduction, regulation of gene expression, and for normal growth and development [45]. The interest on copper metabolism in humans, began in the late twenties, when its role in haemoglobin synthesis was revealed [46]. The essential role of copper in humans was first reported in 1956 in a paper on malnourished children presenting with anaemia refractory to iron therapy [47]. Further studies allowed a clear definition of the pathological observations related to copper deficiency [48], particularly in newborns [49]. Even if the intimate mechanisms of copper homeostasis are not yet completely understood, a significant amount of insight into the kinetics of copper metabolism has been reached about 80 years after the discovery of its role in human metabolism. The need of copper in adults is generally reported as 1 mg per day [50]. Pipe water, for the widespread use of copper pipes in household plumbing, can be an important dietary source of copper, differently from unpolluted fresh water that contains no or very little copper [42,51]. The guideline value for copper in water for human use is 2 mg/L (WHO Directives [52]). The principal dietary sources of copper are chocolate, animal liver, crustaceans, shellfish, green vegetables, dried fruits and nuts; it is much more bio available from meat than vegetables. Copper concentration in these foods ranges from 20 up to 50 mg/kg, about 500 times higher than in human milk, one of the poorest dietary sources [53,54]. The copper content in human milk largely decreases (50%) from the fourth month of lactation [55,56], so that a prolonged breast feeding could lead to copper deficiency [57]. The recommended dietary allowance (RDA) for copper in infancy is 80 μg per kg/day [58]. Among newborns, premature and low birth weight infants are at higher risk to develop copper deficiency. In clinical practice copper deficiency should be suspected in every infant with prolonged or recurrent diarrhoea [59]. Following the first report on the association diarrhoea-copper deficiency in malnourished infants in a children hospital in Lima [60], several studies underlined the risk of copper deficiency and the relevance of the intestinal mucosa in maintaining a normal copper status [61,62]. Copper bioavailability depends on three main factors: absorption from the gastrointestinal tract, transport in blood, and extraction by hepatocytes from the portal blood supply. A variety of factors may alter copper bioavailability: aging, that decreasing the efficiency of copper homeostasis results in higher serum copper concentrations in elderly population [61], sex, i.e. higher mean serum copper levels are detected in females [62], and hormonal factors, women on contraceptive pill have increased serum copper levels with respect to control women [63].

2.2.1. Copper absorption and transport

Copper is absorbed in the stomach, in duodenum and in the whole small intestine [40]. From a clinical point of view, absorption of copper in humans predominantly occurs in the ileum [64]. The gastrointestinal tract not only receives one milligram of copper per day from the diet [50,64], but higher amounts (~4.5 mg/day) are released through saliva, bile, gastric secretion and apoptotic intestinal cells shedding from the intestinal mucosa [65]. The first step in copper absorption is the uptake of the metal present in the diet by intestinal epithelial cells. The intimate mechanisms by which copper is transported across the brush border of the intestinal cells is not yet well known. The chemical speciation of copper contained in vegetables or in animal foodstuffs represents a key factor in copper absorption: Ledoux et al. reported that, while copper salts (carbonate, acetate, sulfate and chloride) are well absorbed, a much lower ability to cross the intestinal barrier is shown by copper oxide [66,67]. Food treatments also affect copper absorption: salts for food preservation may modify the solubility of copper bound to proteins and its bioavailability [68]. Intestinal pH is probably the most important physiological factor affecting copper absorption since an acid environment is essential for freeing copper ions from complexes formed in foods and in mucosal secretions, setting the conditions for their absorption [69]. The presence in the diet of chelating agents as citric acid, in large amounts in fruits, and lactic acid favours copper absorption [70]. Many divalent cations in the diet act competitively on intestinal copper absorption [65]. The capacity of zinc to halt copper absorption was first described in rats [71] and subsequently confirmed in humans: 3 mg of zinc added to the diet cause copper deficiency [72], leading to severe anaemia and leucopoenia [73]. Zinc interferes on copper metabolism, acting at different sites: displaces copper from the specific carrier on the intestinal cells [74] and increases metallothionein content in the intestinal epithelium, that blocks copper trafficking and favours its loss in faeces through apoptosis of intestinal cells [75]. The molecular pathways by which copper ions enter the enterocytes as well as the biochemical mechanisms responsible for copper uptake at the apical plasma membrane of intestinal cells in mammals are scarcely known [76].

2.2.2. Copper pumps

The most important copper pump from the intestinal lumen into the enterocytes is probably the human cation transporter 1 (hCTR1), a 190 amino acid protein. It is predicted to have three transmembrane-spanning domains, existing in the plasma membrane as a homo-trimer [77], and is considered an essential protein both for early embryonic development and for intestinal copper uptake [78]. Localized on the plasma membrane of enterocytes it acts as a conventional transporter, normally providing a permeation pathway for copper ions. hCTR1 may be internalized in presence of elevated copper levels and it is considered a high affinity copper transporter: its action is time-dependent, saturable, and stimulated by extracellular acidic pH and high potassium concen-

trations [75]. Experimental results in Caco-2 cells have recently shown that the basolateral copper uptake greatly exceeds the apical one in enterocytes [79]; in the same study evidence is given of hCTR1 basolateral localization in T84 cells, a common model for intestinal crypt cells. The basolateral transport is reported to be mediated by hCTR1 [78]. The role of hCTR1 as the major driving factor for intestinal copper absorption in mammals has been shown in the CTR1 knockout mouse, which exhibits severe growth and viability defects, systemic copper deficiency, iron overload, cardiac hypertrophy and severe growth deficit due to intestinal block of copper absorption [80]. The presence of an elevated intraluminal sodium gradient stimulates CTR1-mediated copper absorption by intestinal epithelial cells, by increasing H^+ concentration [81]. CTR1 expression on endothelial cells of the blood brain barrier suggests that copper could be transported from the plasma into the central nervous system via CTR1 [80]. A role in apical Cu(I) transport in intestinal cells is probably played by the dication metal transporter 1 (DMT1), also known as Nramp 2 or DCT1. The DMT1 action in Fe(II) uptake at the apical pole of enterocytes is well known [82]. Recent experimental data suggest that DTM1 is a physiologically relevant Cu(I) carrier in intestinal cells, and indicate that intestinal absorption of Cu(I) and Fe(II) are intertwined [83]. Copper supplementation in Caco-2 cell culture is able to up regulate the expression of DMT1 as well as that of ferroxidase hephaestin (HEPH) and of the iron transporter ferroportin-1 (Fpn1). These data support a major role of copper status in the modulation of iron uptake in intestinal cells [84]. The reduced copper absorption in rats, due to the introduction of Fe(II) in the diet, leads to hypothesize a competition between iron and copper for the same transporter [85]. The molecular bases for these data have been recently clarified by the demonstration that copper could compete with iron for uptake via DMT1 [86]. This copper–iron competition should induce, in clinical practice, to verify copper status in patients suffering from chronic anaemia and in women with hyposideremia during gestation, in which iron enters as a therapeutic agent in abnormally high amounts. Copper uptake in the intestinal lumen via DMT1 has been shown to be also sensitive to pH: its decrease at the brush order of intestinal epithelial cells can create a suitable environment for copper uptake and transmembrane transport [81]. A possible role of ATP7A in regulating copper absorption by intestinal cells raises several questions on its behaviour in intestinal epithelium [87]. In rat, iron deprivation results in a strong induction of the ATP7A gene in duodenal epithelium, followed by a significant copper overload in liver and in intestine, suggesting an increase in intestinal copper transport during iron deficiency [88]. In the same study translocation of ATP7A from the physiological site at the apical domain of enterocytes to the brush border and to the basolateral membrane domains after iron deprivation was observed.

