Environmental Copper as a Modifiable Risk Factor in ALS

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Sara Ghandour
DIVISION OF EXPERIMENTAL MEDICINE, FACULTY OF MEDICINE AND HEALTH SCIENCES, MCGILL UNIVERISTY,
MONTREAL, QC, CANADA
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Abstract:

Amyotrophic Lateral Sclerosis (ALS) is an adult-onset, progressive, fatal neurodegenerative disease with no known cause or cure. There are currently no clinically available biomarkers to monitor disease progression, and although heavy metal-induced oxidative stress has been implicated in ALS, the role of copper toxicity in disease progression remains largely unknown. Contemporary research has uncovered a role for environmental copper (Cu) in the development and progression of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD), with some AD research implicating non-ceruloplasmin bound copper (NCC) in the aggravation of neurodegenerative phenotypes. The exchangeable copper (CuEXC) biomarker, a process-based measurement of NCC, has been used in Wilson's Disease (WD) to monitor disease progression and Cu toxicity. Our research explores the role of Cu in ALS progression through monitoring of CuEXC and other Cu status biomarkers, and through analyses of Cu metabolism gene expression. Changes in ALS related genes and antioxidant status genes are also investigated. We hypothesized that ALS progression would correlate with increases in CuEXC, changes in Cu metabolism genes, and increases in oxidative stress. Results thus far show that ceruloplasmin oxidase activity is moderately decreased in ALS patients, and there is indication of modest alternations in Cu metabolism gene expression. CuEXC appears as a nonredundant biomarker of Cu status in patients and controls and increases with ALS disease progression.

Résumé :

La Sclérose Latérale Amyotrophique (SLA) est une maladie neurodégénérative progressive et mortelle de l'adulte sans cause ni remède connus. Il n'existe actuellement aucun biomarqueur cliniquement disponible pour surveiller la progression de la maladie, et bien que le stress oxydatif induit par les métaux lourds ait été impliqué dans la SLA, le rôle de la toxicité du cuivre dans la progression de la maladie reste largement inconnu. La recherche contemporaine a découvert un rôle pour le cuivre environnemental (Cu) dans le développement et la progression des maladies neurodégénératives telles que la maladie d'Alzheimer et la maladie de Parkinson, certaines recherches sur l'Alzheimer impliquant du cuivre non lié à la céruloplasmine (NCC) dans l'aggravation de phénotypes neurodégénératifs. Le biomarqueur de cuivre échangeable (CuEXC), une mesure de NCC basée sur le processus, a été utilisé avec la maladie de Wilson (WD) pour surveiller la progression de la maladie et la toxicité du Cu. Notre recherche explore le rôle du Cu dans la progression de la SLA par la surveillance de CuEXC et d'autres biomarqueurs du statut du Cu, et par des analyses de l'expression des gènes du métabolisme du Cu. Les modifications des gènes liés à la SLA et des gènes du statut antioxydant sont également étudiées. Nous avons émis l'hypothèse que la progression de la SLA serait corrélée avec des augmentations de CuEXC, des changements dans les gènes du métabolisme du Cu et des augmentations du stress oxydatif. Les résultats jusqu'à présent montrent que l'activité de la céruloplasmine est modérément diminuée chez les patients SLA, et qu'il y a des indications d'alternances modestes dans l'expression des gènes du métabolisme du Cu. CuEXC apparaît comme un biomarqueur non redondant du statut en Cu chez les patients et les témoins, et augmente avec la progression de la SLA.

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Contribution of Authors

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List of Abbreviations

AAS: Atomic Absorption spectroscopy
AD: Alzheimer's Disease
ALS: Amyotrophic Lateral Sclerosis
ALSFRS-R: ALS Functional Rating Score- Revised
apoCp: Apo-ceruloplasmin
ATOX1: Antioxidant 1 Copper Chaperone gene, previously known as HAH1
ATP7A: copper-transporting P-type ATPase 7A (also known as Menke's protein MNK)
ATP7B: copper-transporting P-type ATPase 7B (also known as Wilson's Disease Protein WND)
BBB: Blood-brain Barrier
C9orf72: chromosome 9 open reading frame 72 gene
CCS: Copper Chaperone For Superoxide Dismutase
CHUM: Centre Hospitalier de l'université de Montréal
CNS: Central Nervous System

COX17: Cytochrome c oxidase copper chaperone gene
Cp: Ceruloplasmin
CTR1: Copper transport protein 1
Cu: Copper
CuEXC: exchangeable copper
DMN: distal motor neuropathy
EFSA: European Food and Safety Association
ETIC: Endemic Tyrolean Infantile Cirrhosis
fALS: familial ALS
GSH: Glutathione (reduced)
GSSG: Glutathione (oxidized)
GSTM1: Glutathione S-Transferase mu 1
GSTP1: Glutathione S-Transferase pi 1
GSTT1: Glutathione S-Transferase theta 1
holoCp: Holo- ceruloplasmin
ICC: Indian Childhood Cirrhosis
ICPMS: Inductively coupled plasma mass spectroscopy
ICT: Idiopathic Copper Toxicosis
LOAEL: Lowest observed adverse effect level
MCI: mild cognitive impairment
MD: Menke's Disease
mSOD1: mutant SOD1
MT: metallothionein
NCC: non-ceruloplasmin bound copper
NOAEL: No observed adverse effect level
OD: Optical Density
OHS: Occipital Horn Syndrome
PBMC: Peripheral Blood Mononuclear Cells
PD: Parkinson's Disease
REC: Relative Exchangeable Copper
RNS: Reactive Nitrogen Species
ROS: Reactive Oxygen Species
sALS: Sporadic ALS
SOD1: Cu/Zn superoxide dismutase
SOP: standard operating procedure
TARDBP or TDP-43: TAR DNA binding protein 43
Total Cu: total amount of copper in serum
UdeM: Université de Montréal (University of Montreal)
USEPA: United States Environmental Protection Agency
WD: Wilson's Disease
WHO: World Health Organization
XRF: X-ray fluorescence

1. Introduction

The role of environmental copper in ALS will be examined through the lens of copper toxicity in neurodegenerative diseases. In the following literature review, the function of copper (Cu) in biological processes, particularly in the nervous system, and the mechanisms of Cu toxicity, principally oxidative stress, are assessed. Routes of environmental exposure to Cu are reviewed, and diseases of Cu dysregulation and the role of Cu in select neurodegenerative diseases are explored, revealing what is known about Cu toxicity and neurodegeneration. Finally, available Cu status biomarkers used in monitoring Wilson's Disease (WD) are assessed to select a biomarker to be implemented in our clinical study.

1.1 ALS

Amyotrophic lateral sclerosis (ALS) is an adult-onset, progressive and fatal neurodegenerative disorder with no known cause, no effective treatment, and no known cure. Through the degeneration of the upper and lower motor neurons from the brain to the spinal cord, progressive atrophy of the muscles throughout the body causes paralysis and death (Chiò *et al.*, 2009). The median survival time from onset to death ranges from 20 to 48 months, while 10-20% of ALS patients have a survival longer than 10 years (Chiò et al., 2009). ALS cases can be sporadic (90-95% sALS) or of family origin (5-10% fALS) with an overall incidence rate in Europe and North America of 2-3 cases per 100,000 (Wang *et al.*, 2017), a prevalence in Canada of 10 per 100,000 (PHAC, 2014), and a lifetime risk of 1:400 (Hardiman *et al.*, 2011). The prevalence of ALS is increased among Gulf War veterans and Italian soccer players (Barber & Shaw, 2010; Goodall & Morrison, 2006; Hardiman et al., 2011). The mechanism by which motor neurons are affected is not completely understood. However, aspects of their structural and metabolic specialization may partially explain their particular vulnerability to oxidative stress including their large size, high metabolic activity and energy demand, sensitivity to mitochondrial dysfunction, elevated neurofilament content, and reduced capacity to buffer calcium (Barber & Shaw, 2010; Goodall & Morrison, 2006). Little progress has been made in identifying non-genetic risk factors with any degree of certainty, although geographic clustering of sALS suggests that environmental risk

factors are implicated, the most important being heavy metal and organic chemical exposure (Bergomi *et al.*, 2002; Kamel *et al.*, 2005; Sheykhansari *et al.*, 2018; Wang et al., 2017).

1.1.1 Diagnosis and Monitoring of ALS

ALS has a considerable variability in outcome and its prognostic factors are not well defined. Age is a strong prognostic factor in ALS, and men have a higher risk than women, leading to a male-to-female ratio of 1.2–1.5 (Chiò et al., 2009; Fang *et al.*, 2015). No definitive diagnostic test for ALS exists. Diagnosis is made on clinical grounds, using internationally recognized consensus criteria, after exclusion of conditions that can mimic ALS (Hardiman et al., 2011). The ALS functional rating scale- revised (ALSFRS-R) is the most widely used functional scale for ALS (Table 1). A patient's ALSFRS-R is significantly related to outcome, and the respiratory sub-score is the most significant component (Chiò et al., 2009)

Table 1. ALSFRS- R criteria

Speech	Salivation	Swallowing
Handwriting	Cutting Food	Climbing Stairs
Dyspnea (difficulty Breathing)	Orthopnea (shortness of breath while lying down)	Breathing insufficiency
Dressing & Hygiene	Turning in bed and adjusting bed clothes	Walking

Types of physical functioning assessed in the ALS functional rating scale score- revised (ALSFRS-R). This scale is used as an ongoing measure of ALS disease progression in patients. It is calculated as the sum of the ranking of different aspects of physical functioning on a scale from 0 to 4, where 0 is no capacity to function and 4 is perfectly normal. This gives a minimum score of 0 indicating completely impeded physical function or 48 for complete normal function (ACTSGroup, 1996; Cedarbaum & Stambler, 1997).

One of the most difficult tasks in diagnosis and prognosis of ALS is the identification of biological markers of disease progression (Bowser *et al.*, 2011; Chiò et al., 2009). To date, there are no clinically available biomarkers for ALS (Chiò & Traynor, 2015). The identification of biomarkers for ALS would assist in rapid diagnosis, improved monitoring of disease progression, and would provide insights into its pathophysiology (Bowser et al., 2011).

1.1.2 Disease Etiology

Genetic defects are implicated in familial ALS cases, one of the most common being mutations of the SOD1 gene (15% of fALS), a copper dependant enzyme involved in scavenging reactive oxidant species (Elliott, 2001). In addition to SOD1, mutations in the genes coding for C9orf72 (80% of fALS) , TARDBP (5% of fALS and 1% of sALS) and several other genes are closely associated with ALS (Ahuja *et al.*, 2015; Davidson *et al.*, 2016; Enge *et al.*, 2017; Fang et al., 2015; Farg *et al.*, 2014). Copper is proposed to be involved in ALS motor neuron degeneration mechanisms by mutation of SOD1, TARDBP protein aggregation, and mitochondrial dysfunction (Barros *et al.*, 2018).

SOD1

Wild type Cu/Zn superoxide dismutase (SOD1) is a ubiquitously expressed antioxidant protein in mammalian cells and comprises about 1% of total protein in the brain where it is located primarily in the neuronal cell bodies and dendrites (Elliott, 2001). Copper is used in SOD1 to catalyze an antioxidant reaction that converts superoxides to molecular oxygen and hydrogen peroxide (Ahuja et al., 2015). Different mutations of SOD1 have different effects on the age of onset of symptoms and on the rate of progression of the disease (Chiò et al., 2009). 20% of familial cases are due to dominantly inherited mutations in SOD1 (Brasil *et al.*, 2018; Llanos & Mercer, 2002; E. Tokuda *et al.*, 2009) and over 100 distinct missense SOD1 mutations have been identified in fALS patients spanning all domains of the enzyme promoting toxic SOD1 function (Elliott, 2001; Gaggelli *et al.*, 2006). Mutant SOD1 (mSOD1) has the capacity to catalyze the production of reactive oxygen species (ROS), such as superoxide anions, peroxynitrite and hydroxyl radicals (Elliott, 2001; Goodall & Morrison, 2006). Peroxynitrite, formed from the reaction of nitric oxide and a superoxide anion, is an alternative substrate for SOD1 to yield a potent nitrating species capable of adding a nitro group (-N02) to tyrosine residues of essential neuronal proteins such as neurofilaments (Elliott, 2001). In addition to oxidative stress, other hypotheses have been proposed to explain SOD1-mediated toxicity, including mitochondrial dysfunction, glutamate excitotoxicity, and defects in axonal transport (Figure 1) (Ahuja et al., 2015).

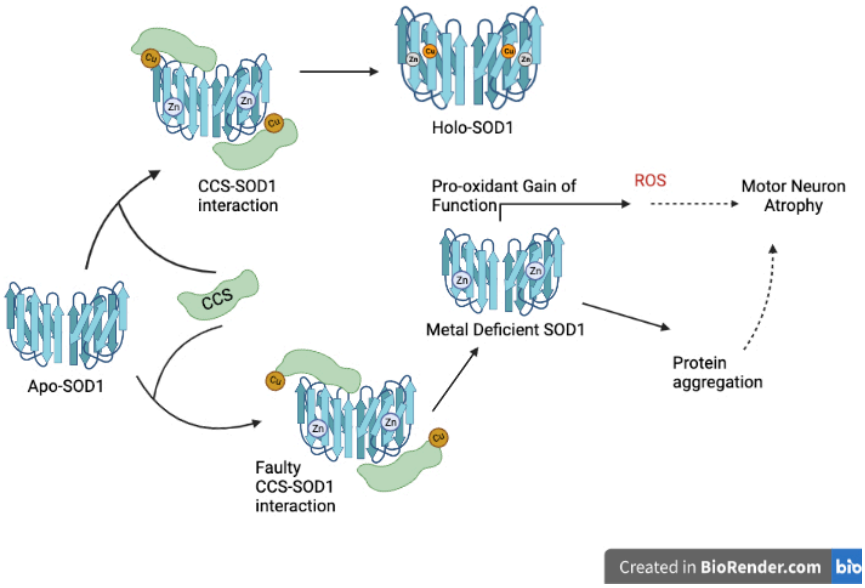


Figure 1. Hypothetical model of mSOD1 mediated excitotoxicity.

Apo-SOD1 is loaded with copper by Cu chaperone CCS. Every SOD1 monomer requires 1 zinc and 1 Cu atom to function. When bound to Cu and Zinc, SOD1 is considered functional holoSOD1. If bound to only one type of metal, SOD1 is metal deficient and can promote motor neuron cell death via ROS generation or protein aggregation. Figure adapted from (Gil-Bea et al., 2017) and created with BioRender.com

Most ALS patients have normal serum copper levels, but some may have copper dysregulation (ALSUntangledGroup, 2018). The strongest evidence for this hypothesis comes from transgenic fALS mouse models caused by SOD1. For example, one ALS mouse model expressing mutant SOD1, hSOD1G93A mice, showed disturbance in the homeostatic control of intracellular copper ions with greater disturbance correlating with greater neurological damage (Eiichi Tokuda & Yoshiaki Furukawa, 2016).

C9orf72

Mutations in the C9orf72 gene resulting in a large hexanucleotide expansion has been associated with 80% of familial ALS (Davidson et al., 2016; Farg et al., 2014). The mutation is an expansion of a hexanucleotide repeat sequence, GGGGCC, in a non-coding part of the gene. In ALS patients, the number of repeats can >10000, in contrast with 2–30 repeats in control populations (Fang et al., 2015). Most patients carrying the C9orf72 mutation are also found to have TDP-43 (TAR DNA binding protein 43) aggregation at autopsy (Mackenzie *et al.*, 2013; Sampognaro *et al.*, 2019). The normal physiological role of the C9orf72 gene remains largely unknown, however, high WT

C9orf72 expression is found in microglia, which may elude to a role in immune function (Trageser *et al.*, 2019). C9orf72 is thought to play a role in ALS pathogenesis via gain-of-function mechanisms, such as the toxicity of its dipeptide repeat proteins and sense and antisense RNA foci (Haeusler *et al.*, 2016).

TARDBP (TDP-43)

A variety of protein aggregates have been described in ALS, the most common of which being majorly constituted of TARDBP (also known as TDP-43) (Barber & Shaw, 2010; Gil-Bea et al., 2017). Wild-type (WT) TDP-43 is a particularly conserved DNA- and RNA-binding protein encoded by the TARDBP gene (Bartoletti *et al.*, 2019; Li *et al.*, 2010) with cell-specific essential roles in RNA splicing in addition to mRNA stability, transport, translation, post-transcriptional modifications, and autophagy (Khosravi *et al.*, 2020; Li et al., 2010; Trageser et al., 2019). Thirty mutations of this gene have been found in about 5% of patients with familial ALS and 1% of patients with sporadic ALS (Fang et al., 2015). When mutated, TDP-43 is cleaved and abnormally phosphorylated, and accumulates in ubiquinated cytoplasmic inclusions in motor neurons of patients with fALS and sALS. ALS patients with mutations in the 3'UTR region of its encoding gene (TARDBP) show overexpressed levels of TDP-43 (Gil-Bea et al., 2017). TDP-43 pathology appears to be a key driver of neurodegeneration through various mechanisms, which may include impaired shuttling between the nucleus and cytoplasm, inhibition of appropriate endocytosis and endo-lysosomal pathway, disturbed ubiquitin-proteasome and autophagy pathways, and TDP-43 aggregation through hyperphosphorylation, ubiquitination, and mutations (Prasad *et al.*, 2015)

Although TDP-43 does not possess the ability to bind copper ions, it has been shown that Cu may influence the aggregation state of TDP-43 (Gil-Bea et al., 2017). Conversely, it has also been reported that the mutant TDP-43 can affect copper homeostasis where overexpression of mutant TDP-43 in the whole CNS disrupted copper homeostasis, increasing Cu content in the spinal cord but not in the brain, resulting in motor symptoms but not cognitive deficits (Dang *et al.*, 2014).

1.1.3 Disease pathology

There is extensive evidence that mechanisms of neurodegeneration in ALS result from redox metal dys-homeostasis, oxidative stress, mitochondrial dysfunction, neuro-inflammation, and glutamate

excitotoxicity (Figure 2) (Barber & Shaw, 2010; Barnham *et al.*, 2004; Sheykhansari et al., 2018). These effects are also apparent in one third of Wilson's disease patients suffering from copper-induced neurological symptoms (EASL, 2012). It is not known if metal interaction is a primary cause or a secondary consequence of neurodegeneration (Sheykhansari et al., 2018). Advanced studies are thus needed to examine the correlation between heavy metal exposure and the development and progression of ALS.

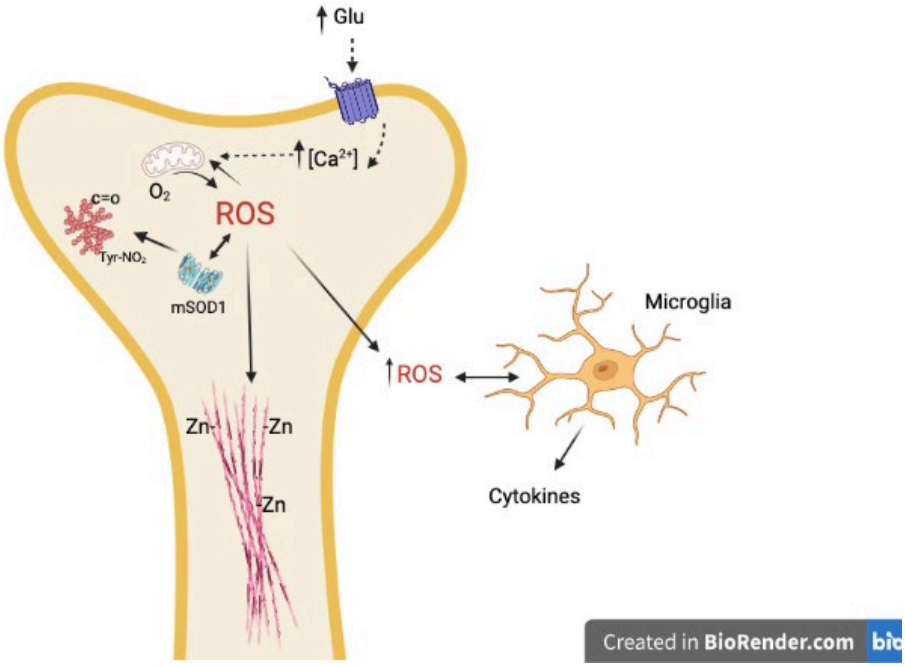


Figure 2. Interplay of oxidative stress mechanisms in motor neurons

ROS can damage and inactivate glutamate uptake receptors. This leads to a buildup of glutamate in the synapse which in turn triggers increased calcium signalling and influx into motor neurons leading to excitotoxicity and neuronal death. ROS can also cross the cell membrane and activate microglia (macrophages of the brain), which respond by releasing cytokines and further ROS. Aberrant oxidative reactions catalysed by mSOD1 increase production of the highly reactive peroxynitrites and hydroxyl radicals, causing nitration and aggregation of proteins including mSOD1 itself, and may also inhibit neurofilament assembly and cytoskeletal transport. ROS damage of neuronal filaments can increase their affinity to zinc. Zinc binding to neurofilaments could deplete zinc binding to SOD1 and exacerbate aberrant SOD1 chemistry, creating even more ROS. Figure adapted from (Barber *et al.*, 2006) and created with Biorender.com

1.2 Overview of normal copper physiology

1.2.1 Copper as an essential element

Copper is an essential element in human nutrition. It is involved in various physiological processes as an enzymatic cofactor. The processes include skin pigmentation, mitochondrial function, collagen synthesis, iron homeostasis, antioxidant defense, and nervous system function (Bost *et al.*, 2016; Gromadzka *et al.*, 2020; Trumbo *et al.*, 2001). Cu's enzymatic role derives from its predominantly catalytic oxidizing capacity. Cu's ability to cycle between states of Cu^{2+} (cupric ion) and Cu^{1+} (cuprous ion) allows it to act either as an electron donor or a recipient (Stern, 2010). In redox reactions facilitated by copper metalloenzymes (cuproenzymes), this characteristic allows Cu to act an intermediary for electron transfer (Trumbo et al., 2001). Key copper-containing enzymes and their functions are summarized in Table 2 adapted from (Manto, 2014; Stern, 2010)

Table 2. Key cuproenzymes and their functions.

Process	Enzyme	Function
Iron homeostasis	Ceruloplasmin (Ferroxidase I or Cp)	Multi-copper ferroxidase essential for iron transport and copper transport. Primary copper containing protein in plasma.
	Hephaestin (Ferroxidase II or Hp)	Multi-copper ferroxidase involved in iron transport across intestinal mucosa into portal circulation.
Mitochondiral function	CytochromeC oxidase (Complex IV or COX)	Terminal oxidase enzyme in mitochondrial electron transport chain converting molecular oxygen to water. Vital for aerobic respiration and energy metabolism.
Nervous system function	Monoamine oxidase (MAO)	Group of enzymes which oxidize primary amines. Involved in pigmentation and neurotransmitter metabolism.
	Dopamine-β-hydroxylase (DBH)	Involved in cadtecholamine metabolism, catylizing conversion of dopamine to norepinephrine. Important for autonomic nervous system regulation.
	Peptidylglycine-amidating-mono- α-oxygenase (PAM)	Multifunction enzyme involved in maturation and modificiation of key neuropeptides (ex. neurotransmitters, neuroendocrine peptides).
Antioxidant defense	Glutathione Peroxidase	Antioxidative defence via conversion of hydroperoxide and hydrogen peroxide.
	Catalase	Antioxidant defence. Conversion of hydrogen peroxide to water and oxygen.

	Superoxide Dismutase (SOD1 and SOD3)	Cu and Zn bound intracellular and extracellular enzymes involved in anti-oxidative defence via conversion of superoxides to H_2O_2.
Collagen synthesis	Lysyl oxidase	Cross-linking of collagen and elastin. Important for stabilization of connective tissues.
Skin pigmentation	Tyrosinase	Production of melanin and other pigments via conversion of tyrosine to L-DOPA.

1.2.2 Normal Copper Metabolism

Since Cu is an essential element, its regulation in the body is tightly controlled by homeostatic mechanisms. Under normal physiological conditions, low Cu intake induces a retention and increase in Cu absorption, while high intake decreases absorption and stimulates excretion (Stern, 2010). In humans with normal intake, 55–75% of ingested Cu is absorbed and actively recycled between the digestive tract, body fluids and tissues (Linder, 2020; Tapiero *et al.*, 2003).

Copper is mainly absorbed in the duodenum, with small amounts absorbed in the stomach and the distal portion of the small intestine (Hordyjewska *et al.*, 2014; Manto, 2014). Organic copper from food intake is absorbed by intestine lining epithelial cells (enterocytes). Enterocyte Copper transport protein 1 (CTR1) allows the transport of copper from the intestinal lumen into the cell. Once inside the cell, Cu is either stored in metallothioneins (MTs), bound to reduced glutathione (GSH), or relayed to other proteins by copper chaperones (Tapiero et al., 2003). There are 3 main intracellular copper chaperone enzymes: (i) CCS: chaperone for Cu/Zn-superoxide dismutase 1 (SOD1), (ii) COX17: chaperone for cytochrome C oxygenase (COX) and (iii) ATOX1: chaperone for the Cu transporter ATPases ATP7A and ATP7B (Figure 3) (Lutsenko, 2010; Manto, 2014). Chaperones deliver copper to their respective targets through direct insertion of Cu into active sites (Schlief & Gitlin, 2006).

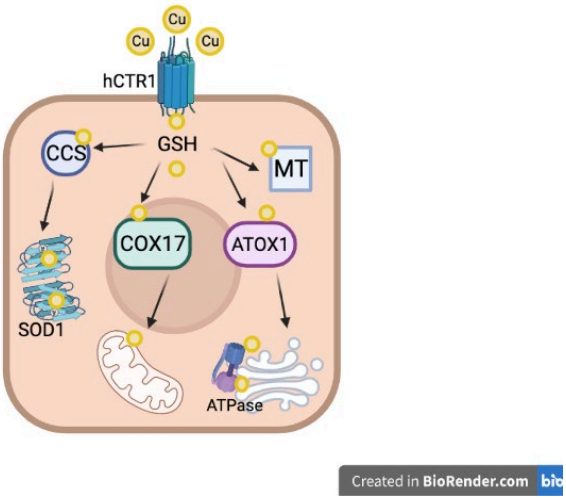


Figure 3. Different fates of intracellular copper

In a generalized cell, Cu enters via human CTR1 and is handed off to copper chaperones ATOX1/CCS/ COX17, which then deliver Cu to their respective target proteins. Figure adapted from (Kaplan & Maryon, 2016) and created on BioRender.com

In the gut, ATOX1 transports copper to the ATPase ATP7A located in the trans-golgi network. Once loaded with copper, ATP7A migrates to the enterocyte's plasma membrane where it acts as a transmembrane transporter using ATP to catalyze the transport of Cu across the epithelial membrane to the hepatic portal vein (Hasan & Lutsenko, 2012). High concentrations of extracellular copper cause CTR1 internalization, and high concentrations of intracellular copper cause ATPase translocation to the cell membrane (Kaplan & Maryon, 2016). In the portal vein, copper is non-specifically bound to proteins, such as albumin or histidine, to be transported to the liver (Roberts & Sarkar, 2008). Once copper reaches the liver, it is transported into the cell by hepatic CTR1 and intracellularly distributed in a similar manner that that in enterocytes.

