

Title: The multifaceted roles of Copper in cancer: a trace metal element with dysregulated metabolism, but also a target or a bullet for therapy

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Abstract:

In the human body, Copper (Cu) is a major and essential player in a large number of cellular mechanisms and signaling pathways. The involvement of Cu in oxidation-reduction reactions requires close regulation of copper metabolism in order to avoid toxic effects. In many types of cancer, variations in copper protein levels have been demonstrated. These variations result in increased concentrations of intra-tumoral Cu and alterations in the systemic distribution of copper. Such alterations in Cu homeostasis may promote tumor growth or invasiveness, or even confer resistance to treatments. Once characterized, the dysregulated Cu metabolism is pinpointing several promising biomarkers for clinical use, with prognostic or predictive capabilities.

The altered Cu metabolism in cancer cells and the different responses of tumor cells to Cu are strongly supporting the development of treatments to disrupt, deplete or increase Cu levels in tumors. The metallic nature of Cu, as a chemical element, is key for the development of anticancer agents *via* the synthesis of nanoparticles or copper-based complexes with antineoplastic properties for therapy. Finally, some of these new therapeutic strategies such as chelators or ionophores have shown promising results in a preclinical setting, while others are already in the clinic.

Keywords: Copper homeostasis, Cancer, Prognostic, Diagnostic, Therapy

Introduction:

Trace elements such as copper (Cu) are involved in many physiological processes. It has been shown that disturbances in copper homeostasis lead to structural abnormalities or loss of certain essential physiological functions. It has been clearly demonstrated that copper homeostasis is deregulated in many cancers. In addition, numerous studies showed that the deregulation of trace element homeostasis may be, at the same time the cause and the consequence of carcinogenesis. Some studies have also revealed that these dysregulations could be of clinical interest as a prognostic and/or predictive biomarker of response to treatment. Accordingly, several therapeutic strategies targeting or using trace elements have been developed. In view of such a rich literature, we present the most significant studies with results and cell mechanisms relating to Cu homeostasis dysregulation and cancer. This review is also an opportunity to present the discrepant results on this subject. Finally, in this work, we review the main therapeutic strategies targeting Cu or using Cu as a central player for cancer treatment.

1. Copper normal metabolism

Cu is an essential trace element with a short half-life of about a month. The required daily intake is 0.8 mg [1]. Cu concentrations fluctuate from 1 to 10 mg/g of tissues and is of approximately 1000 ng/ml in blood plasma. However, Cu concentration may vary depending on various factors [2].

In the body, copper is present in two redox states, Cu(I) and Cu(II). In fluids, it is in Cu(II) state, while in the intracellular reducing environment Cu is mainly found in its Cu(I) state. However, in redox enzymes, Cu is shuttling between Cu(I) and Cu(II). Cu plays an important role for various cellular functions since it is an essential cofactor for many enzymes such as tyrosinases, or antioxidant enzymes such as Superoxides Dismutases (SOD) [3,4]. Cu is also involved in angiogenesis cellular mechanisms as well as in other signaling pathways [5–7].

Although essential for normal cell function, free Cu can induce toxicity to cells. In physiologic conditions, Cu is thus always bound to peptides or proteins to prevent uncontrolled redox activity. Several cellular mechanisms allow precise regulation of the spatial and temporal distributions of Cu (Figure 1A-B). In human cells, Cu is internalized by the Copper Transport Protein 1 (Ctr1) [8]. Additionally, a second Cu transport protein Ctr2 exists, however it has a lower affinity for Cu and its role remains unclear. The Ctr1 protein is trafficking between the plasma membrane and intracellular vesicles to control the entry of Cu into the cell. Thus, in the case of increased copper levels, Ctr1 is internalized in intracellular vesicles. The Ctr1 protein is essential for the stability of Ctr2, and Ctr1 and

Ctr2 have interconnected functions in the Cu homeostasis system [9]. Finally, a model suggests that the regulation of systemic copper and intracellular mobilization involves the cleavage of the ecto-domain of Ctr1 that binds copper. Ctr2 may regulate the formation of the truncated form of Ctr1 (tCtr1) [10]. Moreover, the absence of Ctr2 leads to the accumulation of Cu in the endosomal compartments. Once in the cytoplasm, Cu is distributed through four main pathways (Figure 1A-B).