2.2.3. Copper transport through the cytoplasm of enterocytes

The second step in copper transport across the intestinal epithelium is the passage through the cytoplasm (Fig. 1).

Copper transport across the cytoplasm is mediated by metallothioneins (MTs), a group of proteins identified in the late fifties as cadmium and zinc proteins [89]. The incorporation of copper atoms into MTs is an essential step to protect cell structure from copper toxicity and to prevent oxidative damage [90]. MT is the most important copper transporter from the intestinal cell luminal pole toward the basal pole [74]. Some competition for MTs among different trace elements occurs: cadmium and zinc are the most important ions involved in competition with copper for the binding site of MTs [40]. The relevance of zinc on the binding of copper to MTs was first demonstrated in rat [91]. Cousins reports that Zinc increases MT content in intestinal mucosa cells up to 25-fold, by inducing their synthesis [92], and Botash et al. demonstrate that

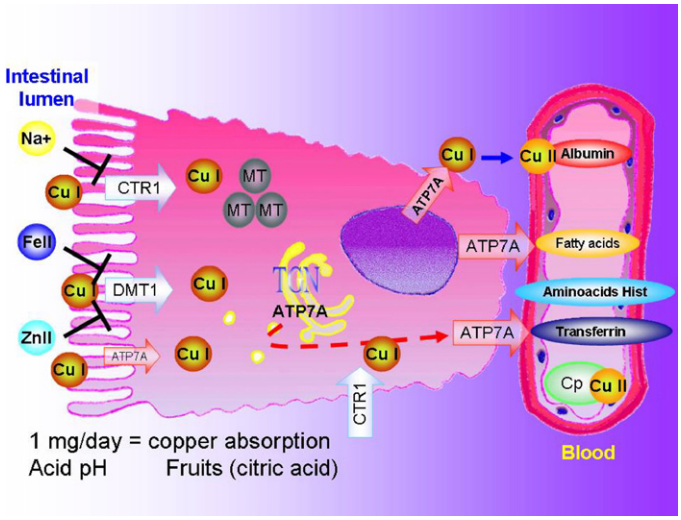


Fig. 1. Dietary copper absorption by enterocytes in the intestinal lumen.

zinc supplementation easily produces copper deficiency in human [93].

2.2.4. Role of ATP7A in enterocytes

Copper ions, arriving to the basolateral pole of enterocytes, are transferred to the main copper transporter through the basal cell membrane. The copper-translocating P-type ATPase ATP7A, also known as Menkes' protein (MNK) [94], has been proposed as the Cu transporter that could play a relevant role in regulating the basolateral transport of copper from the enterocyte into the portal blood [95]. In the structure of ATP7A, three putative domains may be identified:

1. six or eight transmembrane loops that presumably form a channel for metal ion passage;
2. an ATP-binding region;
3. six copper-binding sites [96].

The role of these six copper-binding domains is not fully understood, since two are considered sufficient for copper transport [95,97]. An intriguing role for the remaining four sites should be that of sensors of the intracellular copper concentration [98]. Under copper-limiting conditions, ATP7A is retained in the trans-Golgi-network, but delocalized in proximity of the basolateral membrane [87]. A work of Bremner on the dynamics of ATP7A in intestinal epithelial cells in conditions of copper depletion and copper overload has shown a prevalent localization of the protein in the trans-Golgi-network in copper-depleted cells, and an ATP7A relocalization from the Golgi apparatus to the cell periphery in condition of copper overload [72]. The small quantity (about 10%) detected at the basolateral cell surface raises several questions on the exact role of ATP7A in copper trafficking.

2.2.5. Copper speciation in blood

Copper ions, on entering portal blood, are immediately bound by the two main copper carriers in the way from gut towards liver: albumin, the most abundant plasma protein, and transcuprein (Fig. 1) [99,100]. Copper may be even bound to different aminoacids, peptides, and to fatty acids, but many evidences suggest that these complexes are not relevant in physiological conditions [101]. Human serum albumin is a versatile carrier protein involved that besides fatty acids, vitamins, hormones, xenobiotics transports also metal ions as Cu(II), Ca(II), Zn(II), Co(II), Ni(II) and Cd(II) [102]. Conformational changes of albumin upon contact with

cell surfaces give origin to two subpopulations characterized by different affinity to cell surfaces [103]. Albumin is considered the major copper binder of exchangeable copper in human plasma, binding about 17% of copper thanks to the high affinity for Cu(II) of the sequence Asp-Ala(Thr)-His at the amino terminus [104]. A second binding site, highly conserved during evolution, containing two/three imidazole and one/two carboxylate coordinating groups, does not play a significant role in copper transport (1% of non-ceruloplasmin plasma copper) also being relevant for other divalent cations (Zn, Ni, Cd) [105,106]. Albumin-bound Cu(II) is rapidly reduced by ascorbate to Cu(I)–albumin, that is reoxidized by molecular oxygen [107]. The redox activity of albumin-bound copper is regulated by different factors as follows:

- Cu/albumin ratio < 1:1, copper is virtually redox inactive as long as cysteine-34 is in reduced state;
- the binding with fatty acids facilitates cysteine oxidation and converts copper–albumin complexes from antioxidants to pro-oxidants [108].

Cu(II) complexation causes conformational changes near to the metal-binding site of human albumin. Albumin present in human plasma could bind as much as 40 μg Cu(II)/mL, although it actually binds only 180 ng Cu(II)/mL [100]. This leads to hypothesize the existence of other plasma components with an affinity for copper higher than albumin, such as transcuprein or transcuprein-like proteins, or some specific peptides. The observation of Vrags et al. that analbuminemic rats do not show relevant changes in the distribution of dietary copper to the liver supports this hypothesis [109]. On the other hand, the presence of unsaturated copper-binding sites on albumin might provide some protection against a sudden release of copper ions in blood causing acute intravascular haemolysis, as observed in patients with Wilson disease [110,111]. Some amino acids have a minor role in copper transport in blood, and a small percentage of human plasma Cu is found as histidine complex or as ternary complex of CuAlbHis [112]. A study on the distribution of copper ions between human albumin and transferrin, which has two binding sites for Cu(II), gave evidence that transferrin binds copper more strongly than albumin [113]. The considerably lower concentrations of transferring, with respect to albumin in human blood, prevents its competition with albumin for non-ceruloplasmin cupric ions. Nevertheless, we may speculate that, in the absence of albumin, transferrin could play a relevant role in Cu(II) transport in plasma.

Transcuprein, an alpha glycoprotein discovered in the rat, molecular weight of 270 kDa, is characterized by a higher affinity for Cu(II) than albumin and accounts for about 12% of the exchangeable copper pool in rat serum [114]. It seems likely that humans do not have transcuprein, but employ other transcuprein-like macroglobulins for copper transport that carry about 10% of serum copper [45]. Ceruloplasmin, akin to Pirandello's Six Characters in Search of an Author, has long been a protein in search of a function [26]. It is the major copper-containing protein in human plasma, comprising about 65% of serum copper [115]. It is a glycoprotein, formed by a single polypeptide of 1046 amino acids with several carbohydrate chains attached, it has molecular weight of 132 kDa and carries six copper atoms per molecule [116].