Overall, the liver plays an important role in Cu storage, Cu related protein and energy production, and Cu excretion (Roberts & Sarkar, 2008). Most copper in the body can be found in the liver stored in metallothioneins (MTs). Under normal physiological conditions, the amount of unbound copper within cells is extremely restricted (Schlief & Gitlin, 2006). Cu excretion is controlled predominantly by the liver, where Cu is released mainly via bile (Gromadzka et al., 2020) to ultimately be excreted in feces. A small amount is excreted via urine, however, this excretion may be elevated under pathological or toxic Cu conditions (Roberts *et al.*, 2008).

Ceruloplasmin and circulating copper

In the liver, ATOX1 transports Cu to ATP7B, which then mediates the incorporation of copper into ceruloplasmin, a 132kDa iron oxidizing ferroxidase enzyme that requires 6 Cu atoms for its catalytic activity (Figure 4) (Mohr & Weiss, 2019; Siotto *et al.*, 2010). This complex forms the active version of ceruloplasmin, called holo-ceruloplasmin, which is subsequently secreted into the blood. In the blood, 60-65% of copper is bound to ceruloplasmin (Wirth & Linder, 1985), while the rest is bound to smaller molecules (i.e., albumin, transcuprein, amino acids and low molecular weight compounds), which can pass through the blood-brain barrier, where it may participate in ROS production and exert oxidative stress (Barnham et al., 2004; Chutkow, 1978; Manto, 2014; Wirth & Linder, 1985).

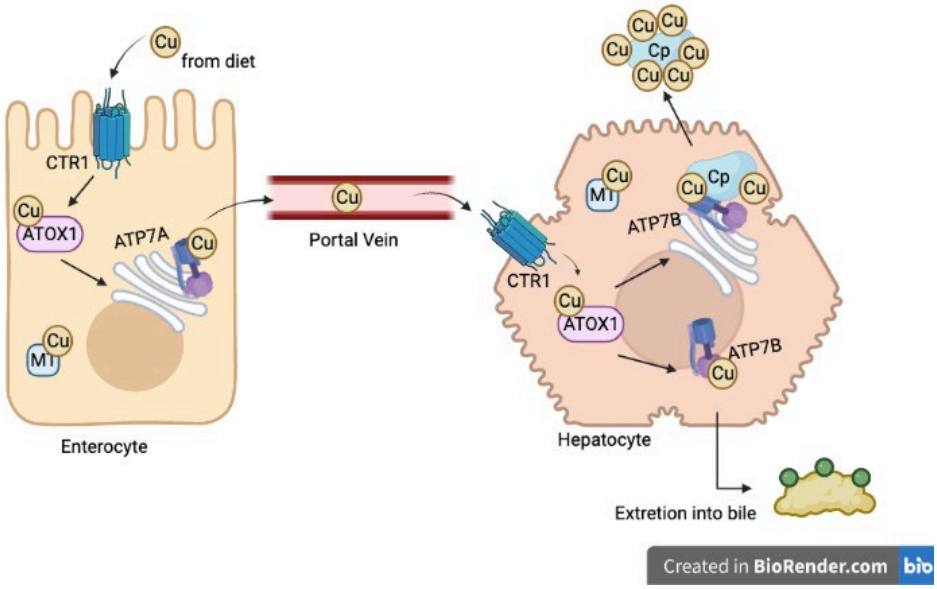


Figure 4. Mechanism of copper transport in the human body.

Dietary copper is relayed from the intestine to the liver. In hepatocytes Cu is loaded onto Cp, stored in MTs, or excreted adapted from (Woimant & Trocello, 2014) and created using Biorender.com

Cu in the nervous system

The brain is the second most copper rich organ in the body (Gromadzka et al., 2020). Although Cu's role in the brain has not been fully elucidated, it has been shown to play an important role in several nervous system processes, such as CNS development, normal mitochondrial function,

neuroinflammation, free radical detoxification, biosynthesis of neurotransmitters, and synaptic transmission and signal modulation (Hartwig *et al.*, 2021; Malosio *et al.*, 2020; Schlief & Gitlin, 2006). It is clear that stable Cu homeostasis in the CNS is essential for normal brain function (Zheng & Monnot, 2012). Both copper excess and deficiency can create oxidative stress, therefore, absorption, transport, and delivery of copper into the CNS, as well as copper homeostasis within neurons are critical and tightly regulated (Barros et al., 2018; Gil-Bea et al., 2017). Currently, the existing knowledge on copper handling by different cell types and its implication in nervous system processes in the brain is rudimentary, however, it is apparent that all key copper handling proteins regulating copper homeostasis in peripheral tissues also exist in the nervous system (Ahuja et al., 2015; Lutsenko *et al.*, 2010).

In a generalized model of a central nervous system cell, copper enters the cell via the copper transporter CTR1 located in the plasma membrane. Copper chaperones CCS and COX17 deliver copper to antioxidant enzyme SOD1 and the mitochondria respectively, while ATOX1 delivers copper to ATP7A located within the trans-Golgi network. When copper is elevated, ATP7A moves from the trans-Golgi network and facilitates copper excretion (Lutsenko, 2016; Lutsenko et al., 2010; Woimant & Trocello, 2014). Cu is not distributed uniformly in the brain, with some areas being more copper rich than others, most likely reflecting a higher metabolic demand for Cu in such regions (Lutsenko et al., 2010).

1.3 Organic vs. Inorganic Copper

Organic copper may be defined as nutritional dietary copper intrinsic to copper containing foods. Inorganic copper is non-nutritional and can be found in sources, such as drinking water, Cu supplements, or Cu from pesticides on the surfaces of foods. For the general population, the principal Cu exposure routes are dietary, involving the ingestion of organic and inorganic Cu in/with foods and inorganic Cu from drinking water (Bost et al., 2016; Husak, 2015). The quantity of ingested organic Cu varies based on the diet (i.e., consumption or not of foods naturally Cu rich, such as meats) and the quantity of inorganic copper ingested with food depends on the agricultural pesticides used in its cultivation, while potable water is another source of inorganic Cu, whose Cu concentrations depends on water quality factors (Brewer, 2010). Organic Cu is metabolized following normal Cu physiological processes; the extent of its absorption and its excretion are

regulated by homeostatic mechanisms. In contrast, inorganic copper is absorbed much more quickly, and is thought to be directly absorbed through the stomach into the bloodstream (Bush *et al.*, 1955).

The chemistry of copper in biological systems is limited to 1+ and 2+ oxidation states. Both these cuprous (Cu^{1+}) and cupric (Cu^{2+}) states can form bonds with organic ligands (Kozlowski *et al.*, 2014). Cu^{2+} is generally considered to be more toxic than Cu^{1+} due to its higher oxidative capacity (Dhir, 2012). Inorganic copper is typically found in the Cu^{2+} state and contributes to the non-ceruloplasmin bound fraction of copper or free copper observed in peripheral blood. This fraction's ability to cross the blood brain barrier (Choi & Zheng, 2009) implicates it in neurodegenerative phenotypes observed in Wilson's disease patients presenting with neurological symptoms (Poujois *et al.*, 2017).

1.3.1 Cu and oxidative stress

When used as an enzymatic cofactor, Cu plays an essential role in normal physiological functioning, however, when Cu exists in an unbound ionic form in the body, it can be toxic. Pathologic effects of copper toxicity manifest as oxidative damage to membranes or macromolecules (Bremner, 1998). Copper can induce oxidative stress by two mechanisms: (1) It can directly catalyze the formation of ROS via a Fenton/Haber-Weiss reaction **(**Equation 1**)** or (2) exposure to elevated levels of copper can significantly decrease glutathione (GSH) levels, a natural antioxidant that regulates Cu ROS activity, consequently leading to an increase in cellular ROS concentration (Bremner, 1998; Jomova & Valko, 2011; Kozlowski et al., 2014). Features of ROS damage which may be observed under toxic Cu conditions include lipid peroxidation in cell membranes, such as the formation of malondialdehyde, direct oxidation of proteins, cleavage of DNA and RNA molecules, as well as DNA adduct formation, including the formation of 8-oxo-2'deoxyguanosine (Jomova & Valko, 2011; Kozlowski et al., 2014).

$$O_2{}^{\cdot -} + Cu^{2+} \rightarrow O_2 + Cu^{+}$$
$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^{\cdot}$$

Equation 1. Fenton/Haber-Weiss reaction generating ROS

GSH acting in concert with its dependent enzymes, known as the glutathione system, is responsible for antioxidant defense via detoxification of reactive oxygen and nitrogen species (ROS/RNS) (Jomova & Valko, 2011; Morris *et al.*, 2014). Decreases in GSH levels and dysregulation of GSH system homeostasis are implicated in neurodegenerative phenotypes and neuroimmune disorders (Morris et al., 2014; Moustapha, 2020). Glutathione-S-transferases including GSTT1, GSTM1, and GSTP1 act by catalyzing the conjugation of electrophiles to glutathione. Polymorphisms in these transferases and in metallothioneins have been identified in individuals less able to excrete metals and other environmental toxins, and who therefore experience higher levels of oxidative stress (Gundacker *et al.*, 2007; Gundacker *et al.*, 2009), as well as in individuals with autoimmune disorders stemming from prolonged environmental toxin exposure (Morris et al., 2014).

Glutathione can suppress copper toxicity by directly chelating the metal and maintaining it in a reduced state, making it unavailable for redox cycling (Mattie & Freedman, 2004) as well as detoxifying the ROS generated from free Cu. The depletion of glutathione may enhance the cytotoxic effects of ROS and allow Cu to be more catalytically active, thus producing higher levels of ROS (Freedman *et al.*, 1989; Jomova & Valko, 2011). Additionally, it has been shown that GSH balance and copper homeostasis are functionally linked and jointly maintain conditions for copper secretion. The GSH/GSSG ratio, the ratio of reduced to oxidized GSH, plays a key role in regulating the redox state of ATOX1, a key secretory copper chaperone protein for Cu- ATPases ATP7A and ATP7B (Hatori *et al.*, 2012). GSH also plays a role in redox regulation of the Cu-ATPases through modulation of Cu binding to the Cu-ATPase cysteine motifs (Singleton *et al.*, 2010).

1.4 Cu Exposure Routes and Guidelines

The estimated average requirement for dietary Cu intake is 0.8-2.4 mg of Cu/day, with an estimated average of 1.5 mg/day (Bost et al., 2016; NRC, 1989). Chronic copper poisoning is uncommon, as the human tolerable upper intake level is considered to be 10 mg/day, a value based on protection from liver damage as the critical adverse effect (Bost et al., 2016; Trumbo *et al.*, 2001). Cu toxicity is generally considered uncommon under normal conditions. However, it can occur in cases of environmental/genetic abnormalities or excessive Cu intake. Easily identifiable toxicity symptoms

of Cu include hemolysis, hepatic cirrhosis/necrosis, and renal damage. Treatment with certain metal chelating agents or zinc is useful to act as an antidote (Brewer, 2010; Gupta & Gupta, 1998).

1.4.1 Cu Pesticides

Cu-based pesticides, in particular fungicides, bactericides and herbicides, are some of the oldest plant protection products in history and remain widely employed in agricultural practice throughout the world today (Arnal *et al.*, 2011; Husak, 2015; Komárek *et al.*, 2010). Most Cu fungicides are applied as foliar sprays and are commonly used in organic viticulture, hop-growing, fruit growing and market gardening to enhance food production by controlling unwanted pests (Arnal et al., 2011; Kuehne *et al.*, 2017).

There are two broad categories of Cu-based pesticides; those that are soluble in water, such as Cu sulfates, and those that are much less soluble in water, such as Cu oxychlorides, hydroxides, and carbonates (Komárek et al., 2010). The active substances in these products are Cu^{1+} and Cu^{2+} ions (European Food Safety *et al.*, 2018). Cu^{2+} exhibits the strongest toxic action, while Cu^{1+} presents a less toxic action in hydroxides $Cu(OH)^{+}/Cu(OH)_2$, carbonates ($CuCO_3$, $CuHCO^{+3}$, $Cu(CO_3)^{2-}$) and chloro-complexes in descending order (Komárek et al., 2010). To date, no cases of resistance to Cu-containing pesticides have been reported, making Cu a unique and invaluable agricultural tool in both organic and conventional farming (Kuehne et al., 2017).

In humans, the extent of exposure to Cu from such pesticides may be greater than previously thought. Notably, a 2018 EFSA (European Food and Safety Association) review of copper pesticide risk assessment determined that an indicative consumer exposure, considering the background levels of copper expected in plant and livestock commodities, resulted in a chronic exposure of 72.3% of the WHO acceptable daily intake of 0.15 mg Cu/kg body weight (European Food Safety et al., 2018).

Soil and Water Contamination from Cu Pesticide Use

Although Cu toxicity in plants due to direct foliar application is relatively uncommon (Gupta & Gupta, 1998), there is concern about its buildup in agricultural soils (Komárek et al., 2010). Cu originating from the intensive application of Cu-based fungicides belongs to the most important

contaminants of vineyard soils, as its concentrations exceed European legislative limits of 140 mg/kg in many vineyards across the world (Komárek et al., 2010).

The residual Cu from pesticide application typically accumulates in the upper 15 cm of the soil, bound to the organic matter and fine clay fraction (Husak, 2015). The primary mobile Cu substance found in these soils is the Cu^{2+} ion, whose mobility and concentration are influenced by pH and the amount of dissolved organic carbon in the soil, respectively (European Food Safety et al., 2018; Kuehne et al., 2017). In acidic vineyards and tilled soils, as well as vineyards located on steep slopes affected by intensive erosion, Cu ion mobility is increased, allowing Cu to migrate through soils more easily (Komárek et al., 2010). Cu applied to eroded vineyard soils can easily reach ground and surface water either as water-soluble species or associated with colloidal soil particles and concentrate in surface water sediments causing groundwater pollution.

1.4.2 Cu drinking water quality guidelines

A prevalent source of Cu exposure in the US and other developed countries is drinking water contaminated via the leaching of Cu from Cu plumbing into tap water (Brewer, 2010). Policy guidelines exist governing the maximum allowable concentration of Cu in drinking water and several retrospective studies examine case studies of Cu toxicity, however, there is no clear consensus on health-based guideline values for Cu in drinking water (Fitzgerald, 1995). Upon investigation of these guidelines, comments and critiques have been made.

Australian researcher DJ Fitzgerald closely examined the World Health Organization (WHO) and US Environmental Protection Agency (USEPA) health-based guidelines for Cu consumption from drinking water (Fitzgerald, 1995). He found that the WHO guideline of 2 mg/L is based on an unpublished 1971-72 Cu-gluconate dietary exposure study conducted on 12 beagle dogs only shared with the WHO at the time of release of this standard. Fitzgerald points to the inadequacy of this study and guideline, arguing that (i) a transcription error on the 1982 WHO JECFA document reporting 5 mg/kg of Cu-gluconate as the no-effect level instead of the study's reported result of 15 mg/kg no effect level had been made and propagated throughout the WHO literature. (ii) The doses referred to in the study refer to Cu-gluconate concentrations but are used as Cu concentrations in the WHO document. A Cu-equivalent concentration should have been calculated

instead, leading to 2.1 mg Cu/kg/day (14% of Cu-gluconate by weight) instead of 15 mg Cu/kg/day as the NOAEL (No observed adverse effect level) dose. (iii) To calculate the Cu guidelines, the WHO uses the erroneous 0.5 mg/kg/day value (assumed to be the 5 mg/kg/day value ÷ a safety factor of 10) to calculate a value of 1.5 mg/day, which is rounded up to 2 mg/day. Using the correct Cu equivalent value of 2.1 mg/kg/day and using the same WHO calculations, the guideline would amount to 0.63 mg/L, a significantly lower dose unlikely to be adopted by policy makers and (iv) although the WHO claims the 0.5 mg/kg value was put forward by the dog study, it is actually found in WHO documents in 1967, 5 years prior, bringing into question the actual origins of this value.

The USEPA value of 1.3 mg/L is based on a 1957 study, which Fitzgerald also argues is inadequate. In this study, symptoms of acute Cu intoxication (nausea, vomiting, diarrhea) were experienced by a group of nurses 0.5-1h after ingesting whiskey cocktails that had been coloured green by adding copper sulfate for Saint Patrick's Day. The drinks were recreated to determine possible Cu concentrations (5.3-32 mg Cu/drink), which the US EPA used to determine a Lowest observed adverse effect level (LOAEL) for setting drinking water guidelines (5.3÷2 (safety factor)/ 2 L=1.3 mg/L). Fitzgerald argues that this interpretation is problematic, as it does not consider the combined effects of Cu, alcohol, and their ingestion on an empty stomach.

Sidhu et. al, in 1995 (Sidhu *et al.*, 1995) recommended that the US EPA national drinking water guideline for Cu needed to be revised to protect children under the age of 10 from potential Cu toxicity. They argue that since infants and children up to 10 years of age do not have fully developed Cu homeostatic mechanisms, they are more susceptible to Cu toxicity, and since they are a significant portion of the population (17%), a new drinking water guideline is needed, which accounts for their safety. Using estimations of safe dietary Cu intakes from drinking water across different child age groups (up to age 10), as well as an adult group, the authors propose a Cu drinking water concentration of 0.3 mg/L (Sidhu et al., 1995).

In 2001, Fewtrell and colleagues examined the science behind the drinking water Cu standards, creating an elaborate quality audit framework to assess the scientific strength and reliability of WHO and US EPA drinking water guidelines, having a maximum total score of 36 (Fewtrell *et*

al., 2001). They build on Fitzgerald's critiques, arguing that the US EPA's LOAEL of 5.3 mg from the cocktail study is in fact a NOAEL, and that since the experiment was a reconstruction of the actual event, with no tested reproducibility, it was of inferior scientific validity. Overall, the US EPA guideline is given a score of 10/36. Fewtrell et al. made similar critiques of the WHO guideline as Fitzgerald, with the additional comments that "the use of such a small-scale unpublished study in an animal known to be a poor model for humans in terms of its Cu metabolism is remarkable", and that the "data behind this standard and the way in which they have been interpreted are likely to bear little relation to the health effects in humans caused by Cu in drinking water". The WHO document was given a score of 6.5/36. Fewtrall and colleagues conclude that these standards are neither clear nor transparent, well founded, robust, or of an acceptable level of risk.

Overall, the literature indicates a potential significant role of inorganic Cu ingested from tap water to increasing levels of free Cu in the blood, leading to Cu toxicity (Brewer, 2010; Eife *et al.*, 1999). Yet, standard Cu drinking water guidelines seem to be based on inadequate scientific studies and conclusions (Fewtrell et al., 2001; Fitzgerald, 1995) and there appear to be controversial claims in peer-reviewed scientific literature dismissing concerns of potential Cu toxicity from drinking water (Taylor *et al.*, 2020). This is concerning, considering that inorganic copper (Cu^{2+}), such as that found in pesticides and drinking water, is toxic at unknown consumed concentrations and could potentially play a role in neurological toxicity and cognitive decline (Brewer, 2010; Morris *et al.*, 2006; Squitti *et al.*, 2006b; Squitti *et al.*, 2009; Squitti *et al.*, 2005).

1.4.3 Implications for Human Health

Occupational Cu Toxicity

Although the EPA does not require data on the teratogenic, mutagenic, carcinogenic, and reproductive effects on mammals for many of the Cu-based pesticides used in common agricultural practices (Husak, 2015), effects have been reported from occupational exposures. Cu-based agrochemicals can indeed affect human health, causing different types of cancer, degenerative diseases, and many immune, hematological, neurological and reproductive disorders (European Food Safety et al., 2018; Husak, 2015). However, the concentrations at which they may prove

toxic and capable of such damage have not been conclusively determined (European Food Safety et al., 2018; Taylor et al., 2020). Irritant effects from occupational exposures to Cu-based pesticides are fairly common, including allergic reactions, itching, and eczema and chronic effects have been reported with vineyard workers, who experienced liver disease after 3 to 15 years of exposure to Bordeaux mixture (Cu sulfate mixture) (Husak, 2015)**.** There have also been cases of workers developing acute and chronic respiratory problems, including lung carcinoma, due to the inhalation of Cu-containing fungicides (Komárek et al., 2010). The EFSA has set an acceptable operator exposure level of 0.08 mg Cu/kg bw per day (calculated to be 50% of the acceptable daily intake of 0.15 mg Cu/kg bw/day in accordance with WHO oral intake guidelines) (European Food Safety et al., 2018). Yet, many occupational workers' exposures exceed this limit, with the extent of exposure also being potentially exacerbated by personal protective equipment dressing guideline adherence (Arnal et al., 2011).

Cu Toxicity in the general population

In 2010, Dr. George Brewer published a paper on Cu toxicity as seen in the general population (Brewer, 2010). Brewer identified significant ingestion of Cu from dietary supplements, which correlates with cognitive decline in the general population (Morris et al., 2006). He notes that authorities have incorrectly assumed that Cu toxicity studies based on Cu in food is adequate to cover all type of Cu intake, which is incorrect as the toxicity of Cu in food (Cu^{1+}) is lower than the toxicity of Cu in water (Cu^{2+}). Additionally, he emphasizes the importance of managing Cu intake, since Cu in drinking water appears to be potently toxic compared to food Cu, and since supplements appear to be a significant source of dietary Cu. The EFSA indicates that the average consumer exposure resulting from copper present in drinking water was estimated at up to 15.1% of the 0.15 mg Cu/kg body weight/day ADI, indicating that chronic exposure may occur due to higher local concentration of copper in tap water (European Food Safety et al., 2018). Brewer also discusses the toxicity of "free" Cu, or non-ceruloplasmin bound Cu as the pathological agent capable of causing cellular damage through the generation of ROS, the recent studies conducted by Squitti et. al linking free Cu with AD progression (Squitti *et al.*, 2006b; Squitti et al., 2009; Squitti et al., 2005), and a parallel between implementation of Cu plumbing and the increase of Alzheimer's disease in developed countries. However, some refute the existence of a toxic inorganic copper species with hazardous effects on human health.

In a 2020 critical review on ingested Cu toxicology conducted by Taylor et. al under the funding of Cu Development Association and International Cu Association, the authors argue that direct evidence in humans consuming excess Cu in drinking water does not support the hypothesis that Cu^{2+} in water elevates non-ceruloplasmin Cu and the more "labile" pool of Cu without providing any evidence or scientific explanation. Taylor et. al also argue that Cu at environmentally relevant exposures would be well handled by most of the population, and that available experimental studies in healthy human subjects do not support the view of Cu dysregulation due to environmentally relevant exposures to Cu since the concentrations at which these effects are observed are unknown. They propose a 2.7 mg Cu/day total Cu intake for the average 70 kg individual in line with the USEPA's 1.3 mg Cu/L drinking water concentration, which they argue is protective of children and adults based on experimental, observational, and case studies of infants exposed to elevated Cu in drinking water (Taylor et al., 2020).

1.5 Diseases of Cu dysregulation

1.5.1 Wilson's Disease (WD)

WD is a hereditary disorder in which the biliary excretion of copper is defective due to a mutation of the ATP7B gene responsible for transporting copper from intracellular copper chaperone proteins into the secretory pathway for both (i) excretion via bile and (ii) incorporation into apo-ceruloplasmin for the formation of enzymatically active holo-ceruloplasmin (EASL, 2012). The disruption of ATP7B activity causes Cu dyshomeostasis in the body, resulting in the accumulation of Cu in the liver (EASL, 2012). When the capacity for hepatic storage is exceeded, cell death ensues, and copper is released into the blood, resulting in the deposition of copper in extrahepatic tissues, such as the eyes and brain (Brewer, 2017a; EASL, 2012; Hoogenraad, 2006).

In WD patients, the accumulation of copper in tissues manifests in initial clinical presentations of liver disease and/or cirrhosis (in 30% of patients), neurological disturbances (in 30% of patients), and psychologic symptoms (in 30% of patients). Keiser-Fleischer rings are found in 95% of diagnosed neurological patients and 50% of diagnosed hepatic patients (EASL, 2012). Some patients, usually identified through family screening, are asymptomatic, with diagnosis confirmed through genetic testing (80% of the time). Any stage of liver disease may be encountered in patients with Wilson's disease with hepatic symptoms ranging from asymptomatic, to disturbance

of biochemical indicators for the liver, to overt cirrhosis/hepatitis or hepatic failure. Less common symptoms may also be observed, such as renal abnormalities, pancreatitis, cardiomyopathy or osteoarthritis (EASL, 2012).

In neurologic patients, excess copper damages parts of the brain which coordinate movement, manifesting as neurologic movement disorders, such as dystonia and incoordination, often accompanied by behavioral abnormalities (EASL, 2012; Hoogenraad, 2006). Neurological symptoms may progress gradually for some patients and rapidly for others to the stage where patients ultimately become severely disabled, usually alert, but unable to speak. Severe cognitive deterioration is observed in some patients with advanced neurological symptoms, but in general, cognitive function is not significantly impaired (EASL, 2012). Although the mechanisms behind Cu-induced neurodegeneration are unclear, increases in Cu concentration in the CNS are evident; significantly elevated Cu levels in CSF are observed in neurological WD patients, and brain stem and glial cell changes are associated with three- to four-fold increases of Cu in the brain (EASL, 2012; Strausak *et al.*, 2001; Stuerenburg, 2000). Cu accumulation can be found predominantly in basal ganglia, subthalamic nuclei, and gray and white matter, and particular lesions are found in cerebral white matter accompanying loss of myelin (Strausak et al., 2001). Astrocytes grow in number and in size as they store large quantities of Cu locally, and abnormal astrocytic cells known as Alzheimer type I glia and Opalski cells are produced. This process impairs normal astrocyte function in the brain, which may contribute to the neuropathology of WD through damage to neurons and oligodendrocytes (Barber *et al.*, 2021).