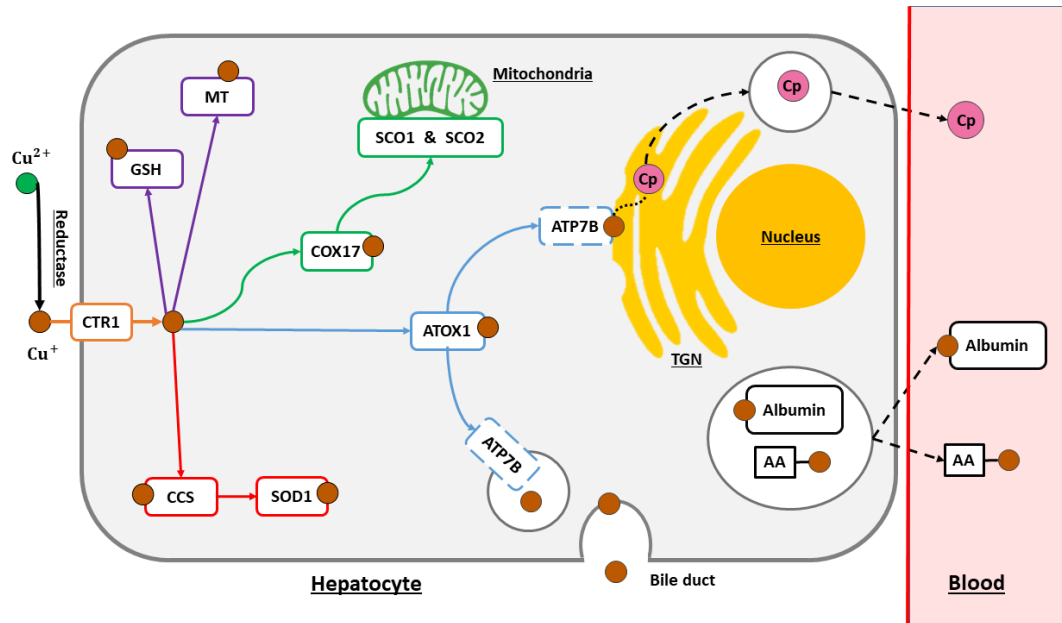


Figure 1A

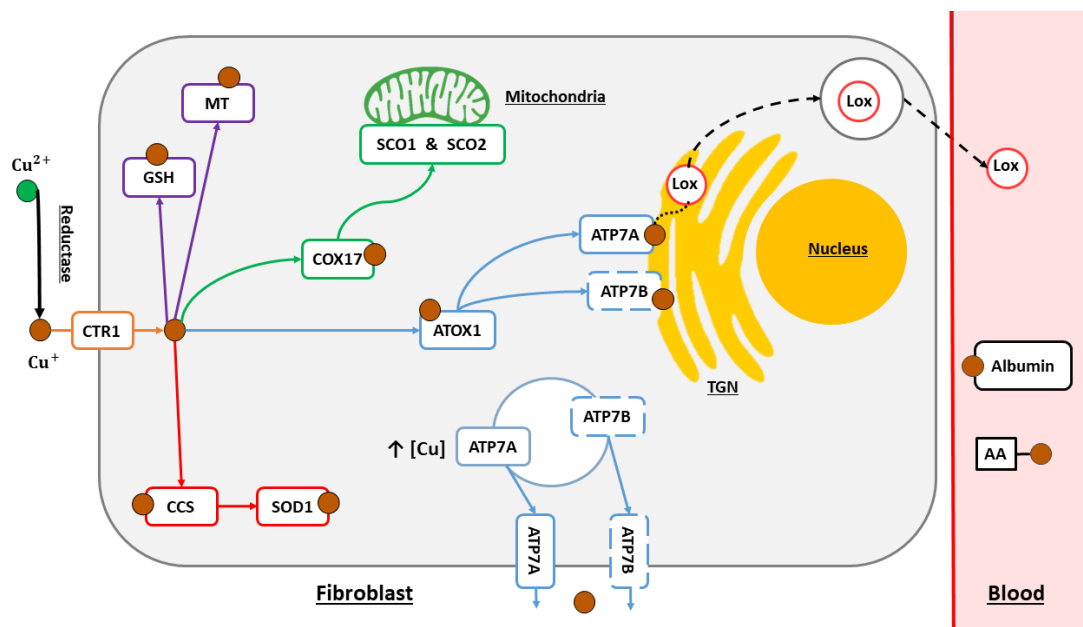


Figure 1B

Figure 1: Cellular copper homeostasis in hepatocytes (A) and fibroblasts (B).

In cells, the internalization of reduced Cu occurs *via* Ctr1. Then, Cu is involved in the control of oxidative stress (red arrows) through copper chaperone for superoxide dismutase protein (CCS) and cytoplasmic

Cu/Zn superoxide dismutase 1 (SOD1). Copper can be stored in its main storage reservoir, the mitochondria, where the proteins Cox17, Sco1, and Sco2 play a role in its incorporation into the complex IV of the respiratory chain (green arrows). Excess of Cu can also be sequestered by metallothioneins (MT) and/or bound by glutathione (GSH) (purple arrows).