2.2.6. Copper uptake by hepatocytes

Cu(II) ions enter mammalian erythrocytes via the band 3-mediated anion exchange pathway in the form of negatively charged complexes with chloride and carbonate [117]. Cupric ions are reduced to cuprous ions on the cell membrane surface, probably by an externally facing sulfydryl group [118]. Copper uptake across the hepatocyte cell membrane occurs at the sinusoidal pole, where it arrives bound to albumin as Cu(II) [119]. Whichever is the substrate for uptake, the hepatocyte has to remove copper ions from high affinity complexes with albumin, histidine and transcuprein-like proteins. The process of copper uptake by hepatocytes is probably initiated by the binding of copper as CuHisAlb ternary complex or as CuHis2 complex, followed by the reduction of cupric to cuprous ions by NADH oxidase on the plasma membrane [120]. It has been calculated that one copper ion is transported for each molecule of NADH oxidized [119]. The copper uptake in hepatocytes is stimulated by the reducing agent vitamin C, that probably speeds up copper reduction [121]. NADH oxidase reduces both Cu(II) and Fe(III): this may explain why increased iron levels in the hepatocytes may halt copper uptake, perhaps by down-regulating the metalloreductase activity on the plasma membrane [122]. The reduction of copper during its passage in the sinusoidal lumen lower the stability of the complexes, freeing copper atoms and allowing their uptake by hepatocytes. Little is known about the molecules and biochemical mechanisms responsible for copper uptake at the plasma membrane of hepatocytes in mammals. New insights into mammalian copper metabolism have been acquired with the identification of the gene family SLC31 (solute-linked carrier 31) [123]. There are at least two members of the SLC31 gene family, the human cation transporter genes (hCTR1) [124] and hCTR2 [24]. Cation transporter 1 (CTR1) is a high affinity membrane copper permease, conserved from yeast to humans, that mediates the physiological uptake of Cu(I) from the extracellular environment, following reduction of Cu(II) by a cell surface metalloreductase [125]. CTR1 binds four Cu(I) ions as cuprous-thiolate polynuclear clusters [126]. hCTR1 is a component of the copper transport machinery at the hepatocytic cell membrane, which transports copper across the plasma membrane with high affinity in a time-dependent, saturable and energy-independent manner [75]. Two methionine-rich domains of hCTR1 regulate copper-stimulated endocytosis: the response to low copper concentrations requires the amino-terminal methionine cluster MMMMPM, while the transmembrane MXXXM motif is required for endocytic response to high concentrations [127]. In conditions of copper overload in vitro, CTR1 is rapidly internalized by endocytosis, ubiquitinylated and degraded by vacuolar proteases [128]. CTR1 is not specific for copper: it may facilitate the transport of other substances, including the three platinum-based anticancer drugs cisplatin, carboplatin and oxaliplatin [129,130]. The role played by hCTR2, identified in a database search, remains unknown; it is expressed in all human tissues examined and its gene is located in 9q31/32 [131]. In Saccharomyces cerevisiae, CTR2 is localized at the vacuole membrane, where it mobilizes vacuolar copper stores towards the cytosol [132]. A study on CTR2 immunolocalization in different human cell lines demonstrates its localization in late endosomes and lysosomes, with a putative role in maintaining copper homeostasis by stimulating copper delivery to cytosol [133]. A study on kidney cells shows that CTR2 is localized on the plasma membrane, where it promotes copper uptake and plays a relevant role in regulating intracellular copper levels [134]. Copper imported by the cell membrane via CTR1 rapidly binds to intracellular carrier proteins (copper chaperones) which deliver copper ions to specific sites within the cell. The human copper chaperone HAH1 plays an essential role in copper trafficking to the secretory pathway of the cell, and interacts directly with ATP7B in the liver and with ATP7A in other cells (Fig. 2) [135,136]. Atox1 has been shown to play a critical role in copper homeostasis, as well as in delivering copper ions to P-type ATPases dislocated in the trans-Golgi-network [137–138]. COX-17, a mitochondrial copper chaperone, is the only protein so far known to exhibit significant primary sequence homology to metallothioneins [139]. It is a key copper donor within the mitochondria, and cooperates with three other copper chaperones, Sco1, COX-11 and COX-2 for delivering copper ions to cytochrome oxidase [140,141]. These

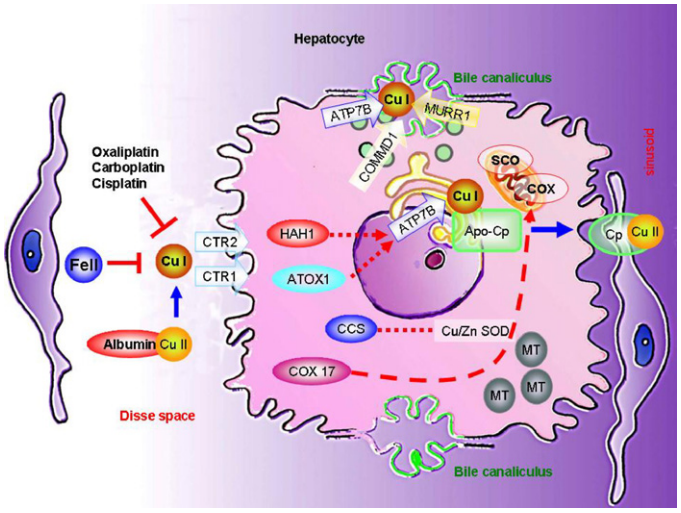


Fig. 2. Copper transport in the hepatocyte.

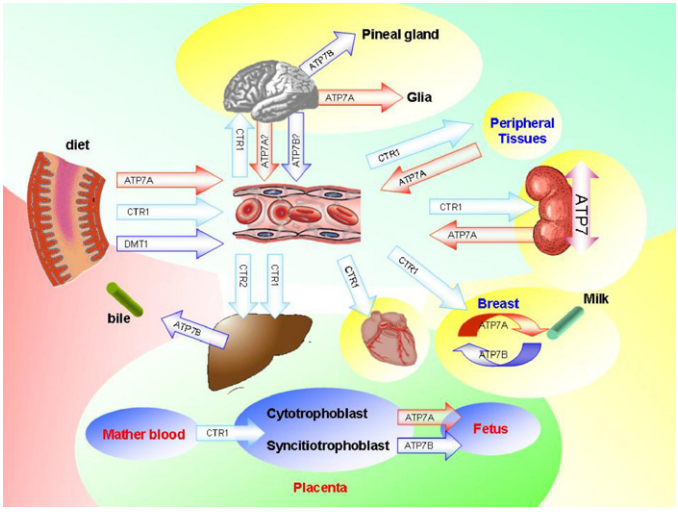


Fig. 3. Proteins involved in copper transport in different organs.

metallo-chaperones utilize a copper pool contained in the mitochondrial matrix [142]. Recent experimental data on human SCO1 and SCO2 suggest that both proteins could act not only as copper chaperones for cytochrome *c* oxidase, but rather as mitochondrial redox probes [143]. A model has been proposed for human SCO, in which COX-17 delivers copper to SCO2, which transfers it directly to COX, the reaction being facilitated by SCO2 [144]. SCO1 and SCO2 could also play additional roles in cellular copper homeostasis, probably as components of a mitochondrial pathway in which both proteins could act as sensors of the intracellular copper status, perhaps by their thiol redox or metal-binding sites [145,146]. Mitochondria could also act as copper deposit, storing copper ions in a distinct ceruloplasmin and in metallothionein, and chaperoning copper to the trans-Golgi compartment and to the secretory pathways when too much copper is accumulated inside the cell [147]. The copper chaperone CCS activates the target essential antioxidant enzyme SOD through direct insertion of copper (prefolded dimers of apoSOD1 serving as substrate for CCS), and it presumably protects the metal ions from binding to free copper intracellular scavengers [148,149]. CCS1 is the metallo-chaperone necessary for delivering Cu(I) to Cu/Zn-superoxide dismutase 1 (SOD1) within the mitochondria in the inter-membrane space [148]. The turnover of CCS in fibroblasts, a cell type characterized by the abundance of this chaperone, has been shown to be related to copper content of the cell, revealing a unique post-translational component of the intracellular copper homeostasis [150]. Mammalian SOD1 may receive copper ions even by an alternative CCS-independent pathway of activation, which involves the reduced form of glutathione or GSH [151]. Conflicting results have been reported on the role in liver cells of metallothionein (MT), a cysteine-rich cytoplasmic protein, localized in the cytosol, in the nucleus, and in the inter-membrane spaces of mitochondria, that strongly chelates copper as well as other metal ions. Mts, binding the metal ions through their cysteine-rich alpha and beta domains and acting as antioxidants, protect hepatocytes and other cells, including duodenal and renal epithelium, against toxic copper excess [152–153]. On the other hand, during oxidative stress in presence of H_2O_2, the protection by MT may disappear, and MT release free copper enhancing the formation of ROS which potentiate cellular damage [154]. MURR1, also called COMMD1, is a gene recently identified which has been hypothesized essential for copper excretion at the biliary pole of hepatocytes, acting downstream ATP7B. Its mutation is responsible for canine copper toxicosis, one of the animal models of Wilson disease [155]. MURR1/COMMD1 encodes a protein without any detectable sequence homology to known proteins. It is readily detectable in all tissues, suggesting a pleiotropic function in copper metabolism in different organs [156]. XIAP is a potent suppressor of apoptosis which is found to interact with MURR1. The reduced copper levels found in cells and in tissues of XIAP-deficient mice, imply a role of XIAP in copper homeostasis through a negative regulation of MURR1 [157].