WD may present symptomatically at any age, however, it often presents between ages 5-35 (EASL, 2012). It is recognized to be more common than previously thought, with a gene frequency of 1 in 90–150 and an incidence that may be as high as 1 in 30,000 (Reilly *et al.*, 1993). No single test is specific to WD, thus, for many patients, a combination of tests is required to assess disturbed copper levels, including measurements of serum ceruloplasmin, non-ceruloplasmin bound copper, urinary copper excretion, hepatic biopsy tests, genetic testing, and MRIs for neurological evaluation (EASL, 2012; Woimant & Trocello, 2014). Measurement of free copper or non-ceruloplasmin bound copper is recognized as a valuable diagnostic test in ensuring that the correct diagnosis of Wilson's disease is made (Brewer, 2017a; EASL, 2012; Hoogenraad, 2006). This fraction of copper can be measured indirectly by subtracting the ceruloplasmin bound fraction of

copper from the total copper content found in blood (clinically available method), or directly, through other recently developed methods (not commercially available) (El Balkhi *et al.*, 2011).

1.5.2 Other Cu related diseases

Non-Wilsonian Cu toxicosis may be observed in other liver disorders such as Indian Childhood Cirrhosis (ICC), Idiopathic Copper Toxicosis (ICT) and Endemic Tyrolean Infantile Cirrhosis (ETIC) (Barber et al., 2021). ICC, ETIC and ICT form a second class of copper-overload disorders that are distinct from WD. Most patients die at an early age due to liver failure following liver cirrhosis. Plasma ceruloplasmin levels are normal, and no neurologic damage is evident (Llanos & Mercer, 2002). Phenotypic expression of ICC, ETIC and some cases of ICT appear to be associated with both an excessive copper intake and an underlying genetic defect (De Bie *et al.*, 2007).

Cu deficiency-related diseases include Menkes disease, occipital horn syndrome, and ATP7A-related distal motor neuropathy, which are all caused by different mutations in the ATP7A Cu-transporter gene (Kaler & DiStasio, 1993). Defective ATP7A function results in the dysfunction of several Cu related enzymes, due to the inability to load these enzymes with Cu via ATP7A, and reduction of Cu elimination from cells in almost all tissues except the liver and brain, where Cu may instead accumulate to abnormal levels (Woimant & Trocello, 2014).

ATP7A related disorders

Menkes disease (MD) is a multisystemic fatal disorder of impaired copper metabolism characterized by neurodegenerative symptoms and connective tissue manifestation (Ahuja et al., 2015). When ATP7A is inactivated by mutation, individuals suffer from systemic copper deficiency due to poor absorption of dietary copper in the small intestine and defective distribution within the body. Copper becomes trapped in the endothelial cells of the BBB and the brain becomes severely ion deficient, leading to profound neurological symptoms and causing death within the first few years of life (Ahuja et al., 2015; Llanos & Mercer, 2002; Shim & Harris, 2003)., The incidence of MD is estimated to range between 1 in 40, 000 and 1 in 350,000 (Møller *et al.*, 2009). Characteristics include progressive cerebral atrophy, seizures, coarse “kinky” hair, and connective tissue abnormalities due to the reduced activity of several cuproenzymes (Ahuja et al.,

2015; Chen *et al.*, 2020). Neurodegeneration occurs likely as a result of a lack/dysfunction of COX, DBH and PAM cuproenzymes (Shim & Harris, 2003; Strausak et al., 2001). Mechanistically, it has been suggested that the synaptic release of copper might competitively inhibit N-methyl-D-aspartate receptors (NMDAR) in a neuroprotective fashion, therefore, dysfunction of ATP7A and copper deficiency might lead to prolonged, potentially deleterious NMDAR activation, inducing degenerative loss of neurons in MD patients (Chen et al., 2020)

Occipital Horn Syndrome (OHS) is a milder allelic variant of Menkes disease, presenting with milder neurological symptoms due to the translation of some functional ATP7A splice variants allowing some ATP7A-mediated Cu transport (Kaler, 2011; Møller et al., 2009). Its incidence rates are unknown (Woimant & Trocello, 2014). OHS shares the hair and connective tissue defects observed in Menke's disease attributable to the impairment in lysyl oxidase (LOX) activity, an enzyme needed for collagen crosslinking (Chen et al., 2020; Kaler, 2011; Kaler & DiStasio, 1993). OHS is characterized by "occipital horns," distinctive wedge-shaped calcifications at the sites of attachment of the trapezius muscle and the sternocleidomastoid muscle to the occipital bone (Kaler & DiStasio, 1993). Patients with this disease have low to normal levels of serum copper and ceruloplasmin, and abnormal plasma and CSF catecholamine levels, which together reflect a deficiency in DBH activity, as in Menkes disease (Kaler, 2011).

ATP7A-related distal motor neuropathy (DMN), also known as X-linked spinal muscular atrophy type 3 (SMAX-3), is an adult-onset motor neuron degenerative disorder resembling Charcot–Marie–Tooth disease (Manto, 2014; Woimant & Trocello, 2014; Yi & Kaler, 2014). Missense mutations induce subtle defects in ATP7A intracellular trafficking, resulting in preferential accumulation of the protein at the plasma membrane of motor neuron cells (Chen et al., 2020). This disorder appears to only affect motor neuron function (Lorincz, 2018). There is no sign of systemic copper deficiency; serum copper and ceruloplasmin levels are normal (Yi & Kaler, 2014). Symptoms include gradual atrophy and weakness of distal muscles in hands and feet, followed by involvement of the upper limbs, reductions in tactile and vibratory sensation, and loss of deep tendon reflexes (Kaler, 2011; Kaler & DiStasio, 1993).

1.6 Cu in neurodegenerative diseases

The dyshomeostasis of copper plays crucial roles in the progression of neurodegenerative diseases (Chen et al., 2020; Trumbo et al., 2001). In addition to neurodegenerative phenotypes observed in known cases of Cu overload (ex. WD) or deficiency (ex. MD), Cu dysregulation has been implicated in the pathology of other complex neurodegenerative diseases. With the exception of AD, there is no literature investigating the role of non-ceruloplasmin bound copper in their pathophysiology.

1.6.1 Alzheimer's Disease (AD)

AD is a complex multifactorial disorder, in which the convergence of polygenic, epigenetic, environmental, vascular, and metabolic factors increases the global susceptibility to the disease and shapes its course (Brewer, 2017a). It is the most common cause of dementia in the US and other developed countries (Brewer, 2012), the seventh leading cause of death in the world (WHO, 2020), and the only disease in the top 10 causes of death with no effective treatment (Brewer, 2017b). One of the risk cofactors of AD is the dysregulation of brain metals, including Cu (Sensi *et al.*, 2018).

Although the role of copper in the progression of AD is yet to be conclusively determined, many correlations have been made between Cu deposition in the brain and AD progression. Specifically, excess copper concentrations in the brain have been correlated with a decrease in microglial activity and an increase in brain inflammation, both of which are indicators of AD (Patel & Aschner, 2021). Accumulated copper deposits have also been found in regions targeted by AD, such as the hippocampus, cerebral cortex, cerebellum, and brainstem, concomitant with adverse effects on memory, information processing, motor skills, and regulation of autonomous functions in AD (Patel & Aschner, 2021).

The leading theory behind AD is the amyloid cascade hypothesis. It posits that aberrant accumulation of the Aβ peptide, a derivative of the Amyloid Precursor Protein, in the brain is the initial step in AD pathogenesis (Patel & Aschner, 2021; Sensi et al., 2018). In addition to Aβ peptide accumulation, intracellular neurofibrillary tangles (tau proteins) are also observed and

postulated to play a neurotoxic role. Increased Cu concentrations have been found to colocalize within Aβ and neurofibrillary tangles and have been shown to regulate APP expression, suggesting a significant role for Cu toxicity within the amyloid cascade hypothesis' framework (Patel & Aschner, 2021; Sensi et al., 2018). Two alternative models describe how Cu can affect Aβ neurotoxicity. In the gain-of-function model, copper, given its high affinity for Aβ plaques, binds to the plaques at two distinct copper-binding sites, where it cycles between a Cu^{1+} and Cu^{2+} state, promoting ROS formation and leading to the formation of Aβ oligomers and their precipitation within plaques as well as lipid peroxidation (Huang *et al.*, 1999; Patel & Aschner, 2021). The loss-of-function model proposes that Aβ peptides act as protective buffers against the toxic availability of metals within the brain (Kepp, 2016). According to this model, Aβ works as a physiological chelator that exports excess Cu across membranes, thereby protecting neurons from Cu overload. The defective Cu chelation may also lead to the loss of soluble, metal-binding, functional Aβ monomers, which, in turn, aggregate in toxic oligomers and then in fibrils (Kepp, 2016). Notably, all clinical trials targeting Aβ plaques have failed to date.

Free Copper in AD

Although Cu toxicity is implicated in AD and WD neurodegeneration, it is unlikely that it plays the same role in both diseases (Brewer, 2017a). While AD involves memory and cognition loss, and the formation of extracellular amyloid plaques and intracellular neurofibrillary tangles, WD involves neurological movement disorders, rarely a cognition effect, and no extracellular or intracellular protein aggregates. In AD, copper toxicity is not intense enough to damage the basal ganglia, however, when working in tandem with aging, a small and consistent toxic copper load may be able to produce AD.

Brewer and Hoogenraad have observed that the nature of the copper species exerting a toxic effect in AD is the Cu^{2+} ion, referred to as free copper, or non-ceruloplasmin bound copper, and advocate that its control is essential to treatment (Brewer, 2012, 2017a, 2017b; Brewer *et al.*, 1993; Hoogenraad, 2006, 2011). Brewer suggests that in relation to the amyloid cascade hypothesis, Cu^{2+} increases the risk of aggregation and/or the toxicity of plaque formation (Brewer, 2017a).

In his 2012 review, Brewer presents his hypothesis and arguments based on the relationship between inorganic copper exposure and the development of Alzheimer's disease. He hypothesizes that ingestion of inorganic copper (Cu^{2+}), for which drinking water, primarily leached from copper plumbing in homes, is a source (Ceko *et al.*, 2014), as is copper from mineral/vitamin supplements, is at least partially causal of AD (Brewer, 2012). To support this argument, he cites a 2003 study conducted in a rabbits, where it was found that the addition of only 0.12 ppm copper to the distilled drinking water (much lower than the US EPA limit of 1.3 ppm) greatly enhanced AD-type brain pathology and cognition loss in a cholesterol-fed rabbit model of AD (Sparks & Schreurs, 2003). He also references a large cohort study examining nutrient intake and cognition over a period of 6 years, where it was found that participants with a high fat diet and taking copper supplement pills showed a rate of cognition loss 6 times higher than any other groups (Morris et al., 2006). Moreover, he discusses a series of studies conducted by the Squitti group on AD and Cu showing that (i) the mean blood free copper level is increased in AD (Squitti et al., 2005), (ii) high free copper levels in AD patients are correlated with lower measures in cognition in AD patients (Squitti *et al.*, 2006b), (iii) free copper predicts the rate of decrease of cognition scores over time in AD patients, that is, the higher the free copper level, the greater is the rate of cognition loss (Squitti et al., 2009), and (iv) the higher the blood free copper level, the greater the risk of mild cognitively impaired (MCI) patients, the precursor state to AD, to convert to full AD (Squitti, Ghidoni*, et al.*, 2014).

In addition to the data cited by Brewer, the Squitti group has also shown that the non-ceruloplasmin bound copper fraction in blood is expanded in AD patients and at comparable concentrations to WD patients when compared with controls (Squitti *et al.*, 2018). The process appears to be specific to a subpopulation of AD, who are carriers of selected ATP7B gene variants (Squitti *et al.*, 2013; Squitti, *et al.*, 2017). These results suggest the existence of a 'copper dysfunction' phenotype of sporadic AD with a genetic basis. They also suggest that free copper is a risk factor in AD, modulating additional pathways leading to the disease cascade (Squitti, et al., 2017). Sensi et. al (2018) propose that certain Cu abnormalities may serve as biomarkers of AD and have a role in its progression for a subset of AD patients, namely those who have been found to possess the ATP7B variant and exhibit copper dyshomeostasis (Sensi et al., 2018). It was recently demonstrated that Cu dysregulation affects 50–60% of patients with MCI or AD (Rozzini *et al.*, 2018; Squitti *et al.*, 2017). Additionally, the progressive increase of the non-ceruloplasmin bound copper pool in the

AD brain is consistent with the parallel presence of an expanded pool of exchangeable nCp-Cu in the blood (Rembach *et al.*, 2013; Squitti *et al.*, 2014).

1.6.2 Parkinson's Disease

Parkinson's Disease (PD) is the second most prevalent progressive neurodegenerative disorder after AD. The two pathological hallmarks of PD are the accumulation of protein aggregates (Lewy bodies), which are mainly composed of α-synuclein fibrils, in the cytosol and the gradual degradation of dopaminergic neurons in the substantia nigra resulting in extrapyramidal disorders after loss of 50-60% neurons (Bisaglia & Bubacco, 2020; Karpenko *et al.*, 2018). The principal clinical features are resting tremor, muscular rigidity, bradykinesia, and postural instability (Gangania *et al.*, 2017; Montes *et al.*, 2014). 90% of PD cases are sporadic with the etiology of PD rooted in a combination of genetic susceptibilities and environmental factors. General consensus exists that there is a role exerted by oxidative stress and mitochondrial dysfunction in the disease progression, however, exact mechanisms explaining pathogenesis remain largely theoretical (Bisaglia & Bubacco, 2020). The role of copper in Parkinson's disease is controversial, with some evidence indicating increased serum Cu levels, while other results show the opposite (Montes et al., 2014). However, it is important to note that most studies of Parkinson's disease patients have reported total serum or plasma copper levels, rather than non-ceruloplasmin bound copper (Davies *et al.*, 2016).

Some studies associate increased copper levels with increased PD risk, however, this correlation is still controversial and far from definitive, as Cu's pathological role in PD is complex (Bisaglia & Bubacco, 2020). On one hand, free copper is associated with increased oxidative stress, oligomerization of alpha-synuclein protein at physiologically relevant concentrations, and formation of Lewy bodies via Fenton and Haber–Weiss reaction. On the other hand, Cu acts as a cofactor of important antioxidant enzymes such as Cu/Zn-SOD, which reduces oxidative stress (Bisaglia & Bubacco, 2020; Davies et al., 2016; Gaggelli et al., 2006; Gangania et al., 2017). Labile pools of copper ions concentrated in the substantia nigra are also suggested to be a major source of dopamine oxidation leading to a variety of potentially toxic species, such as dopamine-quinones and ROS (Bisaglia & Bubacco, 2020). Additionally, altered ceruloplasmin activity could

be a susceptibility factor for iron-induced oxidative stress since 80% of ceruloplasmin ferroxidase activity is lost in the substantia nigra of idiopathic PD cases (Ayton *et al.*, 2013; Pal *et al.*, 2014).

1.6.3 Prion Disease

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are fatal infectious neurodegenerative diseases in both animals and humans including Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker syndrome, and mad cow disease (Brown, 2002; Millhauser, 2007). Prion diseases result from the accumulation of a misfolded form of the endogenous prion protein (PrP), a copper binding protein located predominantly at the synapses in the brain, spinal cord and peripheral tissues (Hordyjewska et al., 2014). Normal PrP binds copper to be used for antioxidant activity similar to that of SOD1 and might have a role in copper uptake in neurons and delivery of copper to specific target proteins (Brown, 2002; Gil-Bea et al., 2017; Llanos & Mercer, 2002). The exact mechanism by which PrP can cause neurodegeneration and cell death is elusive and the role of Cu^{2+} ions in the pathogenesis of prion disease is complex (Gaggelli et al., 2006; Tapiero et al., 2003). It has been found that exposure to large non-physiological concentrations of Cu^{2+} ions induces endocytosis of this protein and brings its copper into the cell potentially playing a protective role (Gaggelli et al., 2006; Hordyjewska et al., 2014; Kozlowski *et al.*, 2012).

1.6.4 Aceruloplasminemia

Aceruloplasminemia is a rare autosomal recessive disorder caused by the absence of holoceruloplasmin. The absence of ceruloplasmin and its ferroxidase activity leads to pathologic iron overload in the brain and other organs (Hordyjewska et al., 2014; Montes et al., 2014; Woimant & Trocello, 2014). Despite an absence of ceruloplasmin in their serum, patients have no evidence of copper deficiency or abnormalities in copper metabolism (Shim & Harris, 2003; Tapiero et al., 2003). Instead, iron homeostasis is severely disrupted due to the absence of Cp's ferroxidase activity and patients present with diabetes mellitus, retinal degeneration, and a progressive neurological syndrome including Parkinson-like symptoms (Manto, 2014; Shim & Harris, 2003). Elevated ferritin levels are seen along with deposits of iron in certain brain areas like the basal ganglia (Shim & Harris, 2003).

1.7 Biomarkers of Cu Status

To assess the effect of copper on ALS patients, one or several suitable Cu status biomarkers are needed. Several candidate enzymatic and hematologic Cu biomarkers have been examined to determine the best indicator of Cu toxicity. The following were found to be inadequate indicators of Cu status, primarily as the relatively few existing studies have resulted in contradictory or inconclusive results: erythrocyte Cu, platelet Cu, Cu/Zn SOD activity, glutathione peroxidase activity (GPX), cyt c-oxidase activity, urinary pyridinoline and urinary deoxypyridinoline (bone marrow biomarkers), plasma glutathione, and diamine oxidase (DAO) (Harvey *et al.*, 2009). The few biomarkers found to be useful in assessing changes to copper status are used in the treatment and management of WD. They are discussed below.

1.7.1 Urinary Cu excretion

In untreated WD patients, the 24 h urinary excretion of Cu is elevated and correlates with the non-ceruloplasmin bound Cu fraction in serum (EASL, 2012; Mohr & Weiss, 2019). Thus, urinary Cu has an established role in diagnosis, however it was found for only about 85% of diagnosed WD patients in a retrospective study (Merle *et al.*, 2007) . Since traditional chelating agents, such as d-penicillamine and trientine act by enhancing urinary Cu excretion, urinary Cu excretion serves as a valuable tool in monitoring treatment efficacy (Brewer, 2017a; EASL, 2012).

1.7.2 Liver Biopsy with quantitative Cu determination

A liver biopsy is warranted when other clinical and biochemical markers fail to establish the final diagnosis of WD (Mohr & Weiss, 2019). In such cases, such biopsies can contribute to the diagnostic WD Leipzig score, which assesses the severity of 7 WD markers (Ferenci *et al.*, 2003), often revealing parenchymal damage with fibrosis and subsequently, a development of cirrhosis (EASL, 2012). However, copper accumulation in the liver is very heterogeneous and, hence, low values cannot rule out WD (Ferenci *et al.*, 2005).

1.7.3 Ceruloplasmin (Cp)

Copper in the blood is bound to ceruloplasmin (60-65%), transcuprein (15%), albumin (15%) and to other low molecular weight components (10%) (Wirth & Linder, 1985). When Cu is bound to

Cp, Cp is enzymatically active, and is referred to as holoCp, whereas Cp without Cu bound to its active sites is inactive, and referred to as apoCp (Walshe, 2003). In addition to its role in Cu transport, holoCp acts as a ferroxidase and is functional in iron metabolism, antioxidant defense, coagulation, and angiogenesis (Mohr & Weiss, 2019). As an acute-phase protein, in cases of infection or inflammation, the body responds with increased Cp levels in plasma (Danzeisen *et al.*, 2007; Mohr & Weiss, 2019). Its concentrations are also subject to seasonal changes (lowest concentrations during winter) and increase under the influence of oestrogen, oral contraceptives, and pregnancy (Danzeisen et al., 2007).

Cp levels are currently measured using two immunologic assays: the immunoturbidimetric assay and nephelometric assay (Mohr & Weiss, 2019; Siotto et al., 2010). These assays rely on the use of a Cp-specific antibody to quantify Cp concentrations in blood; however, this antibody is not capable of differentiating between apoCp, holoCp, and Cp fragments (EASL, 2012; Merle *et al.*, 2009; Mohr & Weiss, 2019; Siotto et al., 2010; Walshe, 2003). Thus, the quantity of Cp calculated using such immunological assays often overestimates the true value of Cp-bound Cu present in blood. Despite these limitations resulting in a 6% predictive value (EASL, 2012), Cp quantification is considered to be a biomarker in the diagnosis of WD. Low Cp is a prominent biochemical finding in serum in WD, when not raised due to other infections (Mohr & Weiss, 2019). Also, diagnosis of WD must not be based on low Cp alone, as 20% of healthy heterozygous subjects have Cp levels below the lower reference limit, and normal or near normal Cp concentrations are reported in WD patients (Mohr & Weiss, 2019).

Cp activity assays

As Cp activity is contingent on its Cu content, measurement of its enzymatic function is regarded as a more reliable method of measuring Cp-bound Cu content (Merle et al., 2009; Mohr & Weiss, 2019; Siotto et al., 2010; Walshe, 2003). Determining Cp activity allows for accurate Cu content quantification and subsequent free copper calculation, reducing the rate of false negatives (EASL, 2012).

In the "*p*-phenylendiamine (PPD) method", one of the most commonly Cp enzymatic assays, Cp catalyzes the oxidation of PPD to yield a colored product, which is measured at 530 nm. The rate

of formation of the colored oxidation product is proportional to the concentration of serum active Cp (Siotto et al., 2010). In the (Schosinsky *et al.*, 1974) method, the substrate is oxidized is *o*-dianisidine dihydrochloride. This reagent is converted to a yellowish-brown reaction product at pH 5. Acidification with 9M sulfuric acid stops the enzymatic reaction, and a stable purplish-red solution is formed, which is measured at 540 nm. In Erel's method (Erel, 1998), serum samples are incubated with a known amount of ferrous ammonium sulfate at pH 5.8; then a chromogen (3-(2-pyridil)-5,6-bis(2- [5-furylsulfonic acid])-1,2,4-triazine) is added that forms a highly colored complex with ferrous ion, but does not react with ferric ions. The remaining non-oxidized ferrous ions are measured spectrophotometrically at 590-610 nm. This ferroxidase assay may be a suitable replacement for *o*-Dianisidine assay in for WD patient detection, as its results correlate well with those of the Schosinsky method, and its reagents are cheap and stable at room temperature (Erel, 1998; Siotto et al., 2010).

1.7.4 Serum Cu and Non-Ceruloplasmin Bound Cu (NCC)

Total Serum Cu

Blood Cu not bound to Cp is bound to smaller molecules (i.e., albumin, transcurein, amino acids), which can pass through the blood-brain barrier (Barnham et al., 2004; Choi & Zheng, 2009; Chutkow, 1978). This fraction of Cu is referred to non-ceruloplasmin bound Cu (NCC) or free copper. The total amount of Cu present in peripheral blood, or total Cu, encompasses both the NCC fraction and the ceruloplasmin bound fraction of Cu. Counterintuitive to the Cu overload causing WD, total serum Cu is decreased in WD (Mohr & Weiss, 2019). In a 2009 review of studies of copper supplementation, Harvey et. al undertook a statistical analysis to find trends in changes of potential biomarkers of copper. The only biomarker in which a trend was observed was total serum Cu (population normal range 10 -27 µmol/L), for which an increase of 1.11 µmol/l (a 6% increase) was observed for "copper replete" individuals. However, the significance of these results is of questionable value as studies of biomarkers for WD have found that the non-ceruloplasmin bound copper is the toxic moiety (Brewer, 2017a; Danzeisen et al., 2007; Hoogenraad, 2006; Squitti et al., 2009) and total serum copper is usually decreased in WD (i.e. situation of copper excess) (Danzeisen et al., 2007; EASL, 2012).

Analytical methods for measuring total serum Cu (bound and free) in biological and environmental samples include atomic absorption spectrometry (AAS), X- ray fluorescence spectrometry (XRF), inductively coupled plasma-mass-spectrometry (ICPMS), and colorimetric automated methods. As discussed by Siotto et. al, all these methods have both advantages and disadvantages (Siotto et al., 2010). For example, ICPMS has a high power of detection (ppb detection thresholds), can detect several metals in one sample, and is useful for isotope analysis and metal imaging, making it the best qualitative method of analysis. However, it also requires a high technical skill level, is costly, and an aliquot of the sample is used up in the process of analysis.

Indirect Non-ceruloplasmin bound Cu (NCC) quantification

In WD, serum NCC is used as a tool to estimate of the free (toxic) portion of total serum Cu and assess the decoppering treatment progression of patients (Danzeisen et al., 2007; EASL, 2012; Hoogenraad, 2006; Roberts et al., 2008) The following formula can be used to calculate NCC in µmol/L: NCC = total serum Cu (µmol/L) – 0.049 x Cp (mg/L). This calculation expresses free Cu in µmol/L and calculates it by subtracting the estimated amount of Cu bound to Cp (6 Cu atoms/Cp molecule) from the total amount of detected Cu (EASL, 2012; Walshe, 2003). The usefulness of this calculation in WD has been the subject of intensive debates in the field since this method results in an underestimation when Cp concentration is determined immunologically rather than enzymatically (Mohr & Weiss, 2019; Siotto et al., 2010; Walshe, 2003).

Exchangeable copper (CuEXC)

To directly determine the concentration of NCC or an estimate thereof, as opposed to indirectly estimating it by subtracting assumed Cp bound Cu from total Cu, direct methods of quantification have also been developed. Exchangeable copper (CuEXC) is defined as the plasma fraction that is labile, that is not tightly bound to ceruloplasmin, transcuprein or albumin, and understood to be representative of the damage causing fraction. The El Balkhi method is a process-based measure whose determination has been standardized for use in the diagnosis of WD (El Balkhi *et al.*, 2009) and is being investigated for diagnosis of AD (Siotto et al., 2010). Using EDTA as high affinity Cu chelator, serum is incubated for 1h, selected as a standard time during which Cu is chelated. The incubated serum is centrifuged in ultrafiltration devices with 30kDa cut-off filter membranes, separating the serum into two fractions: a ceruloplasmin/protein rich fraction that remains above

the membrane and an ultrafiltrate containing the exchangeable Cu (CuEXC), which has been understood to be an approximation of, or proportional to, the NCC fraction. This fraction's Cu content is determined using atomic absorption spectrometry or ICPMS (Siotto et al., 2010). The method has shown good repeatability and a very low limit of detection (0.7 nmol/L) (El Balkhi et al., 2009). When used on serum from WD patients, this method showed good correlation between both CuEXC and the clinical and biological features of the patients, and the CuEXC/Total Cu ratio referred to as the relative exchangeable Cu (REC) value was found to have an ideal prognostic accuracy in identifying WD yielding 100% sensitivity and 100% specificity in WD identification, with no false positives or negatives (El Balkhi et al., 2009).