(A) In hepatocytes, copper can be secreted *via* the bile duct or released in the blood stream, after transport by ATOX1 and ATP7B proteins (blue arrows) and transit in the Trans-Golgi Network (TGN), bound to ceruloplasmin (Cp). Cu is released into the blood stream, and travels bound to Cp, amino acids or albumin.

(B) In fibroblasts, ATP7A carries Cu from ATOX1 to the TGN for its integration into Lysyl oxidase (LOX) before its release into the blood stream. In addition, excess of intracellular Cu can be removed from the cell through ATP7A and ATP7B.

1.1 The secretory pathway:

The cytoplasmic Cu Chaperone, Antioxidant-1 protein (Atox1) brings Cu to copper transport ATPases, ATP7A and ATP7B. Both are present at the trans-golgi network level, importing Cu into the Golgi apparatus lumen for the maturation of Cu-dependent target enzymes such as Ceruloplasmin (Cp) or Lysyl oxidase (LOX), both secreted by exocytosis into the blood stream. Cu bound to Cp is the main form of Cu in the blood (80-90%). Cp is mainly synthesized in hepatocytes, but also in kidneys, placenta, breast, or brain. Macrophages and mononuclear cells from blood can also produce Cp during inflammation [2]. The LOX enzyme ensures the reticulation of collagen and elastin to preserve rigidity and structural stability of the extracellular matrix.

As soon as intracellular Cu concentration increases and is in excess, both ATP7A/B proteins are relocated to the plasma membrane to excrete Cu out of the cell [8].

The expression levels of ATP7A and ATP7B vary depending on the tissue. The ATP7A protein (Menkes' protein) is expressed in all cell types except hepatocytes. The ATP7B protein (Wilson's protein) is mainly expressed in the liver, but also in the kidney, placenta, heart, brain, and lung tissues [11].

ATP7B is central for Cu homeostasis in the liver and thereof at the organismal level since liver regulates Cu concentrations in blood. Indeed, this protein ensures the excretion of copper from hepatocytes to the bile in response to Cu overload, which is the main process to decrease Cu level in the organism.

In addition, in plasma or other fluids, Cu is bound to different amino acids, peptides or proteins such as histidine, albumin, transcuprein or metallothioneins [12–15].

This pathway can be subject to disruptions due to mutations in the genes coding for the proteins ATP7A or ATP7B leading to Wilson's and Menkes' diseases, respectively. The former is an inherited autosomal recessive genetic disease resulting in copper accumulation in the body, principally in the liver and the brain and triggering hepatic and neuropsychiatric symptoms [16]. Menkes' disease is an X-linked

recessive disorder in which patients have a lack of Cu, which induces growth delays and nervous system alteration.

1.2 The cytosolic pathway:

The copper chaperone for superoxide dismutase (CCS) protein transports Cu towards the Cu/Zn Cytoplasmic Superoxide Dismutase 1 (SOD1) (Figure 1A-B). SOD1 is a key player in the process of defense against oxidative stress because it catalyses the degradation of superoxide anions into hydrogen peroxide and oxygen [7].

1.3 The mitochondrial pathway:

Cu is actively transported to the mitochondrial matrix where it is required to mature in a first instance cytochrome c oxidase (COX), which is the complex IV of the respiratory chain. The identity of the chaperone driving Cu through the mitochondrial membranes is not clearly established but it could be either COX17 or a non-protein metallophore of unknown identity and named Cu ligand [17]. Within mitochondria, the metallochaperones Synthesis Of Cytochrome C Oxidase 1 (Sco1) / Synthesis Of Cytochrome C Oxidase 2 (Sco2) (Figure 1-2) are then involved in further Cu insertion into the COX. Being the main intracellular Cu storage reservoir, mitochondria host a Cu-dependent energy production *via* oxidative phosphorylation [8,18].

1.4 The metal detoxification pathway:

In cells, Cu is also stored in lysosomes. Metallo-reductases, such as the human 6-transmembrane epithelial antigen of prostate (STEAP) family proteins are necessary to maintain copper in its Cu(I) state because lysosomes are an oxidative environment. These reductases, principally STEAP3 and STEAP4, are located in intracellular vesicles and are involved in many biological processes, such as regulation of cell proliferation and apoptosis [19,20].