2.2.7. *ATP7A and ATP7B*

Two P-type ATPases, ATP7A and ATP7B, are key molecules for the regulation of copper homeostasis in mammals (Fig. 3) [158]. In the liver, they play a dual role: when located in the trans-Golgi-network they provide copper ions to essential cuproenzymes, while under copper stress ATP7A and ATP7B sense the increased copper levels and translocate to the cell membrane to excrete the excess of intracellular copper [159]. In human hepatocytes, the specific copper transporter ATP7B is located to the trans-Golgi-network when extracellular copper concentration is low (below 1 μmol/L), and redistributes to vesicular structures and to biliary canaliculi at increased copper levels [160]. A new intracellular transporter of ATP7B has been recently identified: it is the Niemann-Pick disease type C protein (NPC1), a late endosome protein which regulates intracellular vesicle traffic of ATP7B [161]. A recent study on ATP7B immunolocalization in hepatoma cells (HepG2) gives evidence that the final trafficking destination of ATP7B is a paricanalicular vesicular compartment, rather than the canalicular membrane of the hepatocyte as previously reported. According with this report, ATP7B could be responsible of copper accumulation in vesicles, which subsequently undergo exocytosis, releasing excess copper across the plasma membrane [162]. The final destination of ATP7B in hepatocytes during the copper-induced trafficking process is still under debate [163]. Although the ATP7B translocation is conserved among non-hepatic cell lines, no co-localization with MURR1/COMMD1 was detected, suggesting that the translocation of ATP7B could take place independently [164]. ATP7B transports copper into cells against a gradient: to enter this transport pathway cupric ions have to be reduced since copper ATPase7B transports cuprous ions or Cu(I)–histidine complexes. Increased expression of ATP7A and ATP7B genes has been recently observed in some human cancer specimens, and may be associated with tumorigenesis and chemotherapy resistance [165]. In neuroblastoma cells, ATP7A expression has been shown to be regulated by retinoic acid receptor β and it affects the intracellular copper levels, revealing a link between the anticancer action of retinoids and copper metabolism

[166]. ATP7B stability is partially regulated by COMMD1 (MURR1), the protein deleted in Bedlington terriers, animal models of copper toxicosis [167,168]. COMMD1 exerts its regulatory role in copper homeostasis specifically interacting with newly synthesized ATP7B and decreasing its stability [169]. COMMD1 specifically binds Cu(II) and may cooperate with ATP7B to facilitate biliary copper excretion [170,171]. Glutaredoxin (GRX1) has been also proposed as an essential factor for ATP7A and ATP7B function; it catalyses the reduction of disulfide bridges and may reverse the glutathionylation of the cysteine residues within the six copper-binding motifs MXCXXC, facilitating copper binding for subsequent transport [172]. An additional protein, the dynactin subunit p62, has been shown to interact with ATP7B, and not with ATP7A. The ATP7B/p62 interaction is a putative key component of the pathway that delivers ATP7B-bound copper to subapical vesicles of the hepatocyte for the removal of excess copper into bile [172]. The function of ATP7B in tissues other than liver is unclear [43]. The role of ATP7A in copper physiology has been well studied in transgenic mice over expressing the human Menkes protein. The protein was hyper expressed in heart, smooth muscle of the lung, distal tubules of the kidney, intestinal enterocytes, hepatocytes, as well as in the hippocampus, cerebellum and choroid plexus of the brain [173]. In mice over expressing human ATP7A, copper concentration was reduced in most tissues, particularly in heart and brain, suggesting a relevant role of ATP7A in copper efflux from cells and tissues.

2.2.8. Copper distribution to peripheral tissues

The knowledge on molecules involved in copper distribution to peripheral tissues (Fig. 3) is very low. Ceruloplasmin has been proposed for a long time as the principal copper transporter to peripheral tissues [174], and its role in copper transport has been reported also recently [175]. Copper chaperoned to the trans-Golgi-network is transported into the Golgi apparatus by ATP7B and then incorporated into ceruloplasmin in vesicles that cross the plasma membrane and release ceruloplasmin-bound copper into the plasma [147]. Ceruloplasmin synthesis requires Cu(II), and Cu(I) undergoes oxidation at some stage before its incorporation into the protein [176]. Intrahepatocytic chloride channels are involved in copper metabolism, by promoting copper incorporation into ceruloplasmin, probably by improving the efficiency of ATP7B [177], even if many researchers judge that this protein plays no essential role in the transport or metabolism of copper [26,178,179].

2.2.8.1. Brain. The mechanism of copper transport into the brain is unclear. The expression of copper transporters has been shown to be higher in brain barriers than in brain parenchyma; as a consequence, copper transport through the blood–brain barrier into the brain is mainly achieved by free copper ions [180]. New functions of ceruloplasmin have been recently proposed in the central nervous system, where it could play an important role in neuropathological conditions by stimulating various neurotoxic molecules, including nitric oxide (NO) in microglial cells [181]. The ceruloplasmin relevance in the striatal metabolism of catecholamines is well underlined by the observation that the lowest content of serum ceruloplasmin is observed in the most serious neurological forms of Wilson disease [182]. CTR1 is probably the most important factor which mediates copper uptake in the vast majority of peripheral tissues, including brain, tubular cells of the kidney cortex, choroid plexus, heart, spermatozoa, mammary gland ductal epithelium and retina [183].

2.2.8.2. Placenta. Copper has a significant influence in maintaining normal female reproduction and foetal development [184]. The placenta may be considered a key organ in copper supply to the foetus during pregnancy, being one of the few organs in the human body which expresses both ATP7A and ATP7B, that have distinct functions in copper transport. In human placenta, ATP7A is localized in the cytotrophoblast, in the syncytiotrophoblast and in the foetal vascular endothelial cells whereas ATP7B immunolocalization is restricted to the syncytiotrophoblast [185]. ATP7A is putatively involved in delivering copper to placental cuproenzymes and in transporting the metal ions into the foetal circulation; ATP7B maintains placental copper homeostasis by transporting copper excess into the maternal circulation (Fig. 3) [186]. In placental trophoblasts in culture the two hormones essential during gestation, insulin and oestrogen, may influence copper transport in the placenta by inducing ATP7A translocation to the cell membrane; this results in an increased efflux of copper from the trophoblasts, which could be utilized by the developing foetus [187]. In contrast, levels of ATP7B were reduced in response to insulin, with a corresponding decrease in copper efflux across the apical membrane of the trophoblasts and a lower copper return to the maternal circulation [186]. hCTR1 also exerts a role in copper trafficking in human placenta: in term placenta cells hCTR1 is localized within the syncytiotrophoblasts and in the foetal vascular endothelium in placenta villi, and its immunoexpression is significantly modified by insulin, oestrogen and progesterone [188].