These results have been validated with additional WD patients, in which CuEXC was the only biological marker to be positively correlated with the neurologic disease burden in WD, but did not indicate the severity of liver damage (Poujois et al., 2017). This confirms the study in which worsening neurological condition was associated with significant spikes in serum free Cu levels at the time of deterioration (Brewer, 2009). These data are consistent with the association of increased free Cu and a neurologic deterioration (Mohr & Weiss, 2019).

Other free copper measurement methods

Preliminary studies using electrochemical devices were proposed by Althaus and Kanzer in which apo-enzymes were prepared from Cu-dependent enzymes (Althaus, 2007). The reconstitution of the apo-enzyme and its subsequent activity are dependent upon the concentration of loosely bound Cu in solution. The activity of the reconstituted enzyme is measured and correlated with NCC levels.

In another proposed method, a carbon working electrode was modified for the determination of heavy metals including zinc, cadmium, lead and Cu. In this method, discrimination between free and bound metal is based on the kinetics of metal deposition on the surface of an electrode (Kanzer, 2008). Once deposited, metal detection and measurement are based on a signal generated using square wave voltammetry. Parameters for both deposition potential and deposition time can be finely tuned so that detection of free metal only can be performed in a solution from a mixture that contains both free and bound metal. The concentration of free Cu is then determined from the measured voltametric signals.

The emergence of direct NCC quantification methods shows great prospects for future Cu toxicosis diagnostics in other diseases. Squitti et. al have already demonstrated the utility of CuEXC in AD prognosis, where they establish it as a reliable longitudinal prognostic value in AD progression (Squitti et al., 2009) as well as a predictor for mild cognitive impairment (MCI) to AD conversion (Squitti et al., 2014). Such results highlight the significant potential of CuEXC measurements in studying neurodegenerative diseases implicating Cu toxicity, such as ALS.

2. Research Premise

The ALS literature reports on a correlation between ALS incidence and heavy metal exposure in disease pathology. However, the role of Cu dysregulation in ROS generation and its effect on ALS incidence and progression has not been examined. Recent research on Wilson's Disease uses high levels of non-ceruloplasmin bound copper as a biomarker for diagnosis (EASL, 2012; Hoogenraad, 2006) and to monitor the progression of treatment and modulate treatment dosage (Brewer, 2009; Hoogenraad, 2006).

We hypothesize that ALS patients have high levels of non-ceruloplasmin bound copper, and that levels will increase with disease progression, thereby providing support that environmental copper exposure could be a risk factor in ALS incidence and progression. We also hypothesize that the vulnerability of individuals to environmental copper exposure could correlate with gene expression associated with copper homeostasis and/or antioxidant status.

2.1 Primary aim

The primary aim is to test for a correlation between ALS and high serum non-ceruloplasmin bound copper levels, which would suggest that copper toxicity is implicated in ALS. Non-ceruloplasmin bound copper levels will be analyzed to determine intra and inter-patient variability and to determine if disease progression correlates with increases in non-ceruloplasmin bound copper levels. Correlations will be adjusted for confounders, identified in questionnaires on environmental and lifestyle factors.

2.2 Secondary aim

The secondary objective is to evaluate whether there is an association between serum copper biomarkers and changes in expression (RNA) of genes involved in copper metabolism and antioxidant status.

3. Methods

3.1 Study Design

Data was obtained from a case-control study of 35 ALS patients and 21 controls recruited from a specialized ALS clinic at the CHUM (Centre Hospitalier de l'université de Montréal) under the ethics approval number "CÉR CHUM **20.221**". Study participants were assessed based on the ALSFRS-R to determine ALS disease progression. Blood draws were obtained at approximately three-month intervals for a year, coinciding with normal blood work. Study participants were assessed using a questionnaire at study initiation to determine demographic information and to evaluate exposure to potential confounders (Appendix I). In addition, a short questionnaire (Appendix II) at the time of each blood draw evaluates current exposure to potential short-term confounders. Spouses of patients were recruited to yield roughly age- and sex-matched controls. This group was expected to yield a control group representing the general Montreal population's exposure to Cu.

3.2 Standard Operating Procedure (SOP) Development

Samples were collected from each blood draw to measure copper biomarkers, oxidative stress and investigate genes involved in copper metabolism and oxidative stress. To ensure optimal acquisition and storage of samples for downstream processing, a time sensitive standard operating procedure (SOP) was developed for use in a clinical setting)Appendix III). This SOP serves as a laboratory guide for phlebotomists and lab researchers and includes all relevant information necessary for the collection of blood samples at the CHUM, their treatment, storage, and expedition to external laboratories**.**

To develop the SOP, the following factors were taken into consideration: selection of appropriate blood collection tubes, design of protocol to collect samples according to their priority for the research goal, optimization of the handling and processing of the samples. Since the widespread

distribution of copper is a source of possible contamination (Barberá *et al.*, 2003), TraceMetal grade tubes were used for blood draws to avoid sample contamination from the tubes themselves; BD Vacutainer TraceElement tubes (cat # 14-816-154) were selected for acquisition of the samples analysed by ICPMS and for the samples sent for analysis by AAS. The same TraceElement tubes as well as BD Vacutainer SST tubes (cat # 02-683-145) were used to acquire serum to be sent to LifeLabs for commercial determination of Cu content and ceruloplasmin level respectively. Serum samples were prepared for ceruloplasmin and serum copper determination as per LifeLabs specifications (LifeLabs, 2022). BD Vacutainer sodium citrate CPT tubes (cat # 362761) were selected for acquisition of peripheral blood mononuclear cells (PBMCs) used for DNA and RNA extraction, as well as serum used for ELISAs and the ceruloplasmin colorimetric activity assay. PAXgene Blood RNA tubes (cat # 762165) containing an RNA stabilizing agent were selected for whole blood gene expression analyses.

An order for the sample collection is stipulated in the SOP reflecting the prioritization of the data obtained, with copper biomarkers having highest priority, followed by oxidative stress markers, and finally genetic analyses. Tube coagulation times, centrifugation times and speeds, and storage conditions were obtained from tube manufacturer sites and incorporated into the SOP. Amicon Ultra-4 Centrifugal Filter units with 30kDa cutoffs (Millipore Sigma, cat # UFC8030) were chosen to obtain the serum CuEXC fraction based on results from (El Balkhi et al., 2011) and following the procedure outlined in (El Balkhi et al., 2011). Centrifugation parameters such as time, speed, and temperature, were studied to examine effects on the CuEXC fraction. The optimal centrifugation conditions found were used in the SOP.

To determine the SOP's clinical feasibility, before implementation in a clinical setting, trial runs were performed using healthy donor blood at the LDI clinical research unit (under Protocol #2020-1880 "Adverse effects of environmental exposures" approved by the CIUSSS West-Central Montreal Research Ethics Board). To process the samples, three swing-bucket centrifuges were needed: one kept at 4ºC and two at RT. The duration of the processing protocol from the start of each blood draw until the end of processing required on average 3 hours, including incubation times. The protocol was successfully implemented with blood draws from 3 different subjects at sampling intervals of 30 min. Blood draws at less than 30 min intervals was feasible but not ideal,

as it required additional centrifuges and complicated the procedure. If the samples are drawn within 8 min of each other, they can be processed together at the same SOP timepoints.

3.3 Copper Biomarker measurements

3.3.1 ICPMS copper content analyses

ICPMS was used to analyze digested, diluted samples for total serum copper (Total Cu) and exchangeable copper (CuEXC). ICPMS has a high power of detection (ppb detection thresholds), can detect several metals in one sample, and is able to determine the isotope composition of a sample using less complex pre-treatment procedures than other mass spectrometry techniques, making it the best qualitative method of analysis (Barberá et al., 2003; Siotto et al., 2010). CuEXC samples were obtained using the direct quantification method developed by Elbakhi et al. where serum is incubated with EDTA (1:1) and ultracentrifuged to obtain a CuEXC containing sample quantified by ICPMS. To prevent signal suppression or enhancement in the ICPMS analysis due to non-spectroscopic matrix effects and blockage of the nebulizer and sampler, a thorough sample digestion protocol was developed consisting of the following steps.

After collection and centrifugation, Total Cu and CuEXC samples were acidified with 2 volumes (240 µL) of 67-70% HNO_3 (Plasma Pure Plus Nitric Acid, TraceMetal grade, cat. # 250-036-135). The mix was left to sit in the tubes in a fume hood with the caps loose to allow nitric gases to escape. The following day, 1 volume (120 µL) of 30% H_2O_2 (SigmaAldrich cat. # 95321 for TraceMetal Analysis) was added to the tubes, which were placed in a water bath heated to 80°C and left to incubate for 4 hours (caps slightly loosened). After digestion, the tubes were left in a fume hood for 2 nights to ensure complete, thorough digestion. Next, samples were diluted to 2 mL volume using MilliQ water. The following day, sample volume was completed to 12 mL using MilliQ water, yielding a 200x dilution. The delayed dilution to 200x ensured complete and thorough digestion of samples and avoided ICPMS matrix effects. These samples were capped firmly and kept at 4ºC until transferred for ICPMS analyses performed at the University of Montreal's (UdeM) Chemistry Department.

At UdeM, a Perkin Elmer NexION 300x ICPMS device was used for the quantification of As, Cd, Cu, Mn, Pb and Zn. ICPMS standards certified traceable to NIST were used. Multi-element standard "EPA 200.7 Calibration standard 6", obtained from High-Purity Standards, was used for calibration. For quality control, "Quality Ctrl.Std. 4" (SCP Science), "Quality Control Standard QCP-QCS-3" (Inorganic Ventures), and "Seronorm™ Trace Elements Human Serum L-1 RUO" (cat # 201405) were used. The isotopes 55Mn, 63Cu, 66Zn, 75As, 111Cd and 208Pb were monitored. Y, In, and Bi (Inorganic Ventures) were used as internal standards. ICPMS analyses were performed in triplicate measurements with 20 readings each and an integration time of 1s.

The measured concentration from these samples was multiplied by 200 (to account for dilution) to provide the measured blood content of each metal. Averages of triplicates were used and reported as Total Cu/CuEXC measurements. Relative Exchangeable Copper (REC) values were calculated by dividing the amount of CuEXC by Total serum Cu. To account for potential copper contamination from EDTA, the copper content of the EDTA solution used in sample preparation was also quantified by ICPMS and subtracted from the average copper content measured in the samples. MilliQ water used for dilution was measured and found to have metal concentrations below detection limits. All other reagents added to the sample tubes were TraceMetal grade and were not expected to cause any contamination.

Water samples

Water samples obtained from patient homes were analyzed by ICPMS for metal content to determine if high ingestion of Cu from drinking water was correlated with Cu content in blood and disease progression. Patients were instructed to collect tap water in a 50 ml conical tube every time they used tap water throughout the day (ex. when they made coffee, drank water, prepared food, etc…) and to decant the 50 mL conical tube into a 1 L plastic bottle (trace metal grade). This method provides a composite sample that reflects the copper exposure from the household's drinking water over the day. The composite tap water sample (4,900 µL) was acidified with 100 µL of 67-70% HNO_3 for analysis of the water samples with ICPMS to obtain a 5 mL sample at 2% acidity.

3.3.2 Free Copper Estimation

Free copper values can be calculated from Total Cu concentration and estimation of ceruloplasmin bound copper (Cp-bound Cu). For each serum copper and Cp pair, the amount of copper bound to Cp and the amount of copper not bound to Cp (free copper) are computed using the following calculations (Siotto et al., 2010; Walshe, 2003):

Free copper = total serum copper – estimated Cp-bound Cu

Free copper [µmol/L] = total serum copper [µmol/L] – (ceruloplasmin [mg/L] * 0.0472 [µmol/mg]).

The coefficient 0.0472 = n represents the number of µmols of copper bound per mg of ceruloplasmin. This calculation assumes that the Cp measured is active (holoCp) and has 6 atoms of copper bound to it. It is derived as follows (Walshe, 2003):

$$Cp - Cu\left(\frac{\mu mol}{L}\right) = Cp\left(\frac{mg}{L}\right) * \frac{6\ atoms\ of\ Cu}{molar\ mass\ Cp} * 1000$$

$$= 0.0472 * Cp\left(\frac{mg}{L}\right)$$

However, rounding of the ratio of the molar mass of copper to that of ceruloplasmin from 0.289% to 0.3% leads to the coefficient n = 0.0472, while with no rounding off n = 0.0454. The commonly used n = 0.0472 is used here.

Thus, for a patient with a serum copper concentration of 17.3 µmol/L and a serum Cp concentration of 330 mg/L, Cp-Cu concentration is 330*0.0472 = 15.6 µmol/L, and the free copper concentration = 17.3-15.6 = 1.7 µmol/L.

This estimation is an indirect quantification, which assumes that all Cp is active, and all Cp molecules have 6 atoms of copper bound to them. Calculation of free copper in the same serum sample can be underestimated when an immunological method is employed to determine Cp concentration rather than the enzymatic method. This underestimation occurs because the immunological determination of Cp relies on the use of antibodies, which may detect both

functional, Cu-bound holo-Cp, non-functional apo-Cp, and Cp fragments thus, overestimating the quantity of copper bound to ceruloplasmin (Walshe, 2003) and hence, resulting in an underestimation of free copper. It has been observed that patients with high levels of Cp often result in negative calculated free copper levels (EASL, 2012) indicating that in these cases, there is a relatively high ratio of non-functional or apo-Cp compared to functional holo-Cp. It is noted that Cp is an acute phase reactant.

Nevertheless, as commercial Cp quantification widely available to clinicians uses the immunologic method, this method was included and used to calculate estimated free Cu. Serum samples were sent to a Life Labs, a commercial lab based in Toronto, for immunological determination of Cp content and AAS determination of total serum Cu content. The reported values were used to estimate free copper concentration using the above calculation. Cp bound Cu content was also estimated using values of ceruloplasmin activity determined enzymatically (described below). The estimated value Cp bound Cu was subtracted from the total Cu value obtained from ICPMS analysis to give a more accurate free Cu estimate.

3.3.3 Cp activity Assay

Ceruloplasmin activity was determined using an in-house colorimetric activity assay developed by (Schosinsky et al., 1974). Reagents were prepared as per the authors' instructions. Briefly, two reaction mixes were prepared per patient, each containing 50 µL of serum sample added to 750 µL of acetate buffer pH5 and regulated to 30°C. To each reaction mix, 200 µL of 7.88 mmol/L O-dianisidine dihydrochloride was added to create a stable brown product. For each participant sample, one reaction was reacted for 5 min and another at 15 min. After the allocated time had passed, 2 mL of 9M sulfuric acid was added to stop the reaction, resulting in a purple-pink solution. Optical density (OD) was measured using a Beckman Coulter DU 800 spectrophotometer at 540 nm in 3.4 ml quartz cuvettes (StonyLab, SKU XL-815301). The obtained OD from the 5min reaction was subtracted from that at 15 min, and multiplied by a constant accounting for the concentration of substrate oxidized to obtain the activity of ceruloplasmin in the sample, reported as enzymatic U/L.

3.4 Genetic Analyses

3.4.1 PAXgene RNA extraction

RNA was extracted from PAXgene tubes using the PAXgene Blood RNA Kit (Qiagen catalog no. 762164) as per kit instructions. The following modifications were included (i) Since storage at -80^0C can cause the PAXgene tubes to crack, tubes were placed in 50 mL conicals while thawing to avert sample loss. Samples from cracked tubes were processed in the 50mL for the first few steps. (ii) Microcentrifuge tubes containing proteinase K digestion mix were placed in falcon tubes and taped to remain stable in the shaker-incubator set to 400 rpm. (iii) Elution buffer was left to sit on the membrane at RT for 3min before elution to increase sample yield. (iv) The volume of the second elute was brought down to 30 µL to increase RNA concentration per microliter.

3.4.2 PBMC DNA and RNA extraction

DNA and RNA were extracted from PBMCs using a Qiagen Allprep DNA/RNA Mini Kit (ref 80204) following manufacturer's instructions. The following modifications were included: (i) for RNA extraction, two elutions were performed, the first with a volume of 40 µL and the second 30uL. (ii) For DNA extraction, a dry spin step was added before elution; samples were spun at 10,000 rpm for 1 min and 2 elutions were performed both at a volume of 80 µL.

3.4.3 Nanostring Analyses

Sixteen genes were selected for gene expression analyses. These genes are either related to sporadic ALS (SOD1, C9orf72, TARDBP), involved in Cu homeostasis mechanisms (ATP7A, ATP7B, COMMD1, ATOX1, CCS, CTR1, COX17, MT2A) or involved in antioxidant defense (GSTT1, GSTM1, GSTP1, CAT, HMOX1). Housekeeping genes HPRT1, GAPDH, ACTB, and RPLP0 were included as reference genes for data normalization.

RNA was quantified using the Qubit ™ fluorimeter and the RNA HS Assay Kit (Invitrogen) according to the manufacturers' specifications and stored at -80°C. Gene expression was carried out with the digital hybridization NanoString platform (Seattle, WA, USA). A customized panel including the 16 target genes and 4 housekeeping genes was chosen for the analysis. The NanoString assay was run according to the manufacturer's specifications. In brief, reporter probes

in hybridization buffer were added to 100 ng of RNA and capture probes were added prior to hybridization (65°C for 20 hours). Following hybridization, the samples were loaded to the PrepStation where they were washed with a two-step magnetic bead-purification process and loaded into 12 lane cartridges. Cartridges were next processed using the Digital Analyzer (DA, nCounter, NanoString Technologies Inc., Seattle, WA, USA), and 555 fields of view (FOV) were counted per sample. The NSolver analysis software v4.0 was used for QC and quantitative analysis.

4. Results

4.1 Participant Recruitment

4.1.1 Demographics

A total of 35 ALS patients and 21 controls have been recruited as of May 2022. The patient cohort is composed mostly of males (60%) while the control cohort is composed mostly of females (70%). Amongst patients, females are diagnosed at older ages than males (4 years on average), recruited at an older age on average (5 years older than males) and present with lower ALSFRS scores (around 7 points lower than males) indicating further progression of muscular atrophy at recruitment. Participant demographics are summarized in Figure 6.

A.	**Participant demographics**			
	ALS patients		Cohabiting Controls	
	Male	Female	Male	Female
Cohort composition (%)	60	40	30	70
Age	61.9 ± 9.0	66 ± 9.5	65.7 ± 5.6	60.1 ± 8.5
ALSFRS at recruitment	37.5 ± 5.4	30.9 ± 7.5	N/A	N/A
Age of diagnosis	60.1 ± 9.1	64.5 ± 10.1	N/A	N/A

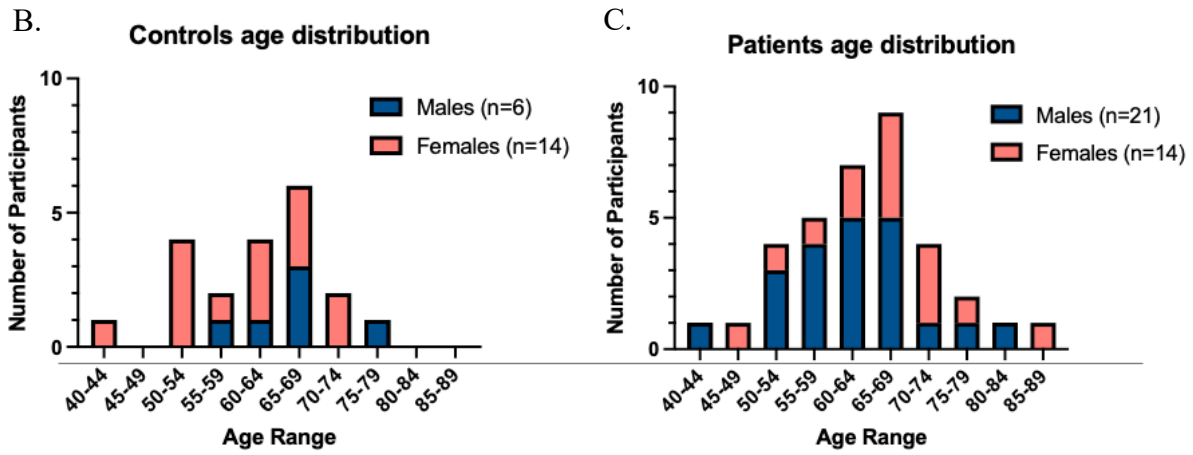


Figure 5. Participant Demographics

(A) Tabulated values include participant cohort composition by sex, age at recruitment (± SD), ALSFRS score and age of ALS diagnosis for patients (± SD) for n=35 patients and n=20 controls. Histograms depict age distribution for controls (B) and patients (C).

4.1.2 Questionnaire for Cu exposure and confounders

At recruitment, participants were administered an intake questionnaire (Appendix I) to assess Cu exposure and identify potential confounders. Table 3 summarizes the questionnaire criteria and distribution of responses in patients compared to controls.

Participants were assessed for family history of ALS. While no controls had a family history of ALS, 15% of patients did, higher than the worldwide average of 5-10% fALS cases (Wang et al., 2017). Participants were asked if they had type 1 diabetes, since this disease has been associated with higher levels of Total Cu and Cp (Squitti *et al.*, 2019). Only one patient out of all participants had this co-morbidity. A question on past physical traumas (such as motor vehicle accidents) was included, since old cerebrovascular injuries (>5 years before ALS diagnosis) are associated with ALS (Wang et al., 2017). Indeed, patients more often had a history of physical trauma compared to controls (37>15%).

Since smoking is a source of metal exposure, questions related to smoker status were posed. Amongst participants who had smoked at some point in their life, the majority (>80%) smoked tobacco, some (>25%) cannabis, and none had a history of vaping. Most participants had smoked in late adolescence to early adulthood, however the majority (>75%) had not smoked in the last 10

years. Fewer patients currently smoke (<5%) compared to controls (<15%). Questions related to Cu exposure from supplements, drinking water, diet, and past occupational exposure were also included. They showed no distinct differences between patients and controls. Usage of reverse osmosis water, tap flushing before use, and kettle filling with hot water were included since these criteria may affect Cu concentrations in drinking water in homes with Cu piping. Cu exposure from ingestion of potable water did not markedly differ between groups. It was found however that more ALS patients (<10%) had a history of occupational Cu exposure compared to controls (≥5%).

Table 3. Distribution of participant responses to questionnaire

Questionnaire criteria and response by in patients and controls. Values are reported in % of individuals from participant subtype who answer "yes" or fill criteria. Missing data points were excluded. Percentages were rounded to the nearest integers.

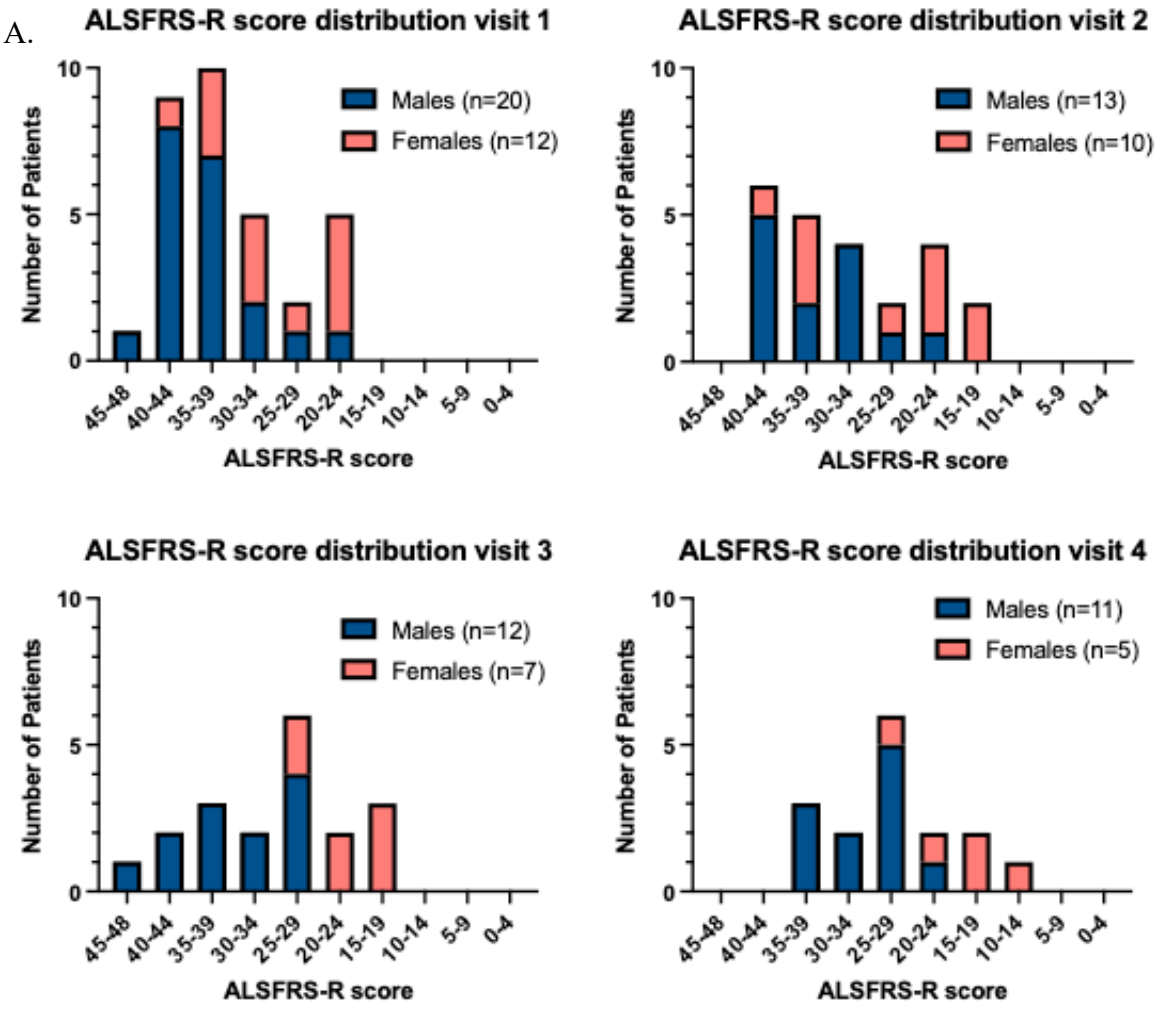
Questionnaire Criteria	**Patients (%) (n=35)**	**Controls (%)** (n=21)
ALS family History	15	0
Have Type 1 Diabetes	3	0
History of Physical Trauma	37	15
Have ever smoked	71	70
Of those who have ever smoked:		
Smoked Tobacco	88	86
Smoked Cannabis	28	36
Vaped	0	0
Age started smoking (± SD)	17.4 ± 3.0	17.6 ± 5.4
Within the last 10 years	16	21
Currently smoke	4	14
Take Copper supplements		
Daily	6	6
Occasionally	6	0
Never	88	94

Drink Bottled Water	31	30
Drink Tap Water	86	85
Municipal water source at home	83	85
Reverse osmosis water source	3	0
Fill kettle with hot water before use	6	10
Flush tap before water use	71	90
Wash Fruit before eating	86	95
Plant Consumption in diet		
Completely plant-based	0	0
Mostly plant-based	23	25
Half plant-based	40	45
Few/no plants	37	30
Meat Consumption in Diet		
Never	0	0
<Once a week	0	0
1-3 times a week	31	40
3-4 times a week	51	45
Daily	17	15
Eat liver or lobster weekly	0	0
Has worked with pesticides	11	0
Has worked with heavy metals	14	5
Has worked with copper	17	5

4.2 Disease progression in patients

At every visit, patients were administered a clinical questionnaire (Appendix II) to determine the extent of disease progression following the ALSFRS-R. Overall, results indicate a decrease in scores and worsening of symptoms with time. Female patients began with worse scores and progressed further than males on average. Results are summarized in Figure 7.