Metallothioneins (MT) and glutathione (GSH) are other important players for intracellular storage and sequestration of excess Cu. Metallothioneins are cysteine-rich cytoplasmic proteins. They play an important role in the homeostasis and detoxification of metals. They have the ability to bind metals such as Cu and zinc (Zn). In humans, there are 4 distinct MT, identified as MT1, MT2, MT3 and MT4. MT1 and MT2 are the main ones, inducible *via* numerous stimuli. The MT3 and MT4 proteins are mainly expressed in the central nervous system [21]. The GSH is involved in several mechanisms such as xenobiotics' metabolism and redox signaling, but also transfer and detoxification of metal ions such as Cu [22,23]. The majority of cytosolic copper is found in Cu(I)-GSH complexes. Indeed, this complex is

considered as a major contributor to the exchangeable pool of Cu in the cytosol. However, this point is subject to debate in the scientific community [24,25].

2. Copper metabolism in cancers

When compared with non-pathological conditions, variations in Cu concentrations or in the Cu/Zn ratios were associated with many cancers. The Cu/Zn ratio is of clinical importance due to its relationship with aging, nutritional status, oxidative stress, inflammation and immune abnormalities [26,27]. Increased Cu levels were associated with decreased Zn levels, in a meta-analysis in bladder cancer [28], as well as in breast cancer, CRC, and prostate cancers [29–35]. Importantly, some discrepant studies reported decreases in Cu levels in CRC and breast cancers [36,37].

Cu is important for functions involved in proliferation or angiogenesis, which are central for tumorigenesis and cancer development. Copper is acting on different molecular pathways leading to a pro-angiogenic response necessary for carcinogenesis processes. It appears that copper also influences the spread and formation of secondary tumors *via* the activation of enzymes responsible for cell proliferation. It is therefore not surprising that Cu concentration is increased in tumors area [38–40]. More recently, it was shown that specific Cu accumulation can be observed in cancer cells themselves [39,41]. It is worth noting that the accumulation of Cu in the nuclear region has been found in breast cancer cells [42].

Besides, early reports described the increases of serum Cu in cancer patients, sometimes even correlated with the grade of the cancer [43]. High serum Cu levels were also found in cancer patients resistant to chemotherapy compared to patients responding to treatment [43]. However, this remains unexplained up to now and data on different types of cancer are plethora and thereof puzzling.

More recently, isotopic fractionation was developed for biological samples, usually measured in blood. It has been shown that isotopic $^{63}\text{Cu}/^{65}\text{Cu}$ ratio is modified in the serum of cancer patients [44], where the lighter isotope is enriched in blood. This phenomenon could be due to metabolism modifications in cancers that shift towards glycolysis, and producing more lactates. This would explain the higher excretion of ^{63}Cu by ATP7A in the blood stream. Besides, it has been shown that Cu isotopic ratio can be used as an early diagnostic biomarker for cancer, usable several months before other classical protein marker. Since Cu turnover is short (i.e. about one month), it is also convenient as a follow-up marker during treatment to monitor the therapeutic efficacy.

Altogether, it is clear that Cu is central for cancer development at each step from tumorigenesis to metastasis. Cancer cell metabolism is also impacting Cu metabolism. Therefore, it is expected that prognostic and diagnostic markers for cancer can be identified in relation with Cu.

3. The use of Copper proteins as cancer biomarkers

As discussed earlier; it is now widely accepted that disruption of copper homeostasis occurs in several cases of cancer. This can be linked with increases or decreases in mRNA and protein levels. Copper homeostasis proteins appear to be promising candidates as predictive biomarkers of treatment response or as prognostic biomarkers and their clinical use may guide the choice for optimal therapeutic strategy in a near future (Table 1).

Table 1: Cu regulators and their role in cancer.

Altered player	Regulation	Sample	Cancer	Prognostic	Ref.
Atox1	+	Tissue	Breast	poor	[45]
Atox1	+	Tissue	Melanoma	poor	[47]
Atox1	+	Tissue	Skin		[47]
Atox1	+		Blood		[47]
Atox1	+	Tissue	Colorectal cancer	poor	[50]
ATP7A	+	Tissue	Pancreatic cancer		[52]
ATP7A	+	Tissue	Breast	poor	[54]
ATP7A	+	Cell lines/Tissue	Lung cancer	poor	[56]
ATP7A	+	Tissue	Colorectal cancer		[46,55]
ATP7A	+	Cell lines/Tissue	Ovarian cancer	poor	[57,58]
ATP7B	+	Tissue	Oesophageal Carcinoma	poor	[62]
ATP7B	+	Tissue	Endometrial Carcinoma	poor	[130]
ATP7B	+	Tissue	Breast	poor	[131]