2.2.8.3. Kidneys. Kidneys are of considerable importance in copper metabolism and their copper content is regulate very effectively, being ATP7A and ATP7B the most important copper transporters that contribute to this regulation (Fig. 3). They are co-expressed in epithelial cells of the proximal and distal renal tubules, where ATP7A contributes to export copper excess and to protect the renal parenchyma against copper overload [189]. ATP7A and ATP7B are also expressed in the glomeruli, remarking their responsibility in the regulation of copper levels in the filtrate. ATP7B has been also immunodetected in the epithelial cell lining the loops of Henle in the renal medulla, where it may have a role in copper reabsorption [190]. In HEK 293 cells, a model of human embryonic kidney cells, hCTR1 was shown to be expressed in the plasma membrane, where it provides a permeation pathway for copper acting as a conventional transporter [77].

2.2.8.4. Mammary glands. Mammary glands present a marked avidity for copper, enhanced during lactation, when most of the absorbed copper is diverted from liver and kidney to mammary glands [191]. Three copper-specific transporters ATP7A, ATP7B, and hCTR1 have been identified in human mammary glands [192], even though their exact role in milk copper secretion is not yet well understood. Kelleher et al. using cultured mammary epithelial cells demonstrated the presence of CTR1 in intracellular vesicles, ATP7A in late endosomes and ATP7B in the endoplasmic reticulum and in the trans-Golgi-network [193]. The levels of CTR1, ATP7A and ATP7B in the mammary gland were found related to zinc status: in particular, a zinc deficient diet induced a significant increase of the copper transporter expression in mammary glands of lactating rats, resulting in higher milk copper levels which expose suckling newborns to copper excess [194].

2.2.8.5. Central nervous system. Copper is essential for brain metabolism, serving as a cofactor to superoxide dismutase, dopamine-beta-hydroxylase, amyloid precursor protein, ceruloplasmin and other metalloproteins essential for normal brain function. ATP7A and ATP7B play a central role in distribution of copper in the various compartments of the central nervous system. ATP7B, expressed in Purkinje neurons in the cerebellum, delivers copper ions to ceruloplasmin, whereas during development ATP7A switches from Purkinje cells to Bergmann glia, the cells supporting neurons in the adult brain [195]. ATP7B is also expressed and functionally active in the pineal gland [196]. The study of many researchers in the next future will be focused on

copper trafficking in the adult human brain, due to the mounting evidence that if copper homeostasis is disturbed in patients affected by Alzheimer disease, this leads to oxidative stress and neurodegeneration [197]. Two proteins related to neurodegeneration, the amyloid precursor protein (APP) and the Prion protein are copper-binding proteins and, at the same time, they are the major regulators of neuronal homeostasis. The relevance of metallothionein in neurophysiological and neuromodulatory functions has been stressed by the very high levels of MT found in the central nervous system and by the identification of a brain-specific isoform, Mt-III [198]. The blood–brain barrier is a key structure in copper trafficking from general circulation into the brain. In the macular mouse, an animal model of Menkes disease characterized by a defective intestinal absorption of copper and by copper deficiency in the brain, the administration of copper by intramuscular injections cannot improve the brain status since administered copper is not transported across the blood–brain barrier. The association of diethyldithiocarbamate to injected copper facilitates the passage of copper ions across the blood–brain barrier, opening a new field of research on copper carriers for the brain with the aim of finding a therapy for children affected by Menkes disease [199]. The human copper transporter hCutC, a member of the Cut family associated with copper homeostasis, was isolated from human foetal brains. Probably it functions as a shuttle in neurons and plays an important role in intracellular copper trafficking [200].

2.2.8.6. Retina. Both copper ATPases have been localized in the human retina, in pigment epithelial cells. ATP7A was also detected in the neurosensory retina [201]. These observations may cast doubts on the pathogenesis of copper deposition in the cornea of patients affected by Wilson disease, leading to the typical Kaiser-Fleischer ring [202]. Retinopathy in Wilson disease, generally related to the abnormal systemic copper deposition, could result from loss of retinal ATP7A and ATP7B, and to the consequent dysregulation of copper levels in the different retinal compartments [201].

2.3. Molecular pathology of Wilson's disease

Wilson's disease is the most important inherited disorder of copper metabolism, clinically characterized by juvenile cirrhosis and by neurological disorders [1,19,203]. Its frequency in the world is estimated about one case out of 35,000–100,000 people [204]. In Sardinia, an Italian island of the Mediterranean sea, WD reaches a higher frequency, with an approximate incidence of about 1:7000 live births [205]. Pathological changes in affected individuals are mainly due to accumulation of copper excess in the liver [13], in the brain [14], and in kidney [206]. Copper concentration in the liver of affected patients normally exceeds 250 mg/kg d.t. (normal values below 50 mg/kg), paralleling the high copper content physiologically observed in newborns [207]. The gene responsible for WD (ATP7B) maps to chromosome 13q14.3; it encodes a protein of 1411 amino acids, a copper transporting P-type ATPase (ATPase7B) and it is highly expressed in the liver, kidney and placenta [8,208]. Much lower levels of the transcript were detected in heart, brain, lung, muscle, pancreas and intestine [36]. The ATP7B gene shows homology to the Menkes disease gene, ATP7A [8]. Defective ATP7B function results in disarrangement of copper trafficking in the hepatocyte as well in other cells, resulting in hepatic, neurological and systemic copper accumulation [209]. The main biochemical peculiarity in WD is the block in biliary copper efflux, for which ATP7B is responsible in physiological conditions. Under basal copper conditions, the localization of ATP7B in the trans-Golgi-network (TGN) has been clearly established [210], suggesting a relevant role in the synthesis of ceruloplasmin [211] and in incorporating copper ions in numerous cuproenzymes (Fig. 2) [212]. Under copper stress,

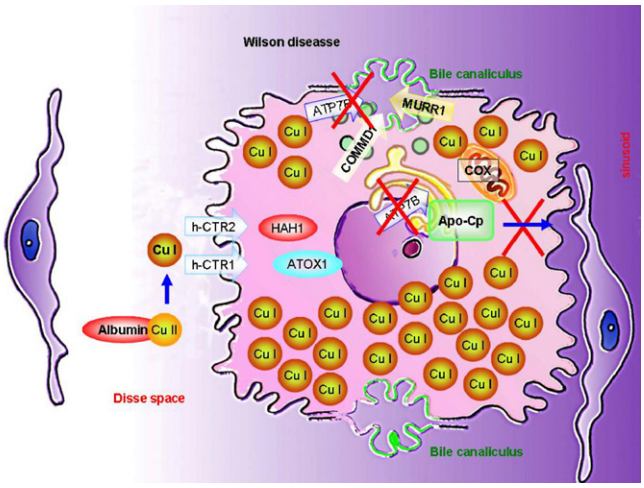


Fig. 4. Copper hepatocytic overload caused by mutations in ATP7B gene in Wilson disease.

ATP7B has been shown to relocalize from the Golgi apparatus to the cell membrane at the biliary pole of the hepatocyte [9], with a switch in function of a protective role, acting as a copper pump that transports the copper excess across the plasma membrane into the bile. The H1069Q mutation in the Wilson gene causes the mislocation of the gene product ATP7B in the endoplasmic reticulum followed by an impairment in copper trafficking resulting in its accumulation and cell death of the hepatocyte (Fig. 4) [213].