A.



B.

ALSFRS score progression								
	Visit 1		Visit 2		Visit 3		Visit 4	
Male	N=20	37.5 ± 5.4	N=13	35.9 ± 5.4	N=12	35 ± 6.4	N=11	30.5 ± 5.2
Female	N=12	30.9 ± 7.5	N=10	27.1 ± 8.4	N=7	22.3 ± 4.5	N=5	19.6 ± 5.2
All	N=22	35 ± 7.0	N=23	32.1 ± 8.5	N=19	30.3 ± 8.5	N=16	27.1 ± 7.3

C.

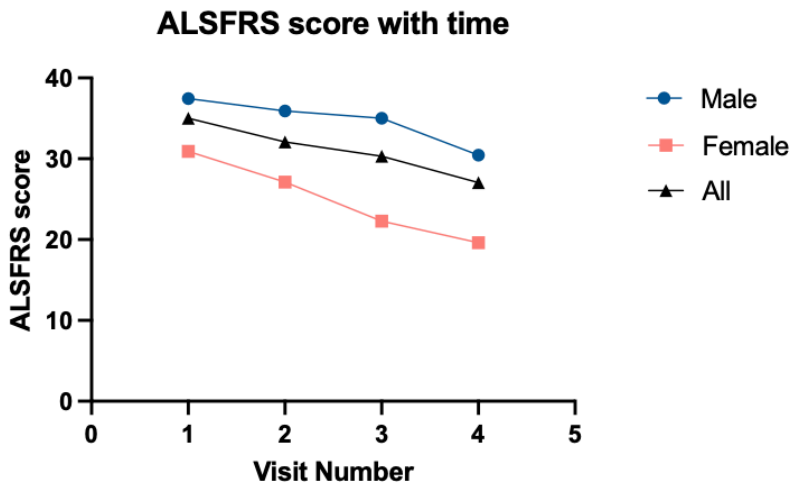


Figure 6. Disease progression in patients

(A) Progression of ALS in patients by age group and sex using the ALSFRS-R. Average scores are tabulated by sex and visit (B) and graphed with time (C). Female scores were lower on average throughout visits.

4.3 ICPMS metal concentrations

4.3.1 Metal concentrations in participant serum samples

Serum samples from participants were analyzed for metal content using ICPMS at the University of Montreal (UdeM). Average metal concentrations and distribution of concentrations by sex for Cu, Mn, Pb, As, Zn, and Cd are reported in Figure 8. Overall, average concentrations in all metals, apart from As, fell within normal ranges for controls and patients. No significant differences were observed between sexes. However, for all metals, there were a few participants with concentrations far above the normal range. Notably, average As concentrations for both controls and patients (visits 1-3) were higher than reference value levels. Overall, no statistically significant differences were observed between controls and patients across visits.

A.	**Average Metal Concentrations in serum (ICPMS)**
	Patient Average

Element	Reference Value	Control average	Visit 1	Visit 2	Visit 3	Visit 4
Cu	11-23umol/L	20.9	18.6	18.5	19.7	21.3
Mn	9.1-21.8ug/L	9.13	13.04	10.5	8.07	12.1
Pb	<50ug/L	27.8	33.9	23	29.9	53
As	<13ug/L	14.1	11.8	15.6	16.4	10.3
Zn	660-1100ug/L	812	858	878	920	987
Cd	<5ug/L	1.59	0.925	1.65	0.9	1.5

B.

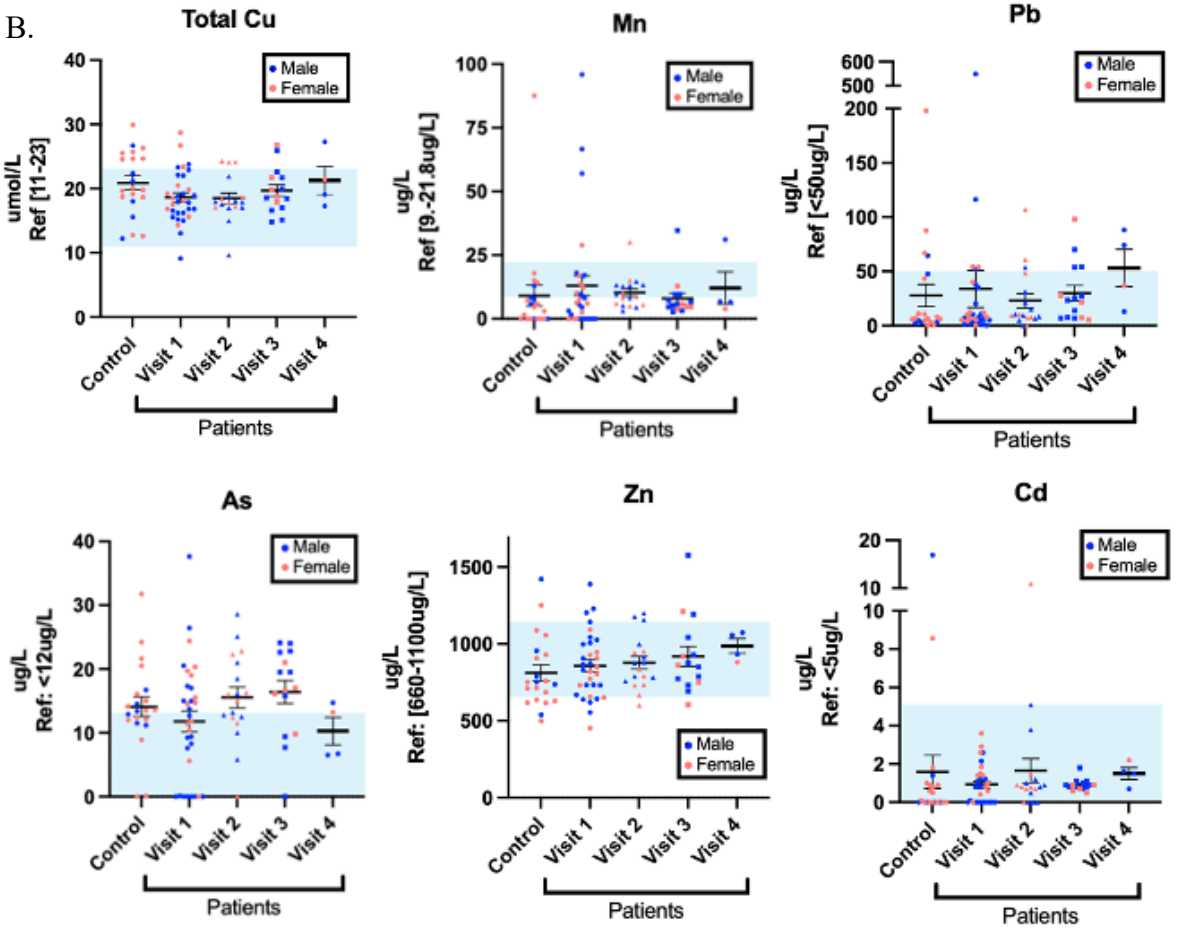


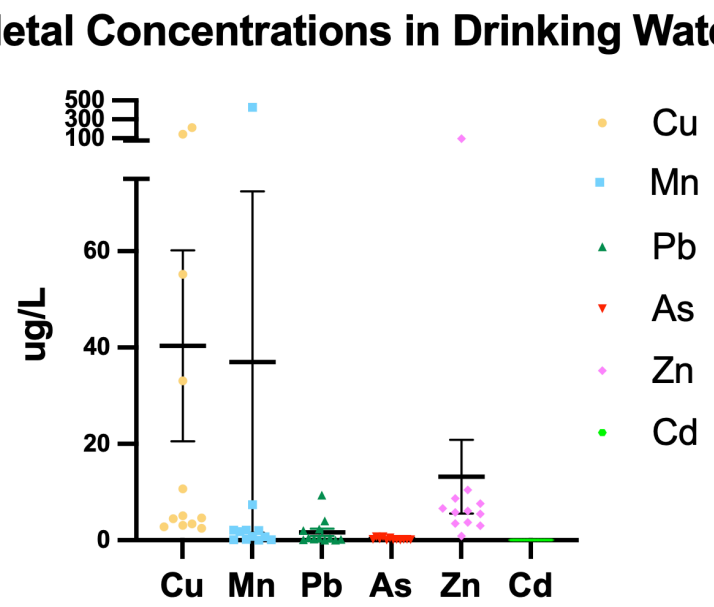
Figure 7. Metal concentrations in participant serum samples

(A) Tabulated metal concentrations are compared between controls and patients across their 4 visits. Reference values were obtained from Mayo Clinic Laboratories online (MayoClinicLabs, 2022) indicating normal ranges for essential metals Cu, Mn, and Zn and levels at which toxicity is observed for nonessential metals Pb, As, and Cd. Datapoints are graphed in (B.) Averages ± SEM are indicated for each metal. Values are reported for n= 21 controls, and n=32, n= 18, n=15, and n=4 patients for visits 1-4 respectively. Reference ranges are indicated in light blue shaded boxes.

4.3.2 Metal concentrations in drinking water

Patient drinking water was analysed by ICPMS to determine if drinking water was a source of environmental metal exposure and if it correlated with serum metal concentrations (Figure 9). Data obtained thus far did not indicate significant contribution of drinking water to metal exposure. All average metal concentrations, apart from Pb, were relatively low compared to Canadian drinking water guidelines (HealthCanada, 2020). Only two patients had water metal levels surpassing Canadian guidelines, one with high levels of Pb, and another with high levels of Mn.

A.



B. Element	Canadian Drinking water guidelines (mg/L or ppm)	Canadian Drinking water guidelines (ug/L)	Average	Median
Cu	2	2000	40.4	4.6
Mn	0.12	120	37	1.9
Pb	0.005	5	1.64	0.36

As	0.01	10	0.262	0.19
Zn	5	5000	13.2	5.8
Cd	0.007	7	0.003	0

Figure 8. Metal concentrations in drinking water

(A) Averages ± SEM for metal concentrations in drinking water obtained from patients' homes are analyzed and plotted for n=12 patients. (B) Averages are tabulated and compared to Canadian Drinking water guidelines.

4.4 Cu content analyses

Total serum Cu was determined using two methods. The first by ICPMS was conducted at UdeM, and the second by AAS was conducted at the commercial lab Lifelabs. The averages for both controls and patients remained in the normal range. However, UdeM values were consistently approximately 10% higher than Lifelabs values for the same samples. Both methods showed similar trends with controls exhibiting slightly, but not significantly, higher serum Cu concentrations than patients. Patient serum concentrations fluctuated within the normal range across visits and appeared to increase more with time in UdeM values compared to Lifelabs. CuEXC was measured only by ICPMS. Both patients and controls had high average levels of CuEXC compared to reference values (Poujois et al., 2017; Squitti et al., 2006b; Squitti et al., 2019; Squitti et al., 2005; Squitti *et al.*, 2021; Woimant *et al.*, 2019) with controls slightly higher on average than patients at the first visit. Patient CuEXC increased with time, particularly at the third and fourth visits (albeit there are only 4 data points for the fourth visit). Despite the evident increase in CuEXC, no statistically significant results were found after conducting an ordinary one-way ANOVA with Tukey's test for normally distributed data (tested with the Shaprio-Wilk test for normal distribution) or using the Kruskal-Wallis test for non-normally distributed data. No sex differences were observed for Total Cu or CuEXC levels. Data are graphed in Figure 10.

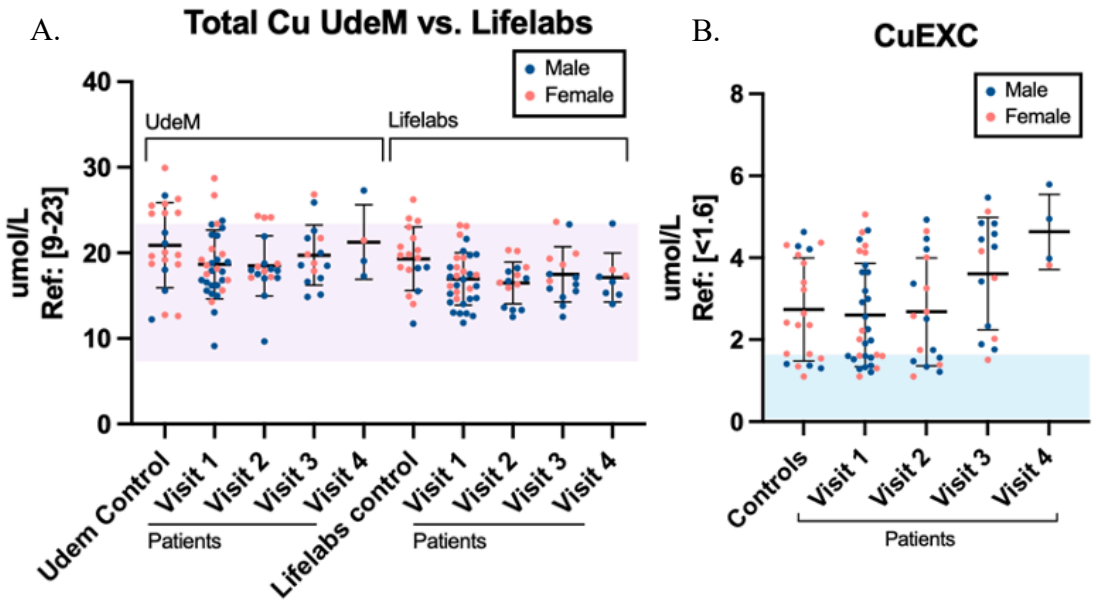


Figure 9. Total Cu and CuEXC

(A) Comparison of serum Total Cu values determined through ICPMS (UdeM) and AAS (Lifelabs). (B) Comparison of CuEXC values. Lightly shaded pink and blue boxes indicate reference ranges. Data plotted indicates averages ± SD. Data sets for UdeM Total Cu and CuEXC represent N=20 controls, and N=31, N=14, N=15, and N=4 patients for visits 1-4 respectively. Lifelabs data sets represent N=18 controls, and N=32, 15, 14, 8 for patients visits 1-4 respectively.

4.4.1 Ceruloplasmin values

Cp concentrations were determined immunologically by Lifelabs. Averages were found to lie in the normal range for both controls and patients across all 4 visits. Patients presented with lower Cp concentrations than controls (by about 10%), which only mildly fluctuated across the 4 visits. Similarly, Cp oxidase activity, determined using an in-house colorimetric assay, was found to be decreased in patients compared to controls (by about 15%) with some fluctuation across the 4 timepoints. All Cp data was found to be normally distributed following the Shapiro-Wilk test, but no decreases were found to be significant following a one-way ANOVA with Tukey's test. Data are plotted in Figure 11. No sex differences were observed.

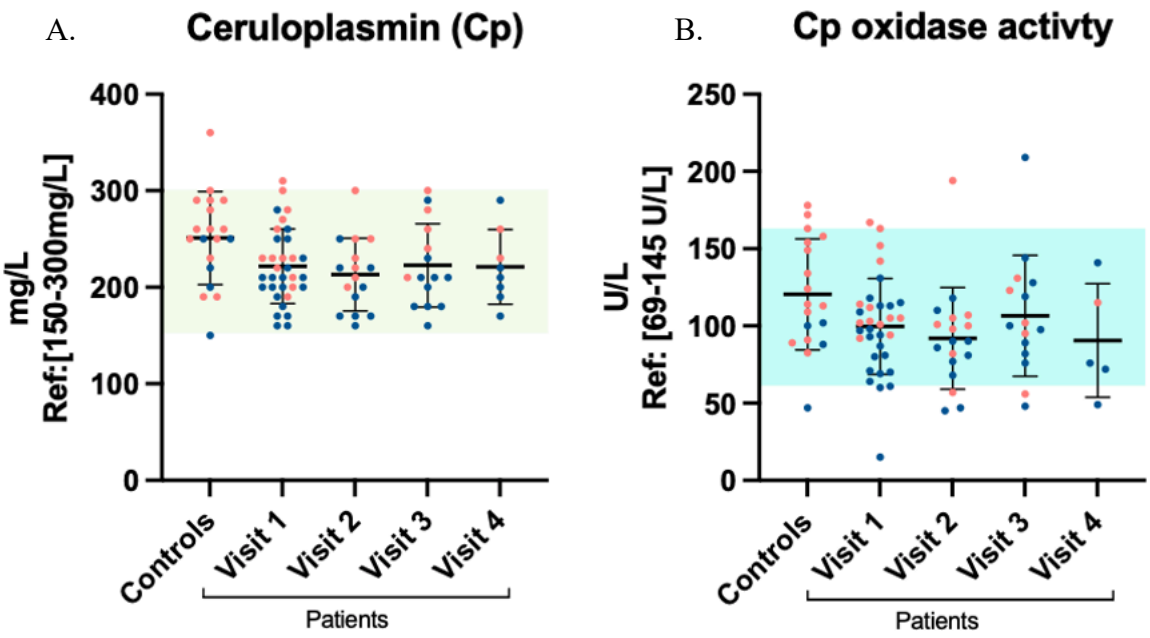


Figure 10. Ceruloplasmin (Cp) measurements

Averages ± SD for Cp concentrations and activity. For Cp concentrations: Controls N=19, Patients N=33, 22, 15 and 8 for visits 1-4 respectively. For Cp oxidase activity: Controls N=34, Patients N=32, 18, 15, and 5 for visits 1-4 respectively. Lightly colored green and blue boxes indicate reference ranges obtained from Lifelabs (A) and (Schosinsky et al., 1974)(B). Male and female participants are indicated with blue and pink data points respectively.

4.4.2 Calculated values: NCC, Proportion of NCC to total serum copper and REC

Non-ceruloplasmin bound copper (NCC) is an indirect calculation used to estimate free copper in the blood concentrations in blood. By subtracting the amount of copper calculated to be bound to Cp (using immunologically determined Cp values), NCC is obtained. Recall that this results in an underestimation of the true NCC, since this method assumes that all immunologically measured Cp, measuring both holo- and apo-Cp, bind coppers, when in fact only holo-Cp is only bound to Cu. NCC values for both controls and patients are exceedingly high (6-8x reference levels), with control levels significantly higher than patients only at visit 1. Patient levels do not appear to change markedly with time. It can be inferred that the true NCC is higher for patients than for controls as the Cp activity value, which measure the holo-ceruloplasmin, is lower for patients than for controls (by about 15%). When NCC is divided by Total Cu (also determined by Lifelabs), the proportion of NCC that makes up Total Cu in serum is obtained and is about 40% for both controls and patients, with no significant changes in patients over time.

Relative exchangeable copper (REC) is determined by dividing the amount of directly measured CuEXC by the amount of Total Cu (measured by ICPMS). In contrast to the NCC proportion observed in participants, REC values lie within the normal range for controls and initially for participants and increases with time for patients, with average for the patients' 4th timepoint outside normal values. Although these results are preliminary and need to be supported with more data points, there is indication that increasing CuEXC levels across timepoints make up a significant portion of Total Cu in patients, with REC values significantly increasing at visits 3 and 4.

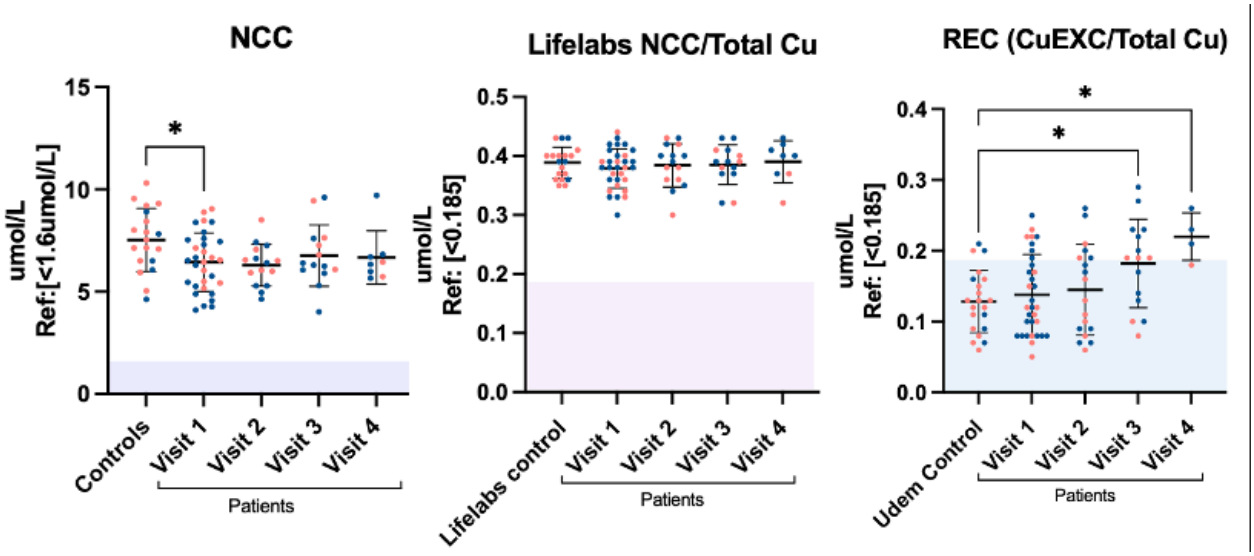


Figure 11. NCC, Proportion of NCC, and REC calculations

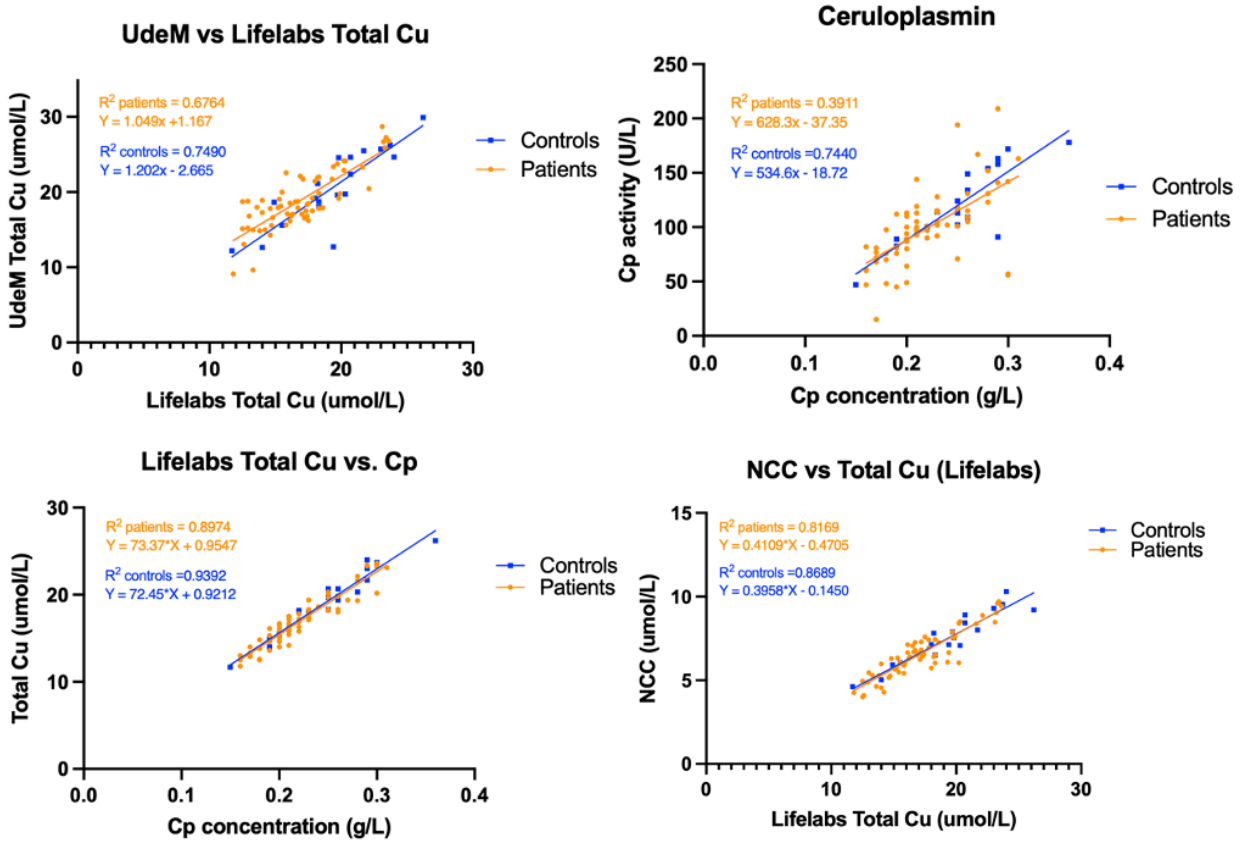
NCC, proportion of NCC: Total Cu, and REC averages ± SD are plotted with controls N=19, and patients n=31, 15, 14 and 8 for patients visits 1-4 respectively for Lifelabs values and n=21 for controls, n=33, 18, 15, and 4 for patients through visits 1-4 respectively for REC values. Males are indicated in blue points, females in pink, and reference values in lightly shaded boxes. A one-way ANOVA with Tukey's test was used for normally distributed data (tested with the Shaprio-Wilk test for normal distribution) and the Kruskal-Wallis test was used for non-normally distributed data, with * indicating $p \leq 0.05$.