ATP7B	+	Tissue	Oral Squamous Cell Carcinoma	poor	[61]
ATP7B	+	Tissue	Gastric carcinoma	poor	[132]
ATP7B	+	Tissue	Hepatocellular Carcinoma	poor	[133]
LOX-1	+	Tissue	Pancreatic Cancer	poor	[134]
LOX-1	+	Tissue	Prostate cancer	poor	[135]
LOX	+	Tissue	Colorectal Cancer	poor	[87]
LOX-2	+	Tissue/Sera	Hepatocellular Carcinoma	poor	[88]
LOX	+	Tissue	Breast	poor	[136–138]
LOX	-	Tissue	Bronchogenic carcinoma	poor	[139]
LOX	-	Tissue / cell lines	Gastric cancers		[140]
LOX	+	Tissue	Head and neck squamous cell carcinoma	poor	[141]
LOX	+	Tissue/cell lines	Lung adenocarcinoma	poor	[142,143]
LOX	+	cell lines	Melanoma	poor	[136]
LOX	+	Tissue	Oral and oropharyngeal squamous cell	poor	[144]
LOX	-	Tissue/cell lines	Basal and squamous cell carcinomas		[145]
LOX	+	Tissue	Renal cell carcinoma		[146]
LOXL1	-	cell lines	Bladder cancer		[89]
LOXL4	-	cell lines	Bladder cancer		[89]
LOXL1	+	cell lines	Lung adenocarcinoma		[147]
LOXL1	+	TISSUE	Salivary gland adenoid cystic carcinoma		[148]
LOXL2	+	Tissue/cell lines	Breast	poor	[149–153]
LOXL2	+	Tissue	Colorectal Cancer	poor	[152,154–156]

LOXL2	+	Tissue/cell lines	Gastric cancers	poor	[152,157]
LOXL2	+	Tissue	Endometrial	poor	[153]
LOXL2	+	Tissue	Testicular	poor	[153]
LOXL2	+	Tissue	Hepatocellular Carcinoma	poor	[153]
LOXL2	+	Tissue	Lung Squamous cell carcinoma	poor	[149,158]
LOXL2	+	cell lines	Melanoma	poor	[136]
LOXL2	+	Tissue/cell lines	Pancreatic Cancer	poor	[153,159]
LOXL2	+	Tissue	Prostate cancer	poor	[160]
LOXL2	+	Tissue	Renal cell carcinoma	poor	[153]
LOXL2	+	Tissue	Laryngeal	poor	[149,153]
LOXL2	+	Tissue	Oesophageal squamous cell	poor	[153,161]
LOXL3	+	cell lines	Breast		[162]
LOXL3	+	cell lines	Melanoma		[136,162]
LOXL4	+	Tissue	Colorectal Adenocarcinoma		[154]
LOXL4	+	Tissue/cell lines	Head and neck squamous cell carcinoma		[163–165]
CTR1	+	Tissue	Colorectal		[46]
SCO1	+	Tissue	Colorectal		[46]
COX11	+	Tissue	Colorectal		[46]
CTR1	+	Tissue	Non-Small-Cell Lung	good	[72]
CTR1	Variable	Tissue	Ovarian		[166]
CTR2	Variable	Tissue	Ovarian		[166]
CTR1	+	Cell lines	Ovarian		[69]
CTR1	+	Tissue	Breast		[45]
Cu	+	Serum	Bladder		[28]
Zn	-	Serum	Bladder		[28]
Cu	+	Serum	Breast		[30,32,37,167]
Cu/Zn	+	Serum	Breast		[30,32,167]
Cu	+	Serum/Tissue	Lung Cancer		[39,41]
Cu/Zn	+	Serum	Lung Cancer		[39]
Cu	±	Serum/Tissue	Colorectal cancer		[29,33,36]
Cu/Zn	+	Serum/Tissue	Colorectal cancer		[29,33,36]
Cu/Zn	+	Serum	Pancreatic cancer		[34]
Cu	-	Serum	Pancreatic cancer		[34]
Cu	+	Serum	Cervical Cancer and Uterine Myoma		[35]
Cu/Zn	+	Serum	Cervical Cancer and Uterine Myoma		[35]
Cu	+	Serum	Prostate		[31]

Cu	+	Serum	Papillary Thyroid Carcinoma	[168]
Cu	-	Serum	Endometrial cancer	[169]
GSH	+	Tissue	Hepatocellular Carcinoma	[65]
CSS	+	Tissue	Brain	[170]
CSS	+	Tissue	Ovarian	[170]
CSS	-	Tissue	Hepatocellular Carcinoma	[170]
CSS	-	Tissue	Prostate	[170]

Large scale studies such as the analysis of TCGA data in breast cancer revealed an increase in several copper-related proteins, including ATP7B and Ctr1 [45]. Moreover, Ctr1, SCO1, and COX11 were increased in a transcriptomic analysis of copper homeostasis genes in CRC samples [46].