When intracellular copper concentrations are reduced, ATP7B shifts its under-stress localization and recycles back to the TGN. The intracellular trafficking of ATP7B requires acyl-phosphate formation [162]. Wilson's disease may be caused by a large number of different mutations in the ATP7B gene, which are listed in a public database (http://www.medgen.med.ualberta.ca/database). The H1069 mutation is the most common in European patients, while the R778L mutation is the most frequently reported in Asian subjects [214]. In patients from Sardinia the 5′UTR mutation in the regulatory part of the gene is the most common mutation [215]. A study on pediatric patients from Italian origin provides evidence that the most common mutations are the missense p.H1069Q and p.M769V, the nonsense p.R1319X, the frameshift c.2299delC, c.2298_2299insC and c.2530delA, and the splice site mutation c.2447+5G>A [216]. No significant correlation between genotype and phenotype has been reported in patients affected by Wilson's disease [217]. A study carried out on a large series of WD patients indicated, for the first time, significant correlations between some mutations and clinical behaviour [216]. In the same study serum ceruloplasmin and copper levels were found lower among the patients' homozygotes for nonsense and frameshift mutations than in patients with missense mutations. A one-step, 3-h, reproducible, and accurate real-time amplification refractory mutation system which can simultaneously detect 28 ATP7B mutations has been recently developed. This rapid and cost-efficient method allows wide mutation coverage, rendering the SYBR-green assay feasible and attractive for large-scale routine application [218].

Several WD mutations are clustered within the nucleotide-binding subdomain (N-domain), including the most common mutation H1069Q. To gain insight into the biophysical behaviour of the N-domain under normal and disease conditions, the wild-type and H1069Q recombinant N-domains have been characterized. The mutant showed only 2-fold lower ATP affinity compared to that of the wild-type N-domain. In the same study, molecular dynamics simulations identified specific differences in both ATP orientation and protein structure that can explain the absence of catalytic activity for the mutant N-domain and can identify changes responsible

for the H1069Q WD phenotype in vivo [219]. In Chinese patients the mutation H1069Q seems to be absent, but R778L has been reported to represent 34–38% of mutations [220]. In a study carried out in Indian patients neither H1069Q nor R778L were detected [221]. In Saudi Arabia a founder effect was observed, with a predominant 4193delC mutation [222]. In a meta-analysis of 557 patients H1069Q was associated with late onset and with neurological disease [223].

3. Therapeutical agents in copper removal: chemical characteristics and copper complexation equilibria

Wilson's disease was a fatal disease until treatments for halting copper storage were developed in the fifties. In times in which the concept of chronic liver disease was emerging, first described in American soldiers after a period spent in the Mediterranean area during World War II, Wilson's disease was the first chronic liver disease for which an effective pharmacologic treatment was discovered. In 1951 2,3-dimercaptopropanol, also known as BAL, was introduced [224], and few years later Walshe introduced penicillamine [225]. While the strong interest on iron chelators for iron overload has lead to a wide literature, and different questions ranging from the mean iron input in humans, its distribution in the living organism, the redox potential of the main complexes in the body, the targets of a chelation therapy have been thoroughly examined [226] this did not happen for the drugs used as copper chelators. The analysis of literature on copper chelators for Wilson's disease shows in fact that these studies are in a less developed stage, and this is surely related to the different spreading of the two diseases [227]. Nevertheless it can be of great utility, when working on copper chelators, to take advantage of the knowledge acquired in the study of iron overload. In the following we will take into consideration separately the copper chelators in use, giving the historic, chemical and pharmacological details.

3.1. Penicillamine

This molecule, molecular weight 149 Da, is characterized by three protonation constants imputable to SH, NH_3^+ and COOH groups, respectively ($\log K_1$ 10.8, $\log K_2$ 8.1 and $\log K_3$ 2.2 obtained as the mean values among the cases reported at 25 °C and μ 0.1 M in the IUPAC Stability Constant Data Base (L.D. Pettit and K.J. Powell, The IUPAC Stability Constants Database, ver. 5.7, Academic Software and IUPAC, Otley, U.K. 2001)). The predominant species in 6.0–7.4 pH range (from the intestinal pH to the human plasma pH) is the zwitterionic form $CH_2SH–CHNH_3^+–COO^-$ (Fig. 5).

In 1968 Peisach and Blumberg [228] were the first authors who pointed out that the chelating properties of H_2Pen alone cannot be responsible for the mobilization of toxic copper in patients with Wilson's disease. To explain this they proposed a mechanism, called reductive chelation, in which unstable Cu(II) complexes are formed that in the end yield Cu(I) and oxidized chelator. One year later this concurrent redox/complexation reaction between Cu(II) and penicillamine was made clear by Sugiura and Tanaka [229] thanks to spectrophotometric and potentiometric methods. They gave evidence that in excess of Cu(II) a red–violet complex is produced whose absorption is more intense than those commonly found in

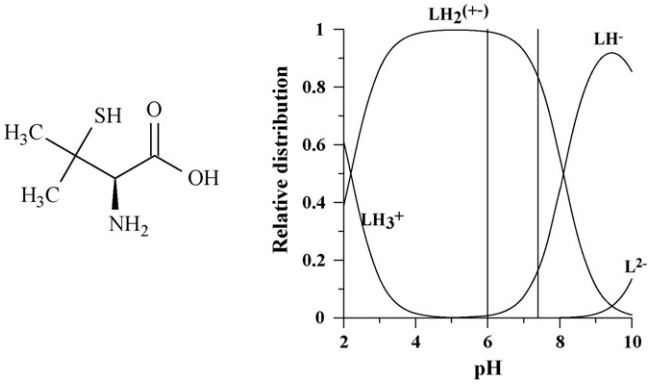


Fig. 5. Molecular formula of penicillamine and distribution curves of its variously protonated forms.

cupric or cuprous complexes, attributed to a mixed valence complex, while in excess of H_2Pen a yellow Cu(I) complex is formed. They also remarked that only those compounds which have a strong copper excretion activity, such as H_2Pen and β-methyl-β-ethylcysteine, form red–violet complexes, contrarily to those which are not effective in copper excretion (N-acetylpenicillamine or cysteine). A red–violet complex formation reaction accompanied by production of oxidized penicillamine was proposed in the form reported in Scheme 1.

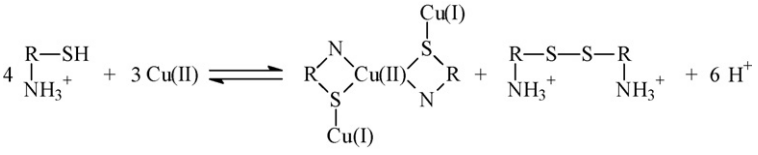
This mixed valence complex was suggested to be implied in copper elimination by penicillamine. The same authors in a successive work [230] proposed a mechanism for copper transfer from the albumin–Cu(II) complex to penicillamine and explained the reductive chelating action of H_2Pen.

In 1977 Birker and Freeman [231] isolated the purple mixed valence cluster complex $[Cu(II)_6Cu(I)_8Pen_{12}Cl]^{5-}$, and obtained crystals with $Co(NH_3)_6^{3+}$, $Ru(NH_3)_6^{3+}$, and Tl^{3+} as counterions. The structure of $Tl_5[Cu(II)_6Cu(I)_8Pen_{12}Cl]^{5-}\cdot nH_2O$ was determined by X-ray diffraction. The complex, while stable for a long period under physiological conditions of pH and saline concentration, decomposes relatively rapidly in urine under aerobic conditions. The known stoichiometry allowed one to rationalize its formation reactions summarized as following:

$$14Cu(II) + 20H_2Pen + Cl^- \rightleftharpoons [Cu(II)_6Cu(I)_8Pen_{12}Cl]^{5-} + 4(HPen)_2 + 32H$$

It accounts for the reaction proposed in Scheme 1 in the limits of experimental errors. The structure determination gives some insights into the chelating action of penicillamine:

1. Cu(II) is in equilibrium with the aqueous medium strongly coordinated by N and S atoms, while Cu(I) is removed from equilibrium;
2. methyl groups of H_2Pen are essential in preventing Cu(I) oxidation;
3. the 12 charged COO^- on cluster surface determine its high aqueous solubility;
4. Cl^- is essential for the formation of the red–violet complex playing an important structural role.