4.4.2 Correlations and trends

Cu status markers

Correlative analyses were conducted between different Cu status markers for controls and for patients over all visits. Total Cu measured by ICPMS (UdeM) and AAS (Lifelabs) correlated more strongly in controls compared to patients ($R^2 = 0.749 > 0.676$). Similarly, stronger correlations

were observed in controls for Cp concentration and activity (R^2 = 0.7440>0.3911), Total Cu and Cp (R^2 = 0.992>0.874), and NCC and Total Cu (R^2 = 0.8689>0.8169) when using Lifelabs Total Cu values. These stronger correlations indicate less disruption of the ceruloplasmin in controls in terms of a more stable value of the ratio of holo- to apo- ceruploplasmin. Total Cu measured by ICPMS correlated more strongly with Cp activity in controls compared to patients (R^2 = 0.6099>0.3109).



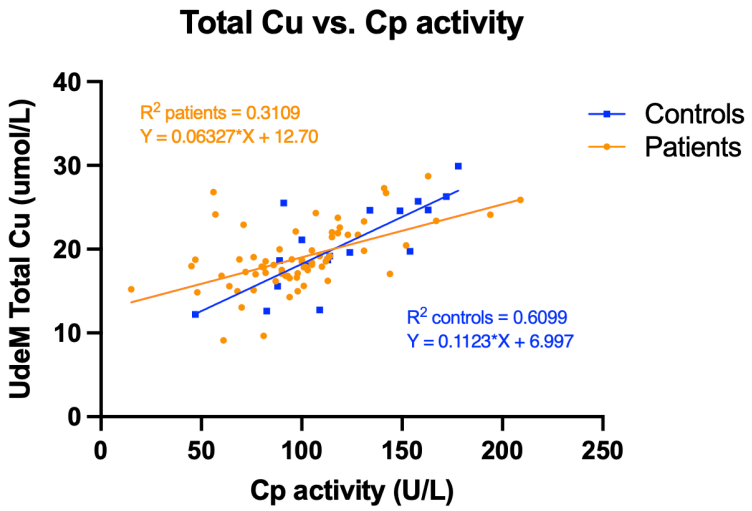


Figure 12. Correlations between Cu status markers.

Datapoints from all patient visits were included. No sex specific differences were observed.

CuEXC correlations

CuEXC did not correlate strongly with any other Cu status marker. The strongest correlation observed was with Total Cu measured by ICPMS and was stronger in controls compared with patients (R^2 = 0.5642>0.3068). Correlations are shown in Figure 14. Figure 15.

Cu markers and disease severity

No Cu markers correlate with ALSFRS score. The only parameter that was found to weakly correlate with ALSFRS score was age (R^2 = 0.0995) (Figure 15). When NCC and CuEXC were graphed for patients across visits (Figure 16.), it was found that NCC did not show any particular trend with most values fluctuating around initial visit concentration, some increasing, and some decreasing. CuEXC did show a net increase across time particularly after the 3rd patient visit, however, a few patients did show a decrease in CuEXC, while others fluctuated around the same concentration as their 1st visit. More patient datasets are needed to draw any further conclusions.

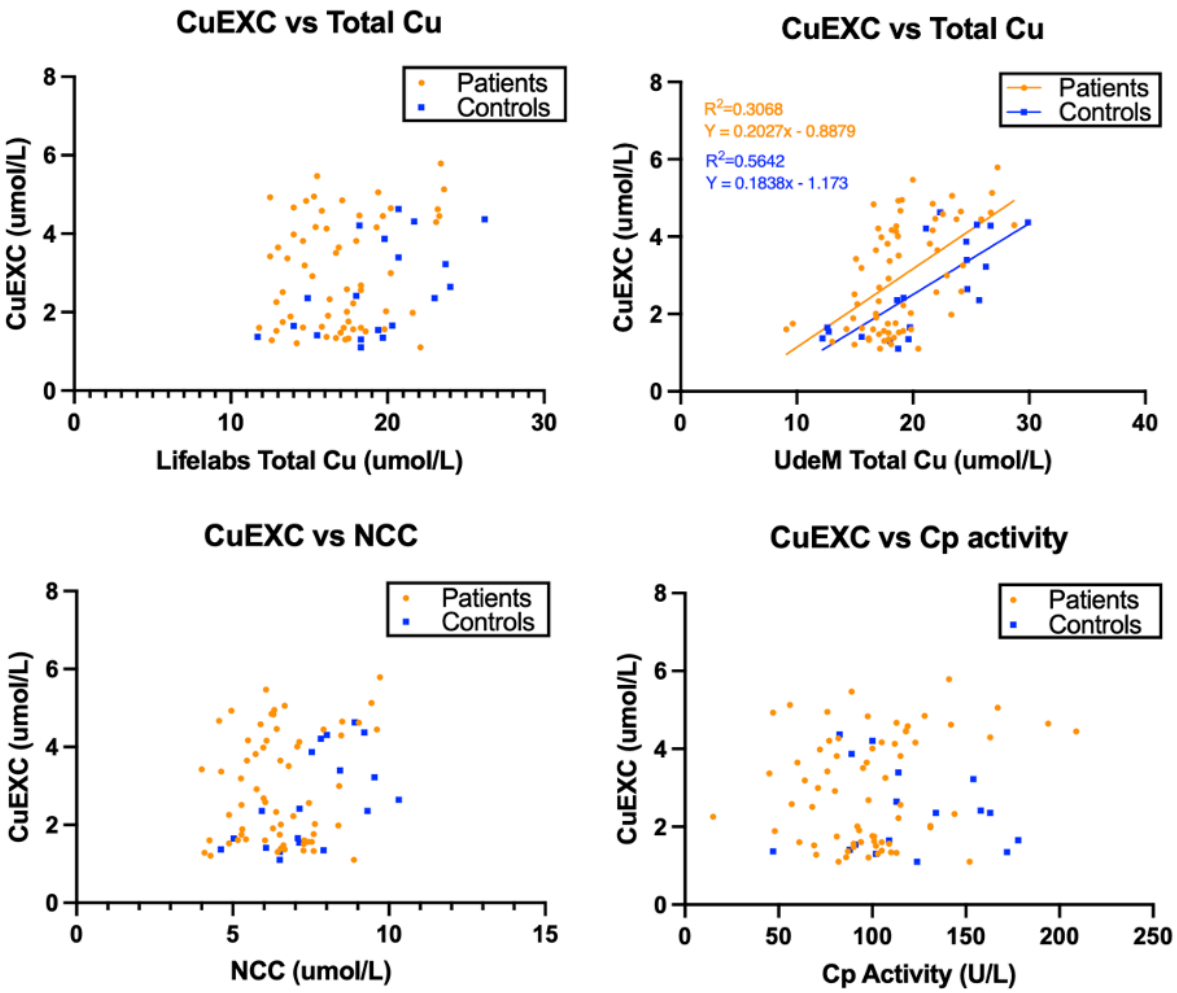


Figure 13. CuEXC correlations

CuEXC correlated with Total Cu measured by AAS or ICPMS, NCC, and Cp activity. Datapoints from all patient visits were included. No sex specific differences were observed.

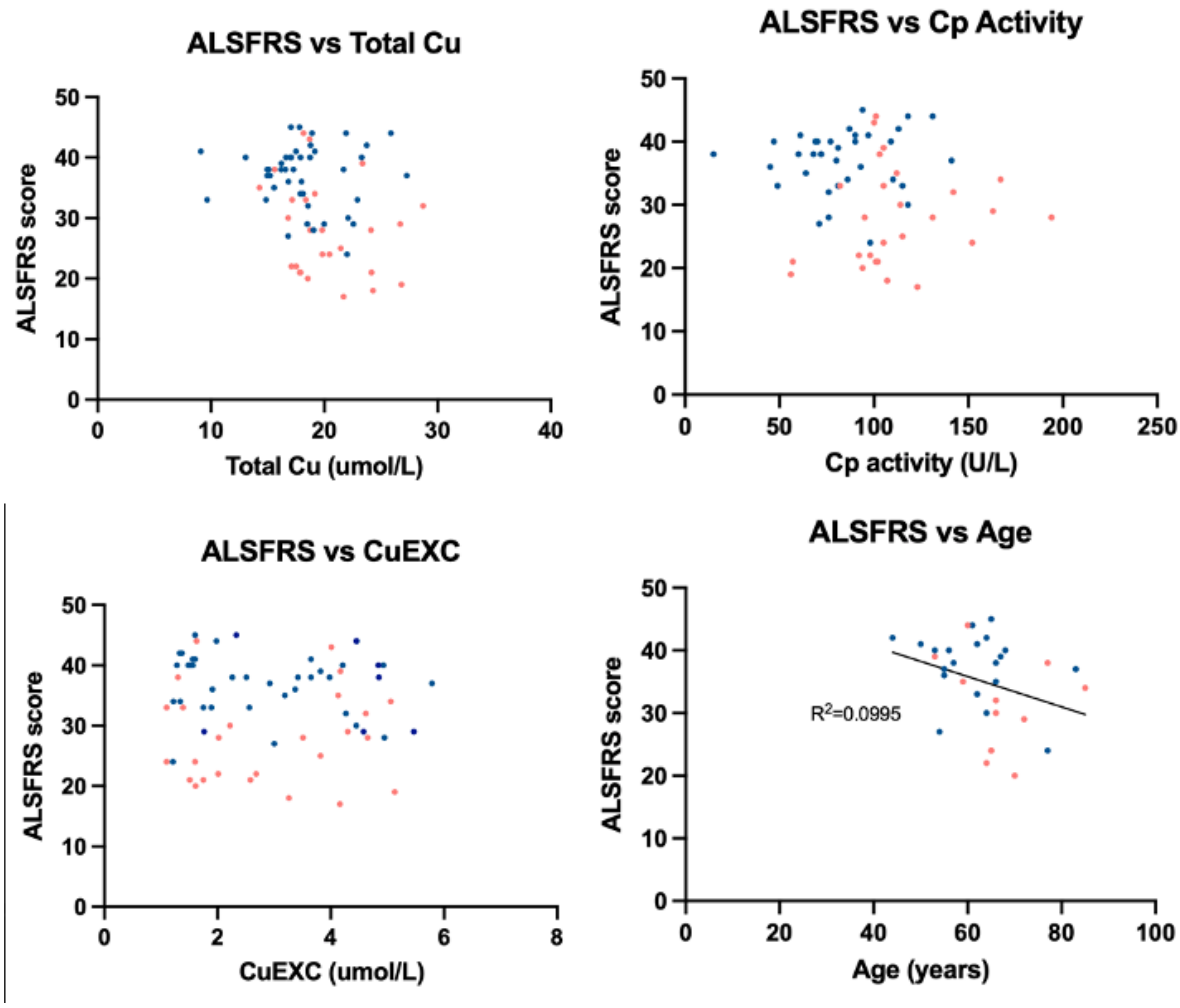


Figure 14. ALSFRS correlations

ALSFRS does not correlate with Cu markers of Total Cu (ICPMS), CuEXC, or Cp activity and weakly correlates with age. Datapoints in pink represent female patients and datapoints in blue represent males. Data from all visits were included for Cu markers. Only data from the first patient visit was included for age.

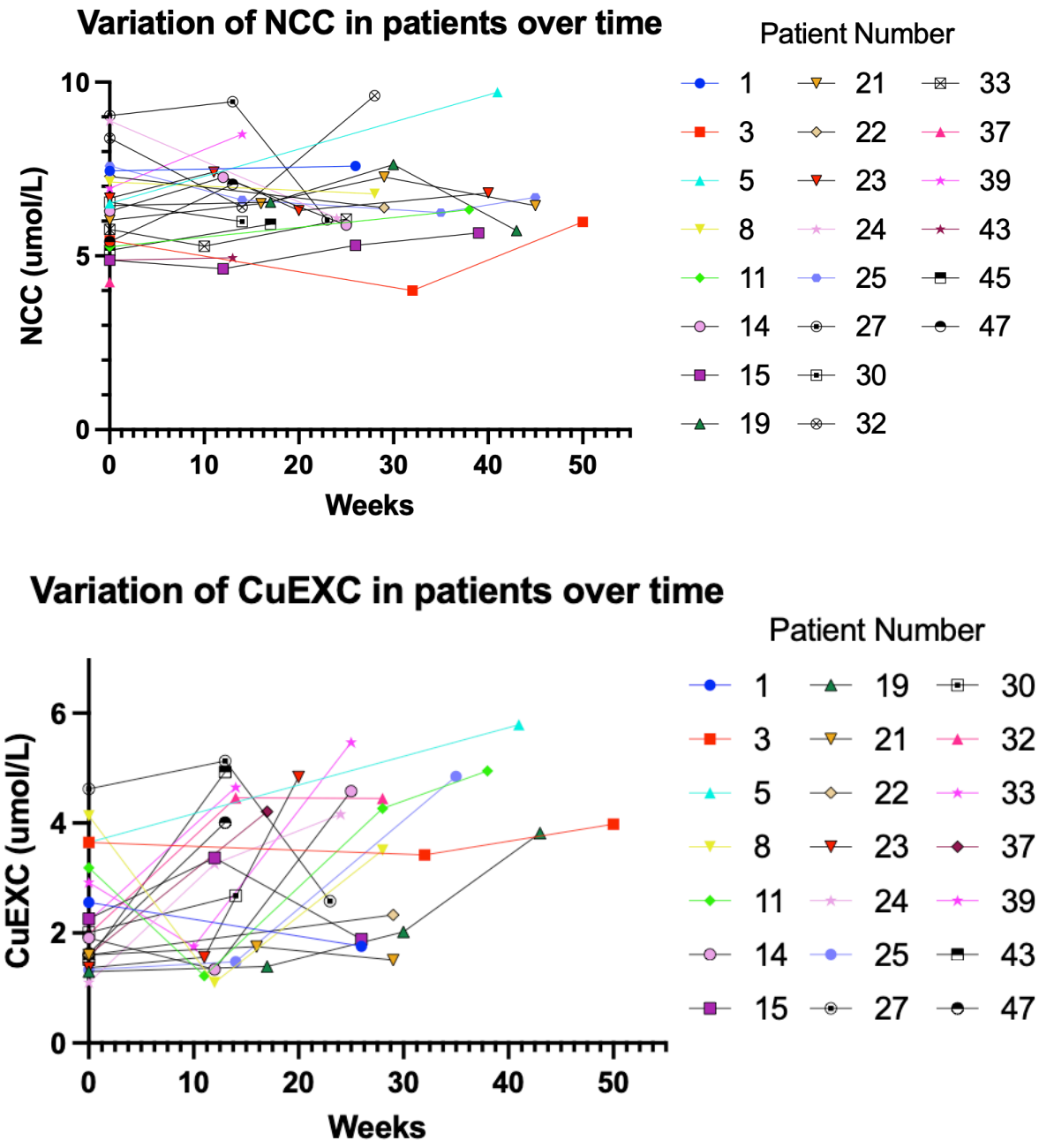


Figure 15. Variations in NCC, and CuEXC with time

Variation on NCC and CuEXC in patients over time. Datapoints indicate patient visits with time.

4.5 Nanostring results

Gene expression levels were determined using Nanostring technology, where molecules of RNA for each target gene were quantified using molecular barcodes. The results in this section represent the number of RNA molecule counts per participant blood sample for each target gene and are normalized to housekeeping genes GAPDH and ACTB (β- actin). HPRT1 and RPLP0 were not used for normalization due to their high coefficients of variations (%CV> 20% difference from GAPDH and ACTB) across different runs.

4.5.1 Cu metabolism genes

ATP7B levels were at background gene expression levels (10-40 RNA counts) and were not included. This is likely due to the fact that ATP7B is preferentially expressed in the liver. ATP7A levels were slightly lower in patients, but not significantly so, and did not show changes across patient visits. ATOX1, the Cu chaperone for ATP7A, also showed initially lower expression levels in patients compared to controls, but also showed further significant decreases observed at visits 2 and 3. Interestingly, MT2A, the form of metallothionein most expressed in human blood in the family of MT genes, also showed decreasing expression levels in patients compared to controls that decreased more across patient visits, with significantly decreased levels observed at visit 3. CTR1 and Cu chaperone protein CCS exhibited gene expression levels slightly but not significantly lower than controls with mild fluctuations across visits. Cu chaperones COMMD1 and COX17 showed overall decreased levels in patients compared to controls, with significant decreases observed at visit 3 for patients, and visit 2 for COX17. No particular sex differences were observed. Overall, there appears to be a disturbance in Cu metabolism in patients compared to controls, however, more datapoints are needed to confirm these findings.

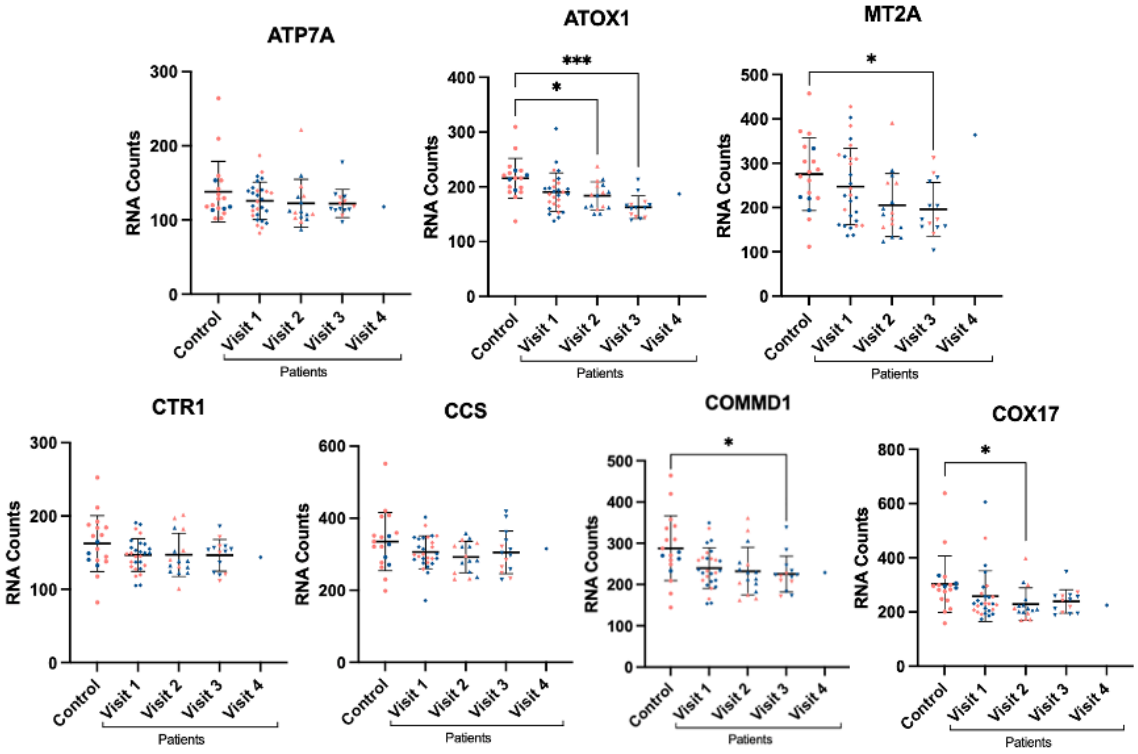


Figure 16. RNA counts for Cu metabolism genes

Average RNA counts + SD for Cu metabolism genes analyzed using Nanostring RNA expression quantification. Number of RNA barcode counts are plotted for n= 18 controls, n=28, 16, 14 and 1 for patients visits 1-4 respectively. Female participants are represented in pink, and males in blue. A one-way ANOVA with Tukey's test was used for normally distributed data (tested with the Shaprio-Wilk test for normal distribution) and the Kruskal-Wallis test was used for non-normally distributed data, with * indicating $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$.

4.5.2 ALS related genes

SOD1, C9orf72, and TARDBP show lower expression levels for patients compared to controls, with expression significantly decreasing at visit 2 for SOD1 and visits 2 and 3 for TARDBP. SOD1's function as an ROS scavenger and its decrease in expression in patients may be linked to oxidative stress in patients. Further genetic analyses are needed to see if gene polymorphisms or functional mutations are also seen in tandem with these changes in expression.

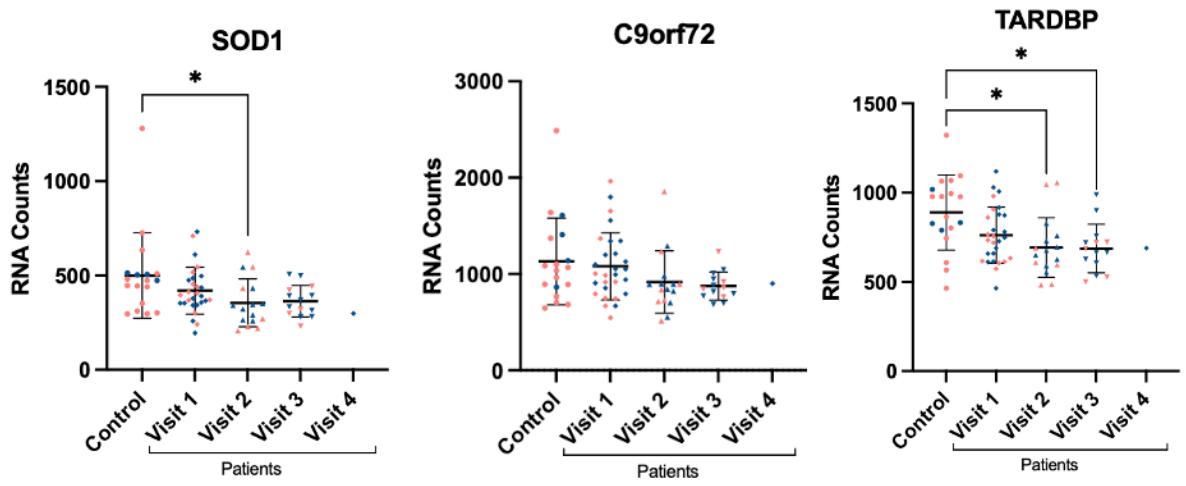


Figure 17. RNA counts for ALS related genes

Average RNA counts + SD for ALS related genes. Number of RNA barcode counts are plotted for n= 18 controls, n=28, 16, 14 and 1 for patients visits 1-4 respectively. Female participants are represented in pink, and males in blue. A one-way ANOVA with Tukey's test was used for normally distributed data (tested with the Shaprio-Wilk test for normal distribution) and the Kruskal-Wallis test was used for non-normally distributed data, with * indicating $p \leq 0.05$

4.5.3 Antioxidant status genes

Decreases in expression of antioxidant status genes are observed for all genes tested on average, but only significantly for GSTP1. HMOX1, GSTT1, and GSTM1 proteins show decreases in patient counts compared to controls from the first visit onwards, and CAT levels show a decrease compared to controls only at visit 2. HMOX1 levels, although lower in patients compared to controls, do not change considerably across patient visits. CAT levels only drop in patients compared to controls at visit 2. GSTP1 is expressed significantly less in patients compared to controls across all visits. GSTM1 shows large variation in expression between participants compared to other genes, with many participants, both controls and patients, showing either background expression levels, or considerably higher expression levels.

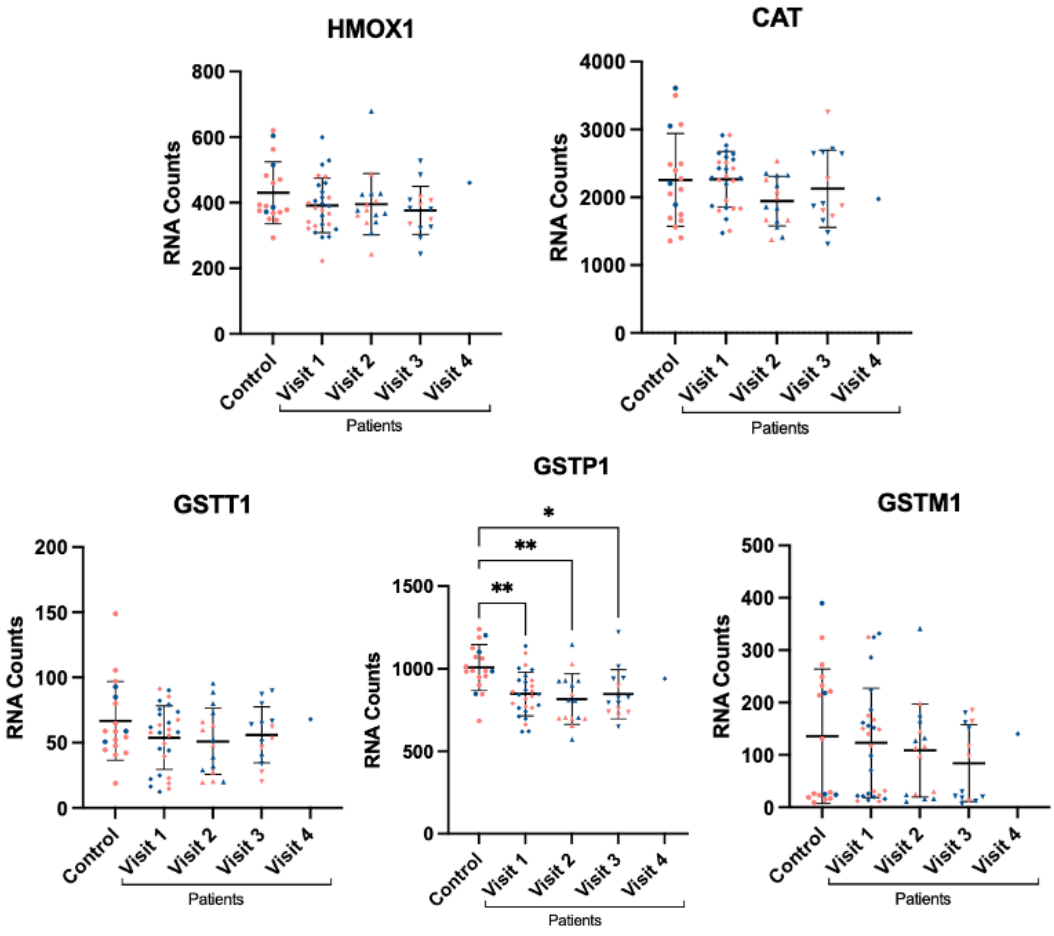


Figure 18. RNA counts for antioxidant status genes

Average RNA counts + SD for ALS related genes. Number of RNA barcode counts are plotted for n= 18 controls, n=28, 16, 14 and 1 for patients visits 1-4 respectively. Female participants are represented in pink, and males in blue. A one-way ANOVA with Tukey's test was used for normally distributed data (tested with the Shaprio-Wilk test for normal distribution) and the Kruskal-Wallis test was used for non-normally distributed data, with * indicating $p \leq 0.05$, and ** $p \leq 0.01$

5. Discussion

To examine the relationship between environmental copper and ALS, we collected serum samples from 35 ALS patients and 21 controls and analyzed them for different Cu biomarkers. Disease progression was monitored clinically using the ALSFRS score, and correlations were made with the different measured Cu status markers. Cu status genes, ALS genes, and antioxidant genes were also analyzed for gene expression. Overall, our findings point to the potential involvement of Cu in ALS, however, further participant recruitment, genetic tests, analyses, and verified CuEXC measurement methods are needed before conclusive remarks can be made.