The Atox1 protein is increased in many types of cancer tissues [45]. In blood, breast and skin cancers the mRNA levels of Atox1 are significantly higher compared to non-pathological tissues (Table 1) [47]. Moreover it has been shown that Atox1 has a role in the migration of cancer cells in breast cancer [48]. This protein also promotes inflammatory neovascularization through its putative action as a transcription factor and as a cytoplasmic Cu chaperone [5]. Atox1 may be a potential prognostic biomarker for estrogen-receptor (ER)-positive and early stages of breast cancers, since increased expression levels of Atox1 correlated with poor survival for stages 1 and 2 breast cancers. However, preclinical studies and clinical trials are required to better understand the carcinogenic role of Atox1 [49]. In melanoma, a correlation between overexpression of Atox1 and poor prognosis was observed, since the knockdown of Atox1 decreased cell growth and BRAF V600E-dependent signaling in human melanoma cell lines [47]. From a tissue microarray, the increased nuclear translocation of Atox1 was observed in metastatic colorectal cancer (CRC), and was correlated with the severity of the disease [50]. The nuclear localization of Atox1 was also correlated with increased migration capabilities in breast cancer cells, in an ATP7A- and LOX-dependent mechanism [51]. Indeed, the co-localization of the three proteins, Atox1, ATP7A and LOX, was observed at the lamellipodia border of the cell. The reason is unclear but it could be a way to maximize LOX maturation close to its excretion site, where it plays a major role in cancer cell migration and metastasis.

ATP7A protein is also deregulated in many cancers, such as in pancreatic cancer where ATP7A is upregulated compared to chronic pancreatitis [52]. The ATP7A protein plays an important role in the formation of metastases in breast cancer and induces the migration of vascular smooth muscle cells [53]. In addition, high levels of ATP7A expression in primary tumors are associated with reduced survival according to publicly available databases [54]. In CRCs, an increase in the level of ATP7A mRNA

was also found [46] and ATP7A may be a promising predictive biomarker of drug resistance in human CRCs [55].

In lung cancers, ATP7A protein is only expressed in 40% of tumor tissues, and patients expressing ATP7A had a poorer response to platinum-based chemotherapy. At the cellular level, both mRNA and protein levels of ATP7A were significantly higher in multidrug resistant A549 human lung adenocarcinoma cell lines when compared with the parental sensitive cells. ATP7A expression may thus be a predictive biomarker of chemoresistance and a negative prognostic factor for survival in non-small cell lung cancer (NSCLC) and ovarian cancer patients treated with platinum-based chemotherapy [56–58].

The ATP7B protein is also deregulated in cancers. In CRCs, low mRNA or protein levels of ATP7B are associated with response to oxaliplatin/5-FU treatment in patients [59]. ATP7B was also a predictive biomarker of platinum-based drug resistance in a NSCLC xenograft model [60], but also in the clinical setting for CRCs [59], in oral squamous carcinomas [61], oesophageal [62] and ovarian [63] carcinomas. In all these clinical studies, ATP7B was not detected in the adjacent non-neoplastic tissues [59,61–63]. Correlations between GSH levels and cancer cell growth were found in melanoma and liver cancer [64,65]. In clear cell renal cell carcinoma, tumor progression and metastasis were linked to increased metabolic activity in both GSH and cysteine/methionine metabolism pathways [66].

Ctr1 is the major Cu influx carrier in human cells. Variations in Ctr1 expression were observed both in non-pathological tissues and in various cancer tissues. However, the absence of Ctr1 expression was also reported in some cancers such as cervical squamous carcinoma, prostate carcinoma, and gastric carcinoma [67]. Ctr1 transporter is also responsible for the absorption of cisplatin, and Ctr1 is logically a key player in resistance and sensitivity mechanisms to platinum-based cancer therapies [68]. Other studies have shown a correlation between increased levels of hCtr1 and better absorption of platinum-based drugs in sensitive cells [69–71]. In addition, Ctr1 overexpression resulted in prolonged progression-free survival (PFS) and improved overall survival (OS) in patients with stage III NSCLC [72]. Finally, many studies have hypothesized that the development of resistance to platinum-based drugs may be due to the defect in glycosylation of the CTR1 protein [73].

Dysregulations of various Cu metabolic proteins can be the cause of resistance to the platinum-based anticancer therapies. These deregulations can be positive or negative whether the protein is involved in the influx or efflux of Cu, respectively. As a consequence, the drug's ability to penetrate cells decreases [74]. This is due to the fact that Cu binding sites are also used by platinum to enter cells *via* Ctr1 [75], but also for intracellular trafficking bound to Atox1 [76,77]. Moreover, therapeutic platinum salts can also be excreted outside cells by the Cu transporters ATP7A or ATP7B [78,79]. This could

explain the down-regulation of Ctr1 to limit platinum cellular entry, and subsequent up-regulation of ATP7A and/or ATP7B and/or Atox1 to induce platinum excretion. To circumvent these resistance mechanisms against platinum complexes, drug screenings were performed to identify molecules able to maintain Cu transport to the Golgi apparatus while inhibiting platinum salts excretion [80].