Scheme 1. Red–violet complex formation as reported by Sugiura and Tanaka [229].

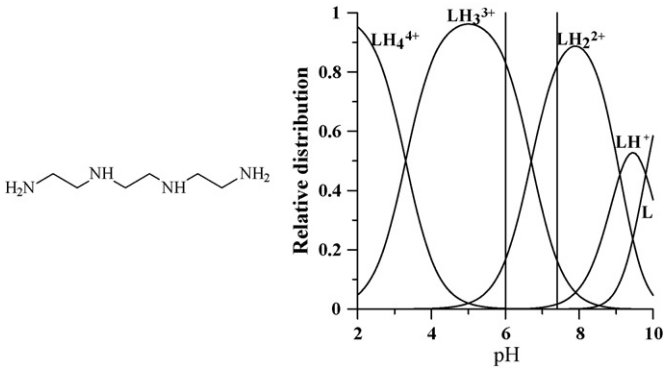


Fig. 6. Molecular formula of Trien and distribution curves of its variously protonated forms.

More recently, Kato et al. [232] studied by ^{1}H NMR spectroscopy the reaction of Cu(I) and Cu(II) with H_2Pen in absence and in presence of glutathione, under aerobic and anaerobic conditions. They confirmed the observations of Birker and Freeman [231], but above all they indicated that the cluster is always the final product under aerobic conditions, regardless the presence of other thiols such as glutathione, i.e. although the cluster is decomposed reductively by thiols, it is reproduced under oxidative conditions. This might be ascribed to the anomalous stability of the cluster with respect to that of any other copper complex with H_2Pen or with other thiols. This peculiar stability determine the efficacy of penicillamine as a drug for Wilson's disease.

3.2. Trien

Triethylenetetramine dihydrochloride (also known as Trientine or Trien 2HCl) was introduced in 1969 in clinical use by Walshe [233] as an alternative for patients presenting intolerance toward penicillamine.

This molecule has, in its dihydrochloride form, molecular weight of 219.2 Da. An oral LD_{50} of 17.1 mmol/kg in rat [234] is reported. It is characterized by four protonation constants ($\log K_1$ 9.8(1), $\log K_2$ 9.1(1), $\log K_3$ 6.7(1), $\log K_4$ 3.3(2)) obtained as the mean values among the cases reported at 25 °C and μ 0.1 M in the IUPAC Stability Constant Data Base.

A very poor intestinal absorption of Trien was reported by Walshe and Gibbs [235]. In Fig. 6 the distribution plot of Trien as a function of pH is presented. The high positive charge of Trien, from +2 to +3 in the gut pH range 6–7.4 [236], explains its poor intestinal absorption according Lipinski criterion [237–238]. Gibbs and Walshe [239] showed that only 6–18% of orally administered Trien was systemically absorbed, in line with Kodama [240] (about 10% of the orally administered trien can be found in urines: ~1% as Trien and ~8% as acetyltrien) and with the more recent results of Lu (urinary recovery ranging from 0.03 to 13.4% in healthy volunteers and from 3.7 to 14.6% in diabetic subjects) [241].

Trien is a very strong chelator toward Cu(II). Its complex formation equilibria have been extensively studied since the work of Schwarzenbach [240], and the stability constant relative to a $[CuL]^{2+}$ complex reported by different authors [243–245] agree on a $\log K$ value 20.3 at 25 °C and μ 0.1 M; minor species of different stoichiometries, proposed by Laurie [246] and by Delgado [247], are present only in a limited amount at pH 7.4. A pCu(II)* of about 18 can be calculated from the stability constant of the 1:1 complex. Trien chelates also other essential metal ions as Zn(II) [242,244,248] and Fe(II) [244], but the stability of their complexes is much lower than that with Cu(II); the stability of Fe(III) complex ($\log K$ 21.94 [249]) is comparable with that of Cu(II) complex, but we strongly suggest a re-examination of the Fe(III)-trien equilibrium. In a paper of 1973 Walshe [250], after discussing the results of a 4-year treatment on the first Wilson's disease patient successfully handled with Trien, presented a comparison of the ability of H_2Pen and Trien to mobilize copper on a pool of 18 patients. In those who had no previous treatment with H_2Pen both agents induced a very large cupruresis, while in patients effectively decoppered by prolonged treatment the cupruretic response was lower. The different trend of copper serum concentration associated with cupruresis induced by chelating agents (H_2Pen causes a fall, while Trien a rise that returns to the base line at 5 h) is attributed by Walshe to the mobilization of copper from different body compartments. Different authors state that the amount of Trien which remains un-absorbed in the gut decreases the intestinal copper absorption by complex formation [251–253].

3.3. Tetrathiomolybdate

The discovery of the anticopper action of tetrathiomolybdate (TM) dates back to observations in New Zealand that ruminants grazing on pastures whose soil was rich in molybdenum, developed a copper deficiency [254–256]. The sulfur-rich rumen converts molybdenum to different thiomolybdates which have anticopper activity in all animals [257]. Among the various thiomolybdates TM is the most potent. TM is toxic toward animals, but all the toxic effects are due to copper deficiency and are reversed by copper supplementation [258–263]. TM, extensively studied in sheep, acts as anticopper agent by forming mixed copper complexes with proteins. These prevent copper absorption when TM is given with food and render serum copper unavailable for cellular uptake forming a mixed albumin complex, metabolized in liver and excreted in the bile [262–268]. Actually only few chemical studies are found on complex formation equilibria between tetrathiomolybdate and copper as well as on ionization equilibria of tetrathiomolybdate, presumably for the difficult preparation of this ligand. Nevertheless some studies are important, above all those of Suzuki and Ogra [269], who pointed out the formation of different complexes copper–TM: when the molar ratio of TM with respect to copper bound to metallothionein is less than unity a ternary complex copper–TM–metallothionein is formed, when it is between 1 and 2 copper is removed from metallothionein as a soluble binary complex copper–TM, and when it is greater than 2 the copper–TM complex becomes insoluble [270]. George et al. have recently studied by X-ray absorption spectroscopy the liver lysosomes from a TM treated LEC rat; on the basis of their data they propose the formation of a copper–molybdenum–sulfur cluster, that suggests that the mechanism of action of TM may be a decrease in the bioavailability of copper or a change in its redox properties, both resulting from cluster formation [271].

The first use of TM in Wilson's disease is always due to Walshe [272] who in 1986 tried it on two patients intolerant to both H_2Pen and trien. The group of Brewer received FDA approval for use of TM in Wilson's patients on the basis of rather extensive animal studies, although formal toxicity studies on humans are lacking. The results of their extensive studies led them to consider TM as a better choice than TRIEN for preserving neurologic function in patients with neurologic disease [273].