5.1 Cu markers and ALS progression

Total Cu quantified by the two measurement methods of ICPMS and AAS correlated strongly (Figure 13) and was found to be 5-10% higher in controls compared to patients across visits 1-3 (Figure 10). The increasing CuEXC levels across patient visits (Figure 10), and decreased Cp activity compared to controls (Figure 11), may suggest the presence of Cu toxicity in patients. The active form of the ceruloplasmin enzyme is bound to 6 copper atoms, which it requires for its catalytic activity. Paradoxically, in cases of Cu toxicity such as in Wilson's Disease, low serum Cu is observed due to inadequate Cu loading of Cp, resulting in both low serum Cu and low Cp levels, as apo-ceruloplasmin has a low lifespan (EASL, 2012). Unlike WD copper toxicosis, the ALS patients do not show significantly low total serum Cu nor low Cp. However, the relatively new copper biomarker used for diagnosis and monitoring of WD, CuEXC and REC do show evidence of copper toxicosis as found in WD. CuEXC is higher in WD and ALS patients than in controls. REC, the relative amount of CuEXC compared to total Cu, is observed to increase in ALS patients to diagnostic levels of WD at more advanced stages of disease progression (Figure 12). Other studies are inconclusive as they only observed the level of total serum Cu relative to controls: they have been observed to be decreased in ALS patients compared to controls (Barros et al., 2018; Kapaki *et al.*, 1997), increased compared to controls (Forte *et al.*, 2017) or unchanged relative to controls (Peters *et al.*, 2016; Roos *et al.*, 2013; Tórsdóttir *et al.*, 2008).

Unlike our observations, Barros et al. observed an inverse relationship between patients' ALSFRS and serum copper levels, indicating increased Total Cu levels with disease severity, and positively

correlated Cp with ALSFRS, indicating decreased Cp levels with disease severity (Barros et al., 2018). However, Peters et al. found no association between ALSFRS-R scores and serum copper concentrations (Peters et al., 2016). Other fluids/tissues have been observed to show increased levels compared to controls, such as CSF (Hozumi *et al.*, 2008; Ihara *et al.*, 2005; Sauzéat *et al.*, 2018), and spinal cord tissues (Kurlander & Patten, 1979).

The observed increasing levels of CuEXC (Figure 10) across patient visits concur with our hypothesis that "true" non-ceruloplasmin bound copper increases in patients with disease progression. Since CuEXC is a process-based measure that represents the damage causing moiety of Cu in the blood (i.e. causes ROS), its elevation in blood would indicate Cu toxicity. These data are in alignment with evidence of elevated divalent copper (Cu^{2+}) levels in ALS patient CSF (Violi *et al.*, 2020). It is however important to note that our controls had levels that were similarly elevated compared to patients at their initial visits. This could be interpreted as the controls and ALS patients having the same environmental copper exposure, as most of the controls were co-habiting with a patient, but had a better ability to metabolize the excess copper or the ROS it generates. We would also have to assume in this case that these households have higher than average exposure to copper, and/or that the percentage of the population that cannot metabolize this level of excess copper is very small (in the order of the incidence of ALS in the population). Alternative explanations are that is due to an error in methodology, inadequacy of reference values for CuEXC (a possibility considering the limited studies in varying geographical populations conducted using this biomarker). CuEXC values only correlated weakly with Total Cu (Figure 13).

Our REC values parallel the increase in CuEXC across patient visits, i.e. over time (Figure 12), indicating a significantly increasing free Cu fraction within circulating Cu. REC values >0.185 are used to determine WD diagnosis and are indicative of high free copper (El Balkhi et al., 2011). Although patient averages remained <0.185 for visits 1-3, there was a clear increase across visits, with select patients in each visit exhibiting REC >0.185. All (n=4) patients had RECs > 0.185 at visit 4. Further participant recruitment and analysis of more samples is required to validate these findings, particularly since significant results are only observed at visits 3 and 4, which have the smallest sample size.

When CuEXC was plotted for each patient against time (Figure 16), an increasing trend was observed, in which several patients' CuEXC levels become much higher at visits 3 or 4. Although some patients did not show any change in levels, and others showed decreases, there was an overall increase in levels over time. However, despite the evident increases in CuEXC with time, there seemed to be no correlation between CuEXC levels and ALSFRS scores (Figure 15). In fact, none of the Cu markers examined showed any correlation with ALSFRS score. Even age, the strongest prognostic factor for ALS, correlated with ALSFRS with an R^2 value = 0.0995. Nonetheless, it is important to note these analyses are influenced by the large range of ALSFRS scores observed and low number of participants. Correlations may be found once a multiple regression analysis is done at the end of the study.

Measurements of Cp concentration and activity correlated relatively strongly with one another (Figure 11) and were both found to be reduced in patients compared to controls (Figure 11). These results indicate that less circulating Cu is bound to Cp in patients, an observation that is also seen under Cu toxicity in WD (EASL, 2012). However, it is important to note that when trying to estimate the amount of Cu bound to Cp, Cp activity assays are far better indicators of Cu-bound status (Walshe, 2003). Cp activity, measuring bound Cu used as an enzymatic cofactor for facilitating oxidative reactions, is indicative of the degree of Cu-binding to Cp. By contrast, Cp concentrations incorrectly assumes that all Cp detected immunologically, that is both holo- and apo-Cp, bind copper, while apo-Cp has no Cu bound to it. Our results of decreased Cp activity and Cp concentrations in patients compared with controls are similar to those observed by others (Domzal & Radzikowska, 1983; Tórsdóttir et al., 2008).

The calculated NCC values (total serum Cu – Cu bound to Cp) did not show any trends or significant changes across patient visits (Figure 12). Although NCC and CuEXC both aim to measure the same fraction of Cu in blood, non-ceruloplasmin bound copper (or labile/free copper), they show vastly different average measurements (Figure 10 and Figure 12). Discrepancies between measurements were expected given that NCC is known to underestimate free copper (EASL, 2012; Walshe, 2003) as it uses the Cp concentration (which includes apo-Cp) in the calculation. However, the remarkably high NCC values observed were surprising. Since NCC is calculated using measurements from immunologically determined Cp concentrations, which may

overestimate Cp concentrations, it is common for NCC to underestimate free copper measurements. In our analyses, NCC levels far exceed normal values for both controls and patients throughout the study. When individual patient values were plotted against time, no trends were observed (Figure 16). High NCC values such as those observed could be taken to indicate Cu toxicity, and toxicity seems likely as it underestimates free copper levels (over-estimation of levels is not possible). Alternatively, there could be measurement error.

We are not able to demonstrate with our results that any of the Cu biomarkers can identify early stages of ALS, however, this is tempered by the observation that our controls were subject to similar levels of Cu exposure. Nonetheless, our SOP demonstrates that CuEXC is a Cu status marker that can be implemented in a clinical setting. Additionally, our Cp activity assay (Schosinsky et al., 1974) can be performed in a standard lab setting with minimal materials and reagents required. An increase in usage of these markers for clinical studies would be useful in better assessing Cu status.

The utility of CuEXC in ALS monitoring is yet to be fully elucidated. Although the correlation between patient visits and increasing CuEXC and REC suggest a causative role of Cu in ALS as CuEXC is higher than in other control groups (Poujois et al., 2017, Squitti et al., 2018), CuEXC may simply be a biomarker of decline, concurrently acting with/resultant of other unknown causative agents. Once a full patient cohort is recruited and a full dataset is obtained, analyses may be conducted to determine the relationship between change in CuEXC and ALSFRS score decline (rate of deterioration). Our current data indicates an increase of CuEXC and REC with patient visits, i.e. over time. This suggests that CuEXC could be used at a more advanced stage to confirm disease progression. However, more data is needed to confirm this trend and observe other potential relationships between CuEXC and disease progression. For example, CuEXC may appear as an indicative factor of rate of deterioration if high CuEXC levels, recorded at the first patient visits, are correlated with more significant ALSFRS decline in subsequent visits.

5.2 Cu Exposure

Our questionnaire revealed that environmental Cu exposure from diet, drinking water, and occupational exposure did not differ in patients and controls (Table 3), primarily as, given that

most controls were co-habiting, their ingestion with food and water would be very similar. In their 2018 study, Barros et. al also did not observe any difference in dietary copper intake between patients and their control group, composed of healthy age and sex-matched adults recruited though local community social media invitations (Barros et al., 2018). Patients did not have any pre-existing conditions that significantly contributed to Cu levels. Analysis of the trace metal content of the potable water samples by ICPMS did not reveal any significant contribution of drinking water to patient copper exposure, although sample size was small (n=12). It is important to note that as our study only examines Cu levels in patients after ALS diagnosis, it is possible that we may be missing the window of exposure to Cu, which most significantly contributes to disease pathology within patients' lifespans.

Although differential Cu exposure was not identified in our patients, they presented with higher CuEXC levels compared to controls, which increased across visits (with time). From the literature on WD, it is known that copper toxicity reduces the ability of Cp to bind Cu, resulting in a progressive increase in NCC that increases rapidly in rate at an advanced stage of disease. Our results also show that as ALS progresses rapidly, CuEXC increases rapidly mirroring the increase in symptom severity. The observation that the initial levels of copper biomarkers in controls and patients are similar, but that patient's health deteriorates rapidly at some point in time, indicates a possible genetic basis for the observed differences in CuEXC levels once a certain exposure level/disease severity is reached.

The congruence of total Cu and CuEXC between patients and controls indicates the potential role of genetic factors in determining how the body responds to high exchangeable copper levels. Underlying genetic susceptibility to Cu toxicity and co-exposure to other environmental factors could explain differences in physiological responses. In Indian Childhood Cirrhosis (ICC), differing genetic susceptibility to Cu toxicity creates different phenotypes where some children develop ICC and experience Cu toxicity, while others, including siblings, are more robust to exposure and do not develop ICC (Barber et al., 2021). In AD, recent research has pointed to differences in susceptibility to Cu toxicity linked to ATP7B polymorphisms. Dr. Rosanna Squitti's group show how certain subpopulations of AD, who are carriers of selected ATP7B gene variants, have elevated CuEXC (Squitti et al., 2013; Squitti et al., 2017) suggesting the existence of a

dysfunction copper phenotype of sporadic AD with a genetic basis. A similar case could be made for ALS, as a multifactorial disease influenced by differing genetic and environmental factors, where environmental Cu exposure and underlying genetic variations, possibly linked to Cu status, interact to produce the observed disease phenotype.

5.3 Genetic Analyses

The preliminary results obtained from our Nanostring Analyses indicate novel discoveries of Cu metabolism gene expression alterations in ALS patients. The most significant and noteworthy changes observed were the decreases in ATOX1 expression in patients compared to controls and across patient visits (Figure 17). Changes in ATOX1 expression in ALS patients may have interesting implications for Cu toxicity; since ATOX1 is the Cu Chaperone for ATP7A/B, the decrease in its expression in patients may point to a disturbance in Cu homeostasis. Without further genetic analyses, it is difficult to determine if decreased ATOX1 levels would affect ATP7A/B Cu secretory function. However, the decrease in ATOX1 may alone signify a profound disturbance in Cu homeostasis. If a decrease in ATOX1 were to be observed with disturbances of ATP7B function in patients, this could explain decreases observed in Cp concentration and activity, as faulty loading of Cp would lead to ATP7B dysfunctionality and faster degradation. Analyses of ATP7B SNPs would be highly beneficial to elucidate any disrupted interactions with ATOX1 potentially contributing to Cu toxicity.

Another noteworthy change observed is the significant decrease in expression of MT2A in patients (Figure 17). MT2A is neuroprotective against copper dyshomeostasis (Scheiber & Dringen, 2013; Tokuda *et al.*, 2015) and metallothionein (MT1 and MT2) overexpression has been shown to prolong survival and restore copper homeostasis in SOD1 G93A ALS mouse models (Tokuda *et al.*, 2014), while its genetic ablation accelerates motor neuron injury and death (Nagano *et al.*, 2001; Puttaparthi *et al.*, 2002). The observed decrease in MT2A coinciding with the increased CuEXC and REC levels points to the presence of Cu toxicity, however, the role of MT2A as either a responsive element to Cu toxicity or as a causal element is difficult to determine. Interestingly, there have been reports of MT overexpression in spinal cords of SOD1 G93A mice (Elliott, 2001; Gong & Elliott, 2000; Nagano et al., 2001; Ono *et al.*, 2007; Tokuda *et al.*, 2007), suggesting a

responsive mechanism to oxidative damage in vulnerable motorn neuron tissues (Nagano et al., 2001). In humans however, MT1/2/3 expression levels have been found to be decreased in sALS spinal cords (Hozumi et al., 2008). Only one SNP has been identified in MT1 in sALS patients (Morahan *et al.*, 2007b). No evidence of genetic changes were found for MT3 or MT2A (Morahan *et al.*, 2005, 2007a), however, this does not exclude the potential of genetic silencing in hindering MT metal detoxification (Bolognin *et al.*, 2014) .

In addition to observed decreases in MT2A, novel discoveries of decreases in expression of Cu chaperone proteins COMMD1 and COX17 were also observed. Taken together, these data point to a clear alteration of Cu homeostasis in ALS pathology. Only one other study has examined changes of expression of Cu chaperone genes in ALS models, where COX17, CCS, and ATOX1 were shown to significantly increase in SOD1 G93A mice spinal cords, paralleling age-dependent increases in Cu levels in the spinal cord (Tokuda et al., 2009).

The observed decrease in TARDBP (Figure 18) is surprising, considering increases in expression of TARDBP have been observed in ALS patients before (Gil-Bea et al., 2017). However, the mechanism of toxicity in ALS includes TARDBP protein aggregation. Perhaps, the observed decreased TARDBP mRNA levels are a negative feedback response to increased protein aggregation. Further genetic analyses are needed to assess the contribution of these examined ALS genes to disease pathology and examine if known polymorphisms, such as the C9orf72 GGGGCC hexanucleotide repeat expansions are present in our patient samples.

There has been an emphasis on researching the effects of SOD1 mutations on ALS in the literature, since this gene is one of the most highly associated genes with fALS (20% of cases) (Tokuda et al., 2009). The status of SOD1 expression in the literature is variable and dependant on many factors, such as sample type, the type of SOD1 mutations in ALS patients, and patient demographics. For example, SOD1 concentrations in CSF of ALS patients may differ by sex, with males presenting with higher levels (Frutiger *et al.*, 2008). Many of the SOD1 mutations associated with fALS appear to increase the likelihood that SOD1 will become a prooxidant, resulting in increased ROS, however it is likely that SOD1 toxicity is not uniform across ALS patients and that different forms of SOD1 alterations may contribute to pathology (Broom *et al.*, 2014). Our results

indicate a decrease in SOD1 expression in patients at their second visit (Figure 19). Whether this observation is a consequence or cause of altered antioxidant status in patients remains unknown.

Antioxidant genes assessed, apart from SOD1, showed decreases in patients compared to controls, but only GSTP1 had significantly lower expression levels. Further assessments of oxidative stress and antioxidant status through genetic analyses are needed to elucidate the extent of implication of oxidative status in patients.

5.4 Limitations

5.4.1 Effect of Covid-19 on study

As the study is based on phlebotomy at regular intervals and on genetic analyses, it has been contingent on ethics approval, obtained from the hospital and university ethics boards, and participant consent, obtained at recruitment. These factors have been significantly impacted by the ongoing COVID-19 pandemic. The first patient was successfully recruited at the end of March 2021. Since then, weekly neurological ALS clinics have been conducted with an average of 2 patients recruited every week. As many patients are still opting to conduct online appointments, the number of patients recruited per week remains low.

5.4.2 Cohort sizes and composition

The most evident limitation in our study is the slow patient recruitment, which has led to a small sample size to date. The large range in ALSFRS scores and variation in CuEXC requires the recruitment of many more participants to consolidate our findings and potentially elucidate trends that are difficult to see currently. The recruitment of more female patients may also be beneficial in elucidating sex specific differences considering that female patients present at older ages with more advanced disease progression and progress more rapidly compared to males.

5.4.3 Validation of Methodology

The rigorous optimization of the ICPMS protocol has presented us with a valid method for the determination of exchangeable copper (CuEXC) in serum. However, the high level of CuEXC observed in both patients and controls compared to reference values calls for additional external

validation of methods. Therefore, aliquots of patient samples have been sent to the independent laboratory of the Ospedale Fatebenefratelli, under the direction of Dr. R. Squitti, an experienced researcher examining the relationship between free copper and Alzheimer's disease, for verification of CuEXC levels. Her lab will perform the analysis of total serum copper and CuEXC by AAS. The reported values will be compared with the values obtained in-house using ICPMS to measure metal concentrations. In addition, the estimations of free Cu obtained using the enzymatic method and the immunological method will be compared. If our methods are validated, and more results concurring with existing observation are obtained from a greater number of patients, evidence would point to a significant role of Cu in the progression of ALS.

5.4.4 Sample Type

While useful in giving a holistic view of Cu status in the body, the use of blood and serum samples is a limitation of our study. The use of CSF or nervous tissue would allow the examination of Cu status markers directly in the CNS, giving a more targeted view of Cu toxicity in ALS pathology.

5.5 Future Directions

The significant decreases in expression of *MT2A*, *ATOX1*, *SOD1*, and *GSTP1* point to a role of oxidative stress in patient disease progression. Increases in oxidative stress markers such as 8-oxo-2'deoxyguanosine (8-OHdG) and decreases in SOD1 activity have previously been detected in ALS patient blood (Ihara et al., 2005). Examination of oxidative stress markers, such as 8-OHdG and malondialdehyde, are needed to support the presence of oxidative stress. Further genetic analyses are also needed examining polymorphisms in genes of interest to assess underlying genetic predispositions to Cu toxicity. Once recruited patients have had all their visits and the dataset is complete, further analyses can be conducted including a change in ALSFRS vs CuEXC regression, and a multivariable regression with copper and zinc analyzed in tandem.

Since most controls recruited thus far cohabit with the patients, their exposure to environmental Cu is very similar. As revealed by our questionnaire, there are no significant differences in exposure between the two groups. Thus, the addition of a second control group, of healthy age and sex matched individuals with no pre-existing conditions, and no relationship to the ALS patients would be beneficial for assessing copper exposure in the general population. It would also be

beneficial to continue collection of water samples from patients' homes to eliminate drinking water as a source of significant environmental Cu exposure.

5.5.1 Treatment for Cu toxicity in ALS patients

The potential implication of Cu toxicity in ALS progression presents with potential treatment avenues. One current trial implicating Cu dyshomeostasis is underway, however this trial assumes a Cu-deficient phenotype, and attempts treatment with Cu^{II}(atsm), which transports Cu^{2+} throughout the body and delivers it when it reaches selectively targeted affected regions (Nikseresht *et al.*, 2020; Violi et al., 2020).

If a Cu toxic phenotype is instead implicated in ALS, treatment inspired by WD Cu toxicosis management would be more appropriate. The history of WD treatment has included the usage of metal chelating agents, such as D-penicillamine, trientine, ammonium tetrathiomolybdate (TTM), or with Zinc (Hoogenraad, 2006, 2011; Squitti *et al.*, 2020). Caution would need to be taken to avoid traditional metal chelators like D-penicillamine and Trientine. Although they are still widely used, they result in neurologic worsening and permanent neurological damage in 25 percent of neurological WD patients (Brewer, 2017; Hoogenraad, 2006).

D-Penicillamine acts by mobilizing copper from complexes in the liver and creating a water-soluble copper complex that is excreted in urine. This low molecular penicillamine-copper complex is also capable of passing the blood-brain barrier, subsequently aggravating neurological symptoms, and causing permanent neurological damage in 25% of treated WD patients (Brewer, 2017; Hoogenraad, 2006). Other side effects include an initial hypersensitivity reaction in 25–35% of patients as well as bone marrow depression, proteinuria, skin damage, and autoimmune diseases, sometimes fatal. Brewer argues that because of all its side effects, penicillamine has no place in the modern treatment of WD, although it is still widely used since a whole generation of physicians are unfamiliar with newer and better treatments (Brewer, 2017). Similarly, Trientine also produces permanent neurologic worsening in approximately 25% of patients when administered to patients presenting with neurologic symptoms, however, it does not share D-Penicillamine's other adverse side effects (Brewer *et al.*, 2006). Conversely, zinc acts by blocking the intestinal absorption of copper by induction of intestinal cell metallothionein, which binds copper from food and

endogenous secretions, and holds it in the intestinal cell until the cell sloughs into the stool with a few days of turnover time (Brewer, 2017; Hoogenraad, 2006, 2011(Hoogenraad, 2006, 2011; Squitti et al., 2020). Zinc may also act by competing with Cu for MT binding, thus decreasing Cu uptake.

Some WD copper chelators have already been tested in ALS mouse models. Tokuda et al. tested the copper chelator ammonium tetrathiomolybdate (TTM) as a strategy to remove the copper accumulation in spinal cords driven by mutant SOD1 proteins in SOD1- G93A mice (Tokuda *et al.*, 2008). They found that TTM treatment prolonged survival of both presymptomatic and symptomatic mice, and decreased motor neuron loss and skeletal muscle atrophy. Other studies have reported beneficial outcomes in transgenic SOD1 mouse models when testing different chelators to remove copper including trientine (Andreassen *et al.*, 2001; Nagano *et al.*, 2003; Nagano *et al.*, 1999), and D-penicillamine (Hottinger *et al.*, 1997) which proved to slow down disease progression or delay onset. The results of WD copper chelating treatments on SOD1 G93A transgenic mice are summarized in Table 4.

Table 4. Results of WD copper chelating treatments on SOD1 G93A transgenic mice

Table adapted from (Tokuda & Furukawa, 2016)

Treatment/condition	Effect on survival	Other effects	Cu level and SOD1 activity	Reference
D-penicillamine (100mg/day) Pre-onset	↑ 8%	Delays onset	Not Examined	(Hottinger et al., 1997)
Trientine (800mg/day) Pre-onset	↑8%	Delays onset	Not Examined	(Andreassen et al., 2001)
TTM (150mg/day) post onset	Not Examined	-	Not Examined	(Nagano et al., 2003)
TTM (5mg/day) pre-onset	↑24%	Delays Onset	↓Spinal Cu level ↓SOD1 activity	(Tokuda et al., 2008)
TTM (5mg/day) post-onset	↑11%	-	↓Spinal Cu level ↓SOD1 activity	(Tokuda *et al.*, 2013)

Targeting metal dysregulation may be a promising avenue for ALS treatment for a subset of patients who are more prone to metal imbalance, similar to AD patients with high-risk ATP7B

variants (Sensi et al., 2018). Preclinical and clinical evidence indicates that Zn supplementation can be a feasible and cost-effective line of intervention to restore the brain Cu balance (Hoogenraad, 2011; Sensi et al., 2018). In his 2011 article, Hoogenraad argues that oral zinc therapy is an effective and safe treatment of type 2 free copper toxicosis in AD, citing studies showing promising preliminary results when treating AD patients with zinc (Brewer & Kaur, 2013; Constantinidis, 1992). Conversely, he warns against the use of metal chelators, such as penicillamine, as its administration in AD patients shows adverse symptoms reminiscent of neurological symptom aggravation in WD patients (Hoogenraad, 2011; Squitti et al., 2005).

Zinc therapy

MTs are involved in antioxidant defence, essential metal homoeostasis and heavy metal detoxification; therefore, manipulation of their expression has a strong therapeutic potential for ALS patients (Hozumi et al., 2008; Morahan et al., 2005). Evidence supporting this includes the accelerated onset of progression of ALS in SOD1 G93A mice with MT1/2/3 knocked out (Hozumi et al., 2008; Nagano et al., 2001; Puttaparthi et al., 2002) and the depletion of Zn in SOD1 G93A transgenic mice accelerating ALS progression, while moderate supplementation provided protection and prolonged lifespan (Ermilova *et al.*, 2005; Trumbull & Beckman, 2009). The usage of Zinc to induce an upregulation of metallothioneins, therefore, presents as a potential treatment avenue for ALS patients. Our data points to the decreased expression of MT2A in ALS patients. By increasing expression with Zinc, excess Cu may be safely stored and made unavailable for uptake by bodily tissues, hindering potential oxidative stress damage. A randomized clinical trial testing could be designed to examine the value of zinc supplements in the treatment of ALS. The effect of a low dose of zinc (150 mg/day) on the free copper concentration in serum, the urinary excretion of copper, and laboratory markers of oxidative stress, could be investigated in a blind, placebo-controlled trial.

6. Conclusion

To examine the relationship between environmental Cu and ALS, a case-control study was conducted examining blood Cu status markers from 35 ALS patient and 21 cohabiting controls. Disease progression was monitored using the ALSFRS-R and gene expression data was examined. Although more data are needed to make conclusive remarks, Cu homeostasis appears to be altered in patients compared to controls. An increase in CuEXC and REC was observed across patient visits as well as decreases in ceruloplasmin concentration and oxidase activity, supporting our Cu toxicity hypothesis. Total Cu and initial CuEXC between patients and controls were found to be congruent, indicating a potential role of genetic factors in determining how the body responds to high exchangeable copper levels. Novel decreases in gene expression of Cu metabolism genes of ATOX1, MT2A, COMMD1, and COX17 were identified in patient samples, supporting this notion. Further participant recruitment, genetic tests, and analyses are needed to validate our findings, which may open the prompt development of novel treatment options for treatment of Cu toxicity in ALS, such as Zinc treatment.