Resistance to cisplatin was also correlated with increased levels of GSH in ovarian tumor cell lines [81]. In various cancer types, the fluctuations of total intracellular GSH level could explain the resistance/sensitivity patterns to cisplatin [82].

The family of copper-dependent lysyl oxidase (LOX) metalloenzymes have a role in tumor metastasis and fibrotic diseases. In the formation of pre-metastatic niches, cancer cells secrete the LOX protein to stimulate collagen cross-linking and fibronectin synthesis [83]. This secretion has the consequence of promoting the migration and adhesion of tumor cells [84,85]. LOX promotes tumor cell migration and adhesion by activating the focal adhesion kinase (FAK1) [84]. To date, several dysregulations of enzymes from the LOX family were reported in breast, colorectal, prostate, gastric, hepatic, pancreatic, head and neck cancers, as well as in skin cancers, including melanoma [86–88]. LOX proteins may play complex and paradoxical roles in the metastatic process, or act as tumor suppressors [86]. In bladder cancer, LOXL1 and LOXL4 inhibited oncogenic RAS-mediated activation of ERK, a tumor suppressive action leading to reduced colony formation [89]. Increased LOX levels may be associated with poor prognosis in different cancers, especially in patients with ER-negative breast cancers [90].

CCS (copper chaperone for superoxide dismutase) delivers Cu to the copper zinc superoxide dismutase (SOD1). This Cu is mandatory for its maturation and its role in the control of reactive oxygen species production. Moreover, CCS could have the ability to promote carcinogenesis. The use of a specific inhibitor of CCS and Atox1 has been shown to reduce cancer cell proliferation and tumor growth. However, the potential presence of other targets that might work in synergy should not be excluded [91]. In patients with breast cancer, CCS protein levels increase. The ability of CCS to promote proliferation may involve the MAPK/ERK pathway [92]. The expression of CCS and Cox17 are generally higher in lung cancer compared to healthy tissue. However, the expression of Cox17 can vary between tumors and cell lines [93].

In the next part of this review, we will present different therapeutic strategies and lines of research based on the therapeutic use or the targeting of Cu and/or Cu-proteins for cancer treatment.

4. Copper as a target or a bullet for cancer treatment

The use of chelators or ionophores is a frequent strategy to target Cu levels in cells [94]. Chelators directly bind and sequester metal ions, while ionophores cross cellular membranes in a Cu-bound form and release Cu on the other side of the membrane generally leading to increase of the intracellular concentrations of metal ions [95].

The first Cu chelators were developed in the mid-20th century for treating patients with Wilson's disease, notably D-penicillamine and trientine that are Cu(II) chelators acting extracellularly. More recently, Cu(I) chelators such as tetrathiomolybdate (TTM) have been developed in order to act inside cells in a more efficient way [96].

It has been shown that D-penicillamine induces inhibition of human endothelial cell proliferation *in vitro* and neovascularization *in vivo* [97]. Afterwards, trientine also showed an antineoplastic effect and caused important suppression of tumor development in murine and human hepatocellular carcinoma cell lines [98,99]. Trientine is considered to have a reduced Cu chelating capacity compared to D-penicillamine, however, it has a more tolerable toxicity profile.

In fact, the availability of cellular Cu is critical for the activity of MEK1 and MEK2 kinases, in the RAS/MAPK signaling pathway. Copper intake promotes the phosphorylation of the MEK1 protein but also ERK1 and ERK2 through a Cu-MEK1 interaction [100]. The activation of the copper-dependent mitogen-activated kinase (MAP) pathway is thus a key player in the promotion of tumor growth, and targeting Cu was proved to be a relevant strategy against cancer progression. In a cornerstone study, Brady *et al.*, demonstrated the link between cancer mutational status and variations in cytosolic Cu content in melanoma [96]. The targeting of Cu with TTM induced antitumor effects in cells with BRAF V600E kinase mutations, which gave a strong rationale for the further development of several secondary studies aiming at disrupting the central role of Cu in other BRAF V600E positive malignancies, such as thyroid, lung, colorectal cancers or hairy cell leukemia [101].