4. Conclusions

The low redox potential of copper which motivates its employment by a multiplicity of enzymes involved in oxidative reactions renders copper an essential metal ion for the human organism, playing numerous different roles in the metabolic processes. This feature at the same time contributes to the production of toxic radicals which oxidatively damage different classes of biomolecules,

such as proteins, lipids and DNA. To avoid these injuries, and the related diseases, living organisms possess firm control of absorption, storage and reuse of copper. In this review a general overview on copper metabolism has been made, presenting up to date research on the key proteins and on the mechanisms involved in the homeostatic control. Further, a brief discussion has been given on Wilson's disease and on the "chelating" drugs used for the removal of copper excess from WD patients. The interest on copper (metal) removal for the treatment of neurodegenerative diseases is now of great relevance, and will be more so in the future, as remarked in two recent reviews by Que et al. and Kozlowski et al. [21,22]. The central nervous system is, in this case, the target organ for the chelating therapy, and the search for proper chelators, which have both to bind copper efficiently and selectively, and to possess suitable transport properties across the BBB and good bioavailability, will be a challenge for the joint efforts of chemical biomedical, and biochemical researchers.

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Glossary

Angiogenesis: Process involving the growth of new blood vessels from pre-existing vessels, typical of wound healing as well as of tumor progression.
Apoptosis: Programmed cell death, caused by the activations of caspases (enzymes: cysteine proteases) in a cascade, responsible for the cleavage of the key cellular proteins, such as cytoskeletal proteins, that leads to the typical morphological changes.
APP: Amyloid precursor protein.
ATP7A: Adenosin-tri-phosphatase 7a.
ATP7B: Adenosin-tri-phosphatase 7b.
Apical uptake: Passage of molecules, such as nutrients, through the apical pole (brush border) of enterocytes.
Basolateral uptake: Passage of molecules, such as nutrients, through the basolateral surface of enterocytes.
Biliary pole of hepatocytes: The hepatocyte is a polyhedral epithelial cell of the liver, highly polarized with transport directed from its sinusoidal pole to the biliary pole, which is the part of plasma membrane that bound the intercellular space constituting the bile canaliculus.
Biliary tree: All the biliary ducts, which originate by the biliary poles of hepatocytes, give rice to bile canaliculi, main bile ducts which carry bile juice out of the liver towards the gut.
Brush border: The name for the microvilli which cover the apical surface (border) of enterocytes bordering the intestinal lumen; on light microscope microvilli can usually only be seen collectively as a fuzzy fringe at the surface of the epithelium, that gave rise to the term "brush border".
Catecholamines: Hormones derived from the amino acid tyrosine and containing a catechol group, released by the adrenal glands and/or sympathetic nervous system in response to stress.
CCS1: Copper-delivering Chaperone required for cytosolic superoxide dismutase (SOD1).
Central nervous system: The central nervous system (CNS) is the part of the nervous system that functions to coordinate the activity of all parts of the body and consists of the brain and the spinal cord.
Ceruloplasmin: Ceruloplasmin is a ferroxidase or iron(II):oxygen oxidoreductase. It is involved in copper, plays a role in iron metabolism. It is produced in liver and secreted into blood.
COX-11: Cytochrome *c* oxidase 11(mitochondrial metallo-chaperone).
COX-17: Cytochrome *c* oxidase 17 (mitochondrial copper chaperone).
COX-2: Cytochrome *c* oxidase 2 (copper chaperone).
CTR1: Cation transporter 1(copper transporter).
Cytotrophoblast: The inner layer of the trophoblast, interior to the syncytiotrophoblast in the chorionic villi of placenta.
Decompensated cirrhosis: Cirrhosis is defined anatomically by the presence throughout the liver of fibrous septa that subdivide the parenchyma into nodules in chronic end stage liver disease. Decompensed cirrhosis is characterized by symptoms such as ascites (liquid accumulation in the abdominal cavity) and rupture of oesophageal varices (dilated veins) with hemorrhage.
DMT1: Divalent metal transporter 1.
Endocytosis: Incorporation of external substances through invaginations of the cell membrane which originates small vesicles that spread into the cytoplasm of the cells.
Enterocyte: Intestinal absorptive cell, simple columnar epithelial cells covering the internal surface of the gut.
ERK: Extracellular signal-regulated kinase.
Erythrocytes: Red blood cells.
Exocytosis: Transport of the content of secretory vesicles out of the cell membrane; the membrane-bound vesicles move to the cell surface where they fuse with the plasma membrane.
Fibroblast: Type of cell that synthesizes the extracellular matrix and collagen; it is the most common cell of connective tissue.
GRX1: Glutaredoxin 1: an essential factor for ATP7A and ATP7B function.
HAH1: Human Atx1p homologue (copper chaperone).
hCTR1: Human cation transporter 1.
hCTR2: Human cation transporter 2.
Hepatocyte: The main cell of the liver, it is involved in protein synthesis, protein storage and transformation of carbohydrates, synthesis of cholesterol, bile salts and phospholipids, and detoxification, modification and excretion of exogenous and endogenous substances.

Intravascular haemolysis: Process of fragmentation of red blood cell (haemolysis) inside blood vessels.
Liver cirrhosis: Chronic liver disease characterized by severe modification of the liver architecture due to liver cell death diffuse fibrous scars (liver fibrosis), fibrous septa that subdivide the parenchyma into nodules.
Lysosomes: The name lysosome derives from the Greek word "lysis", which means dissolution or destruction, and "soma", which means body. They are large, spherical intracellular organelles that contain lytic enzymes (acid hydrolases), with a membrane around them that allow the digestive enzymes to work at pH 4.5. Lysosomes fuse with vacuoles and dispense their enzymes into the vacuoles, digesting their contents.
Mitochondrion: Intracellular membrane-enclosed organelle specialized in ATP synthesis (energy production) for cell functions.
MNK: Menkes' protein, synonymus of ATP7a.
MTs: Metallothioneins, proteins specialized in the storage of metals (copper, zinc, etc.).
MURR1: Also called COMMD1 is a gene which has been hypothesized essential for copper excretion at the biliary pole of hepatocytes.
Neuroblastoma cells: Tumor cells of a brain tumor (neuroblastoma).
Neurodegeneration: Process of degeneration of nervous cells.
NPC1: Niemann-Pick disease type C protein, a new intracellular transporter of ATP7B.
Portal vein: The vein of the abdominal cavity that drains blood from the gastrointestinal tract and spleen to the liver.
Proteases: Enzymes involved in proteolysis, that is protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein.
RDA: Recommended dietary allowance.
Retinopathy: Damage to the retina of the eye.
SCO1: Copper chaperone.
SCO2: Copper chaperone.
SLC31: Solute-linked carrier 31 (copper chaperone).
Syncytiotrophoblast: Multinucleated cells covering placenta villi, the outer syncytial layer of the trophoblasts, outer to the cytiotrophoblast.
Trans-Golgi-network/golgi apparatus: Intracellular organelle found in most eukaryotic cells, located near the nucleus, deputed to the "maturation" of proteins and to their incorporation into vesicles for their secretion out of the cell. The Golgi apparatus is composed of membrane-bound stacks known as cisternae. The cisternae stack has four functional regions: the cis-Golgi-network, medial-Golgi, trans-Golgi, and trans-Golgi-network. The trans face of the trans-Golgi-network is the face from which vesicles leave the Golgi apparatus.
Translocation: The ability of a protein of moving from an intracellular localization to another. It is typical of ATP7B, which when copper intracellular content increases, movies from the trans-Golgi-network to the cell membrane.
Transmembrane protein: Protein with three regions or domains that can be defined: the domain located inside the cell membrane lipid bilayer; the domain outside the cell (called the extracellular domain); and the domain inside the cell (called the intracellular domain).
Ubiquitinylation: Process through ubiquitin attachment to altered proteins can alter the function or location of the protein, or target it for destruction.
Wilson disease: Wilson's disease is an autosomal recessive genetic disorder in which copper accumulates in tissues.
XIAP: X-linked Inhibitor of Apoptosis Protein, a member of the inhibitor of apoptosis family of proteins.