The TTM chelator inhibited the growth of melanoma cell lines resistant to BRAF or MEK1/2 inhibitors and increased the antineoplastic activity of these inhibitors [102]. In addition, in CCR cells carrying BRAF V600E mutations, Cu depletion obtained by pharmacological treatment with TTM reduced the growth of BRAF V600E cells in colon cancers that were resistant to BRAF inhibitors [103]. Nowadays, this chelator is evaluated as an adjuvant therapy in various cancer clinical trials.

Bleomycin (a glycopeptidic antibiotic produced by *Streptomyces verticillus*) and curcumin (a phytochemical agent) are other chelators that gave promising results in oncology [104,105]. Bleomycin is regularly used in combination with other therapeutic agents such as cisplatin and etoposide in testicular cancer [106]. Curcumin may be used in monotherapy or in combination with other anti-cancer agents, but also potentially for the prevention of cancer [107].

Copper ionophores are molecules that transport Cu ions through cellular membranes. Ionophores increase and/or redistribute intracellular Cu levels, often allowing Cu to become bioavailable [94]. These molecules have a high affinity for Cu(II) and a low affinity for Cu(I). The cytosol of the cells being a reducing environment, the Cu entering the cell will be reduced into its Cu(I) oxidation form. Such a release of Cu(I) will poison the cell [108].

In the family of ionophores, several compounds can be found such as docosahexaenoic acid (DHA), disulfiram (DSF), bis(thiosemicarbazone) copper complexes and clioquinol. The clinical use of clioquinol has been discontinued due to its neurotoxicity [109]; however, clioquinol or its analogues are still tested in combination, or with different administration routes, to maintain its anticancer effects while reducing toxicities [110]. The anticancer efficacy of DSF, was demonstrated in *in vitro* and *in vivo* models of inflammatory breast cancer [111], and DSF is currently tested in clinical trials (clinicaltrials.gov id#, NCT04265274 , NCT03323346).

In addition, the combination of DSF and DHA has been shown to promote the death of cancer cells and also reduced the growth of cancer cells *in vitro* and *in vivo* [112]. One study has also suggested combining DSF with a PI3K inhibitor. This combination could be a new therapeutic strategy in breast cancer, particularly for patients with PIK3CA mutations [113]. In addition, co-administration of this drug with copper has shown inhibition of tumor growth in hormone-sensitive and castration-resistant models of disease [114]. Finally, it has to be noticed that only the Cu-complexed form of these ionophores is active as cancer treatment: disulfiram (DSF), bis(thiosemicarbazone) copper complexes and clioquinol because the ligands alone (metal-free compounds) have a minimal anti-cancer effect [115].

Some chelators such as curcumin or D-penicillamine penetrate cancer cells with difficulty due to their physico-chemical properties. The development of innovative delivery systems for Cu-chelating agents should overcome these limitations, but also increase their efficacy and limit potential side-effects [116–118]. Other strategies such as photochemical internalization (PCI) have been used to improve the intracellular delivery of bleomycin [119].

Copper-based nanoparticles (CuNPs) have theranostic applications in oncology, *i.e.* they can be used for imaging or therapeutic purposes [120]. CuNPs can be used in a variety of therapeutic strategies, such as photothermal therapy combined with immunotherapies, to induce systemic immune responses against tumors [121]. The photothermal activity of other CuNPs was successfully exploited to induce the destruction of residual cancer cells and prevent local cancer recurrence, *in vivo* after a single irradiation session [122]. The development of transferrin-based CuNPs loaded with doxorubicin successfully inhibited *in vivo* tumor growth [123].

A long-lasting active research effort has shown that copper-based radioisotopes have a promising future in the field of cancer diagnostics and therapeutics, especially for ^{64}Cu isotope [124,125]. In a model of human CCR in hamsters, ^{64}Cu showed anticancer activity and the survival was significantly increased [126]. Interestingly, the combination of the ^{67}Cu radioisotope with an anti-L1-cell adhesion molecule monoclonal antibody reduced the growth of human metastatic ovarian cancer cells [127]. Metal-based therapies are major players in oncology. In this field, copper-based complexes have a promising future as presented previously. For this reason, the alteration of Cu metabolism in cancer is the basis for the development of copper complexes with antineoplastic characteristics [128,129].

5. Conclusion:

Nowadays, the importance of copper in carcinogenesis and metastasis formation, but also in resistance to treatment has been evidenced. In addition, research work on copper deregulation in oncology and the recent understanding of copper metabolism have also led to the development of numerous therapeutic strategies targeting this trace element. Despite numerous researches and the improvement of the knowledge on copper metabolism over the years, some shadow areas persist. To conclude, specific alterations in Cu metabolism seem to have a promising future in the clinic as prognostic and/or predictive biomarkers. In the coming years, Copper and its metabolism will continue to play a significant role for both cancer diagnosis and therapeutic strategy.

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