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Appendix I.

Research Enrollment Questionnaire

Participant code of identification :
Postal Code (home) :
Date (dd/mm/yy) :

---

1. ____ What was your biological sex at birth?
   a) Male
   b) Female
   c) Intersex

2. ____ What is your age? _____ years

3. ____ Have you received a diagnosis of ALS? If you answered no, then please proceed to question 6.
   a) yes
   b) no

4. ____ What was your age when you received a diagnosis of ALS? _____ years

5. ____ Is there a family history of ALS or has a genetic risk factor for ALS been identified?
   c) yes
   d) no

6. ____ Have you ever smoked tobacco or cannabis? If you answered no, then please proceed to question 11.
   a) yes
   b) no

7. ____ If you have ever smoked tobacco or cannabis or vaped, at what age did you start?
   Age: _____ years

8. ____ If you have ever vaped or smoked tobacco or cannabis, please circle all relevant activities:
   a) tobacco smoking
   b) cannabis smoking
   c) vaping

9. ____ If you have ever vaped or smoked tobacco or cannabis, have you smoked either daily or occasionally within the last 10 years? If you answered no, then please proceed to question 11.
   a) yes
   b) no

10. ___ Do you currently smoke either daily or occasionally?
    a) yes
    b) no

11. ___ Do you have type I diabetes?
    a) yes
    b) no

12. ___ Have you ever had physical trauma including head trauma/injury?
    a) yes
    b) no

13. ___ Do you take a multivitamin tablet or other dietary supplement that contains copper?
    a) daily
    b) sometimes
    c) no

14. ___ Do you drink bottled water at home?
    a) yes
    b) no

15. ___ Do you drink tap water at home?
    a) yes
    b) no

16. ___ Does your home draw water from a well or from a municipal water supply?
    a) well
    b) municipal water supply

17. ___ If you drink tap water at home, do you use a reverse osmosis water purification system?
    a) yes
    b) no

18. ___ If you drink tap water at home, do you fill your kettle at home with hot tap water?
    a) yes
    b) no

19. ___ Do you flush the tap at home before using the water for drinking or cooking?
    a) yes
    b) no

20. ___ Do you wash your fruit/vegetables thoroughly in water before eating?
    a) yes
    b) no

21. ___ How much of your diet is based on plants? (plants include fruits, vegetables, whole grain foods, and foods containing plant proteins such as lentils, split peas, beans, chick peas)
    a) my diet consists only of plants
    b) my diet consists mostly of plants
    c) my diet is about half plants
    d) my diet contains few/no plants?

22. ___ How often do you meals that include meat? (includes processed/sliced meats, chicken, beef, lamb, ham, etc)
    a) never
    b) less than once per week
    c) 1-3 times per week
    d) 4-6 times per week
    e) daily

23. ___ Do you usually eat either liver or lobster on a weekly basis?
    a) yes
    b) no

24. ___ Have you ever worked in landscaping or agriculture and been exposed to pesticides?
    a) yes
    b) no

25. ___ Have you ever been exposed to heavy metals (lead, mercury, arsenic, cadmium) in your work activities?
    a) yes
    b) no

26. ___ Have you ever been exposed to copper in your work activities? Examples: copper-pipe fitter, use of copper sulphate in landscaping or agriculture
    a) yes
    b) no

_____ _____

Signature – research staff **Date**

Appendix II.

Research Clinical Visit Questionnaire

☐ visit 1 ☐ visit 2 ☐ visit 3 ☐ visit 4

Participant code of identification:
Date (dd/mm/yy) :

27. ___ Does the patient have symptoms of a current respiratory infection?
    a) Cough and/or fever
    b) none

28. ___ Is the patient currently receiving treatment for any other type of infection (skin, UTI)?
    a) yes
    b) no

29. ___ Is the patient currently enrolled in another ALS research trial?
    a) yes
    b) no

30. ___ If you answered yes to Question 3, please list the other ALS trials the patient is currently participating in:

_____

_____

_____

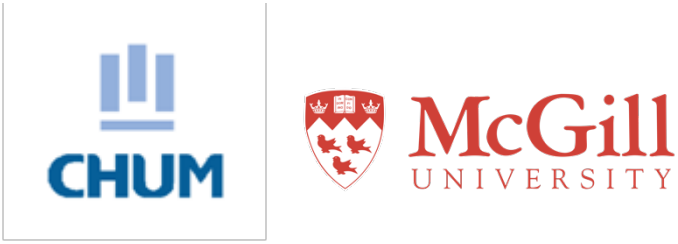
_____

- Please attach a copy of the ALS FRSR assessment for today's clinical visit.

_____ _____

Signature – research staff **Date**

Appendix III.



Environmental Copper Exposure as a Modifiable Risk Factor in ALS

Standards of Procedure (SOPs) for blood collection analysis

Principal Researcher at the CHUM: Dr.Geneviève Matte, neurologist, CHUM

Principal Researcher at McGill University: Dr. Susan Gaskin, Civil Engineering PhD.

Principal Researcher at the Lady Davis Institute: Dr. Koren Mann, PhD, McGill Univ.

A- Introduction

This manual serves as a laboratory guide and includes all relevant information necessary for the collection of blood samples at the CHUM, its treatment, storage, and expedition to external laboratories.

B- Laboratory Material

- PBS (stored in lab on shelf labelled “Sara- ALS Cu”)
- EDTA 3g/L pH 7 (stored in lab on shelf)
- Black Sharpie (thin)
- Racks for Eppendorfs and Falcon tubes (2-4)
- Timer / stopwatch
- Pipettes (1x P20, 1x P200, 1x P1000) + pipette tips (1 box of 20s, 1 box of 200s, and 2 boxes of 1000s)
- Balance tubes for centrifugation (if needed)
- Each participant kit comprised of 2 biohazard bags (Bag 1 and Bag 2) and a kit of spares (Bag 3) containing the following:

- Bag 1: Blood Draw bag – Given to nurse/ phlebotomist
    - Patient timesheet
    - 3 Blue tubes (Blue1, Blue2, Blue3) « Trace Element tubes » - catalog#14-816-154
    - 1 Red Tube « Paxgene tube » -catalog#762165
    - 1 Black and Blue Tube « CPT tube» -catalog#362761
    - 1 Gold tube «Gold SST tube»- catalog#02-683-145
- Bag 2: Sample collection bag- To be used by lab researcher:
    - 1 Blue Tube (Blue3 serum) « Trace Element tubes » - catalog#14-816-154
    - 16 Labelled Eppendorf tubes for sample collection: 2x “Squitti”, 2x “Ceruloplasmin”, 2x “ELISA-8-OH”, 2x “ELISA-Malon”, 2x “ Xtra CPT Serum”, 1x“PBMCs”, 3x“CuEXC”, and 1x “All serum”, 1x “Serum+EDTA”
    - 9 Labelled Falcon tubes for sample collection: “All serum”, “Serum+EDTA”, 3x “Total Cu”, 3x “CuEXC”, “PBMCs”.
    - 3 Labelled pink capped centricons: C1, C2, C3

- 2 Whatman papers for serum spot analysis: 1x “Cu Total” and 1x “CuEXC” with 3 circles on each paper

- Bag 3: Spares collection bag- To be given by lab nurse/phlebotomist and returned for refilling each week.
  - 2 Blue tube « Trace Element tubes » - catalog#14-816-154
  - 2 Red Tube « Paxgene tube » -catalog#762165
  - 2 Black and Blue Tube « CPT tube» -catalog#362761
  - 2 Gold tube «Gold SST tube»- catalog#02-683-145

C- SOP for extracting multiple samples in the clinic

- Indicate the patient number on the labels included on the prelabelled tubes in the kit (ALS- Cu-###) as well as the date.
- Tubes are drawn based on priority (Blue1, Blue2, Black and Blue, Red, Blue3, and Gold).
- Blue1 and Blue2 will be used for ICPMS analysis, Squitti (IGEA) analysis and serum spot analysis. Black& Blue will be used for DNA extraction, oxidative stress measurement by ELISAs, and Ceruloplasmin colorimetric activity determination.
- Blue3 serum and Gold will be sent to Lifelabs for total serum copper and ceruloplasmin analysis respectively.
- Red will be used for RNA extraction.
- All steps are to be performed at RT unless specified otherwise.
- All tubes must be stored at RT prior to use.
- Recorded tube “Start Time” is when the blood has been drawn into all tubes and “End time” is the time tube processing must continue.
- All ICPMS falcon tubes must be made of polypropylene or polyethylene (included in the kits).
- All blood tubes must be filled to maximum volume to obtain the greatest amount of sample possible.
- Keep any serum samples to be frozen in a 4C fridge or on ice until they can be transferred to -80C.

Blood sample collection sequence and instructions

1- Blue1 and Blue2 - 6mL (Trace Element Tubes - catalog#14-816-153)

- Collect 6 mL of venous whole blood into two Blue capped tubes.
- Invert tubes 5 times and allow to sit at room temperature for 30 minutes for blood to coagulate.

2- Black & Blue - 8mL (CPT tube- catalog#362761)

- Draw 8ml of blood into the Black & Blue capped tube.
- Gently invert the tube 8 times to mix and allow to sit at room temperature for 2 hours.

3- Red - 2.5 mL (PAXgene Blood RNA tube-catalog#762165).

- Draw 2.5mL of blood using the full vacuum.
- Gently invert the tube 8-10 times to mix with the RNA stabilizing agent.
- Let tube sit at room temperature for at least 2 hours minimum (72 hours maximum) to ensure complete lysis of cell components.

4- Blue3 - 6mL (Trace element tubes- catalogue#14-816-154)

- Collect 6 mL of blood.
- Invert tube 5 times and allow to sit at room temperature for 30 minutes for blood to coagulate.

5- Gold - 5mL (SST tubes-catalogue #02-683-145)

- Collect 5 mL of blood.
- Invert tube 5 times and allow to sit at room temperature for 30 minutes for blood to coagulate.
- Mark the start time i.e. the time blood has been drawn into this last tube on the patient timesheet.
- Transfer tubes and timesheets to lab for processing.

D- Preparing serum samples: Lab processing:

NB:

- *If there is not enough serum from a tube, prioritize aliquoting one of each type of sample*.
- *Samples to be frozen can be kept on ice or in a 4C fridge till sample manipulation has ended*.

1- **Blue1 and Blue2** – must be processed 30 minutes after collection

**** Note: centrifuge Blue3 and Gold at the same time***

a) Centrifuge "Blue1" and "Blue2" at 1300rcf for 10 minutes in a swing bucket centrifuge. After centrifugation, the blood in each tube should separate into (i)cell components and (ii)serum. The cell components (red layer) should be under the gel and the serum (beige-yellow layer) should be above it.

b) Immediately after centrifugation transfer all serum from both Blue1 and Blue2 into a Falcon tube labelled "All serum". Discard Blue1 and Blue2.

c) Make the following samples from the "All serum" tube:

   i. Aliquot 2x 0.5ml of serum to 2 Eppendorf tubes labelled "Squitti".

   ii. On a Whatman paper labelled "Total Cu", spot 3x 10uL of serum in the center of the 3 pre-drawn circles.

      - Place paper back into its plastic holder and set aside to dry.

d) Aliquot 2ml from the "All serum" tube to a Falcon tube labelled "Serum+EDTA".

   - If there is less than 2ml of serum, keep all serum in "All serum" and perform the following steps in "all serum" instead of "Serum+EDTA", marking down "+EDTA" on the label.

   i. Dilute serum with 2ml of EDTA 3g/l (1:1 dilution) and set aside for 1h. Gently vortex to mix contents. Mark incubation end time on patient timesheet (+1h after adding EDTA solution).

      - If there is not enough serum, measure serum volume and add enough EDTA for a 1:1 dilution.

e) After 1-hour, aliquot 3x 120 uL from "Serum+EDTA" to a falcon tube labelled "Total Cu 1", "…2", "…3" to be used for ICPMS analysis.

f) Aliquot 3x 1ml from "Serum + EDTA" tube to pink-capped centricon tubes labelled "C1", "C2", "C3".

i. Centrifuge the centricon tubes at 2000g for 45min at 4°C in a precooled swinging bucket centrifuge.

ii. Using a P1000, determine the volume of ultrafiltrate and mark it down.

iii. On a Whatman paper labelled “CuEXC” spot 10uL of serum in the center of the pre-drawn circle corresponding to each centricon (3 spots total).

- Place paper back into its plastic holder and set aside to dry.

iv. Aliquot 3x 120 uL ultrafiltered serum, one from each centricon to corresponding falcon tubes labelled “CuEXC-1”, “CuEXC-2”, “CuEXC-3”.

- Transfer any remaining ultrafiltrate (at least 120uL) from each of the centricons to corresponding CuEXC 1”, “CuEXC2”, “CuEXC3” eppendorfs.

g) Transfer any remaining serum from “All serum” and “Serum +EDTA” to corresponding eppendorfs.

h) Freeze the Squitti samples at -80°C, to be later batch shipped to IGEA.

i) Freeze all “CuEXC” and Eppendorfs at -80°C to be used as back up samples.

2- **Blue3 and Gold-** Processed after 30 minutes
Note that these are centrifuged at the same time as Blue1 and Blue2

a) Centrifuge “Blue3” and “Gold” at 1300rcf for 10 minutes in a swing bucket centrifuge. After centrifugation, the blood in each tube should separate into (i) the cell components (red layer) under the gel and (ii) the serum (beige-yellow layer) above it.

b) From “Blue3”, aliquot of all its serum to a new blue topped tube labelled “Blue3 serum”, and cap tightly.

c) Refrigerate “Blue3 serum” and “Gold” tubes at 2-8°C (to be shipped to LifeLabs at the end of the day).

3- **Black and Blue** – Processed after 2h

a) Centrifuge for 20 min at 1500 rcf in a swinging bucket centrifuge. The tube contents should separate into 4 layers. The top layer consists of clear yellowish plasma, followed by a cloudy white PBMC layer, the gel barrier, and the red blood cells at the bottom.

b) Make the following 6 + samples from the serum in “Black and Blue”:

Note: if there is not much serum, prioritize getting at least 1 of each type of sample.

i. Aliquot 2x 0.2ml of serum into 2 Eppendorf tubes labeled "Ceruloplasmin".

ii. Aliquot 2x 0.2ml of serum into 2 Eppendorf tubes labeled "ELISA 8-OH".

iii. Aliquot 2x 0.2ml of serum into 2 Eppendorf tubes labeled "ELISA-Malon".

iv. If there is any remaining serum, transfer it into Eppendorf tubes labelled "Extra CPT serum" (up to 1.5 mL/tube).

v. Freeze all tubes at -80°C till further processing.

c) Remove the PBMCs and transfer them to a 15ml falcon tube labelled "PBMCs".

d) "PBMCs" falcon tube:

i. Add 10mL of sterile PBS to the "PBMCs" falcon tube.

ii. Centrifuge tube at 300rcf for 10 min to pellet the cells.

iii. Aspirate PBS without disturbing the pellet. Discard PBS.

iv. Add 1.2ml sterile PBS. Gently pipette up and down to mix PBS with PBMCs.

v. Transfer this PBMC mix to a 1.5ml Eppendorf labelled "PBMCs".

vi. Centrifuge "PBMCs" Eppendorf at 300rcf for 10min to pellet cells.

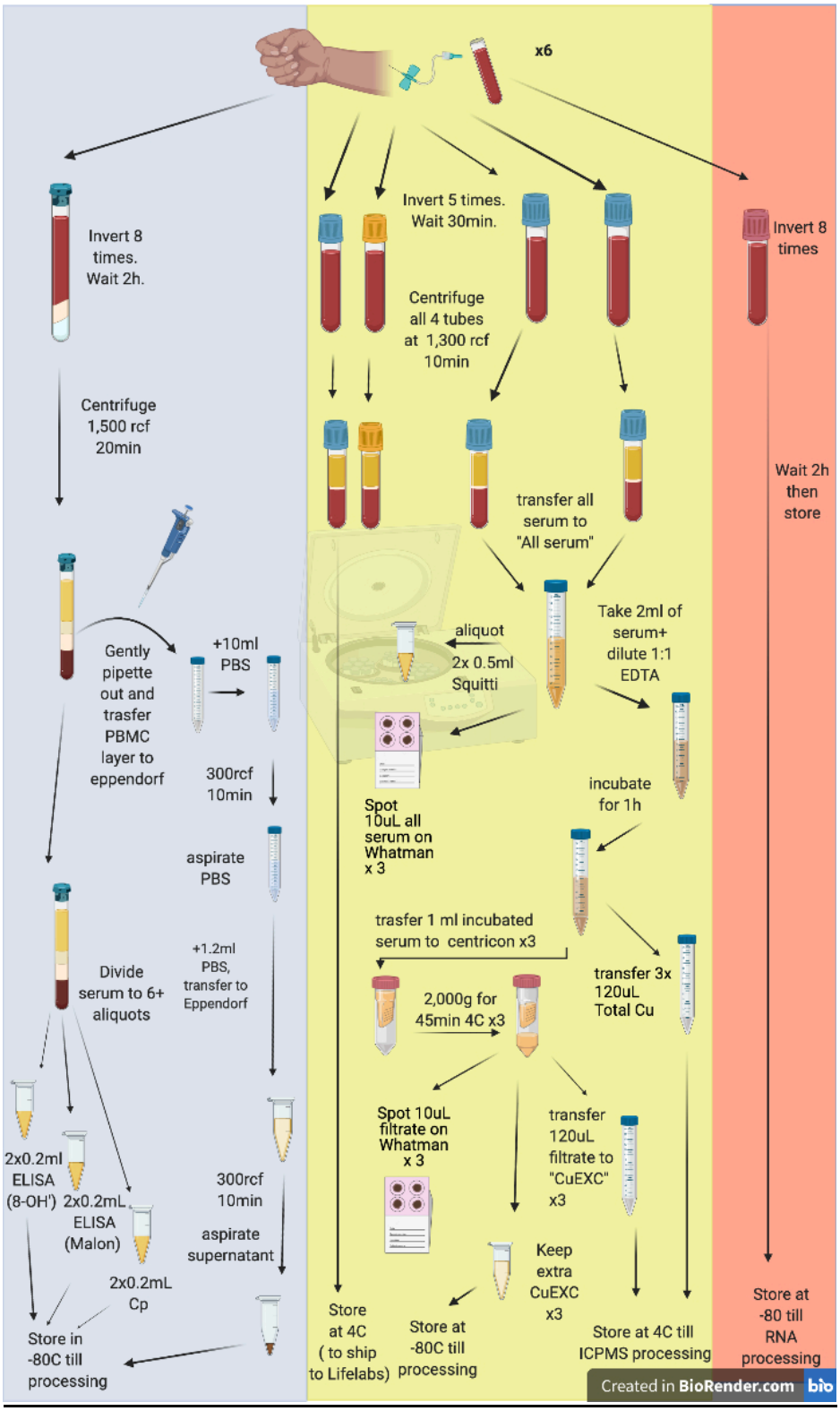
vii. Aspirate PBS without disturbing the pellet.

viii. Freeze cells ("PBMC" Eppendorf) at -80°C till DNA extraction.

4- **Red PAXgene** – <u>processed after at least 2 h and maximum 72 h</u>

a) After at least 2h have passed, transport the Red PAXgene tube at RT and store at -80°C till further RNA processing.

E- Flowchart


x6
Invert 8 times. Wait 2h.
Centrifuge 1,500 rcf 20min
Gently pipette out and trasfer PBMC layer to eppendorf
+10ml PBS
300rcf 10min
aspirate PBS
Divide serum to 6+ aliquots
+1.2ml PBS, transfer to Eppendorf
2x0.2ml ELISA (8-OH')
2x0.2mL ELISA (Malon)
2x0.2mL Cp
300rcf 10min
aspirate supernatant
Store in -80C till processing
Invert 5 times. Wait 30min.
Centrifuge all 4 tubes at 1,300 rcf 10min
transfer all serum to "All serum"
aliquot 2x 0.5ml Squitti
Spot 10uL all serum on Whatman x 3
Take 2ml of serum+ dilute 1:1 EDTA
incubate for 1h
trasfer 1 ml incubated serum to centricon x3
2,000g for 45min 4C x3
transfer 3x 120uL Total Cu
Spot 10uL filtrate on Whatman x 3
transfer 120uL filtrate to "CuEXC" x3
Keep extra CuEXC x3
Store at 4C (to ship to Lifelabs)
Store at -80C till processing
Store at 4C till ICPMS processing
Invert 8 times
Wait 2h then store
Store at -80 till RNA processing
Created in BioRender.com

Timesheet for sample collection

Patient ID: ALS-CU-__ __ __

Has the patient signed a consent form for the genetic sub-study?

☐ Yes
☐ No – Do not collect blood for the following tube: Red (PAXgene) and PBMCs

1- First Visit Date: _____ Start Time:_____

Tubes	Blue1, Blue 2, Blue3, Gold	Black&Blue, Red	EDTA+serum incubation
Incubation time	30min	2h	1 h
End Time			

2- Second Visit Date: _____ Start Time:_____

Tubes	Blue1, Blue 2, Blue3, Gold	Black&Blue, Red	EDTA+serum incubation
Incubation time	30min	2h	1 h
End Time			

3- Third Visit Date: _____ Start Time:_____

Tubes	Blue1, Blue 2, Blue3, Gold	Black&Blue, Red	EDTA+serum incubation
Incubation time	30min	2h	1 h
End Time			

4- Fourth Visit Date: _____ Start Time:_____

Tubes	Blue1, Blue 2, Blue3, Gold	Black&Blue, Red	EDTA+serum incubation
Incubation time	30min	2h	1 h
End Time			

F- Preparation of samples to be transferred to other labs/instructions:

Samples for Lifelabs:

- Blue3 and Gold, transported refrigerated the day of the clinic
- Complete the form CONTRACT NUMBER: AD337 Client reference ALSCu_MGCHUM for the LifeLabs requisition.
- Pack the tubes in a Styrofoam container with a gel pack and into the Lifelabs shipping box for refrigerated transport.
- Make sure to indicate Exempt Human Specimens on the box.
- For collection by FEDEX. Use the Lifelabs account. The box must clearly indicate the address of the shipper and the destination.

Samples for the Lady Davis Institute:

- Falcon tubes: “All serum”
- Eppendorfs: All “CuEXC”, “All Serum”, and “Serum+EDTA”
- Eppendorfs: 2x “Ceruloplasmin”, 2x “ELISA 8-OH“, 2x ELISA-Malon, 0 to 2x “CPT serum“, 1x “PBMCs”
- “Red” PAXgene tube

To be processed at Lady Davis then transferred to UdeM for ICPMS analysis:

- Falcon tubes: 3x “Total Cu”, 3x “CuEXC”

To be later shipped to Dr. Rosanna Squitti at -80°C:

- Eppendorfs: 2x “Squitti”

Note: All final samples may be stored at -80°C untill transport is permitted in the case of pending approval for sample transportation.

G- Laboratory contact information:

Lifelabs, Medical Laboratory Services

- Liason contact:
  - **Lucy Torres**: **Account Manager, Specialty & Contract Services**
    - LifeLabs | 100 International Blvd. | Toronto, ON M9W 6J6
    - Telephone: 416 675 4530 or toll-free 1 800 268 0902 ext. 42761 | F 416-213-4728
    - Cell 416-995-4360
    - Email **Lucy.Torres@LifeLabs.com** ; **www.LifeLabs.com**

- Adress and contact information of the lab receiving our samples:
- Remember to include Lifelabs requisition for each sample set.
  - LifeLabs
    - Attn: Specimen Management
    - 37 Voyager Court North
    - Toronto, ON M9W 4Y2
    - Ph: 416-675-4530 ext. 42693

  Use LifeLabs FedEx account # 649062471 (3rd party account). Select Priority Overnight service

- **Laboratory at the Lady Davis Institute**
  - Contact: Laboratory Miller-Mann-Del Rincon
    - Address : 3755 chemin de la Côte-Ste-Catherine, Montréal (Québec) H3T 1E2 E-504
    - Tel: (514) 340-8260 ext 24257
    - Contact : koren.mann@mcgill.ca, c_guilbert@yahoo.com

- **Dr. Rosanna Squitti's Lab :**
  - Adresse:
    - Rosanna Squitti, PhD
    - Dipartimento di Medicina di Laboratorio, Sezione Ricerca e Sviluppo
    - Ospedale Generale
    - 'San Giovanni Calibita' FATEBENEFRATELLI
    - Isola Tiberina -00186 Roma

- **Laboratory at the UdeM :**
  - Dr. Kevin J. Wilkinson's Chemistry Lab
    - Address : Complexe des sciences 1375 Avenue Thérèse-Lavoie-Roux, local B-5019, Montréal, Québec, H2V 0B3
    - Contact : kj.wilkinson@umontreal.ca
    - Cell : 514 343-6741

H- Table of Visits/sample collection

Patient with ALS	DNA	RNA	Blood			
Visit 1	x	**x**	**x**			
Visit 2		**x**	**x**			
Visit 3		**x**	**x**			
Visit 4		**x**	**x**			
Control Group						
Visit 1	**x**	**x**	**x**			

I- Storage and transfer of samples

Analyses	Tube	Storage	Laboratory
Ceruloplasmin activity	Eppendorf Aliquots « Ceruloplasmin »	-80°C	Lady Davis Institute
Indicators of oxidative stress	Eppendorf Aliquots « ELISA-8-OH », « ELISA-Malon »	-80°C	Lady Davis Institute
Serum Cu, CuEXC	Eppendorf Aliquots «Squitti »	-80°C	Lady Davis Institute and on to Dr. Squitti
Serum Whatman paper spots	From: All Serum and ultrafiltrate of centricons “C1”, “C2”, “C3”	RT in sealed plastic bags	Lady Davis Institute
ICPMS- serum copper, CuEXC	Falcon tubes « CuEXC » , « Total Cu»,	4°C	Lady Davis Institute and later on to UdeM
Serum copper, ceruloplasmin	Blue3 serum and Gold	Between 2 and 8°C	Lifelabs
DNA	Eppendorf « PBMCs »	-80°C	Lady Davis Institute
RNA	Red PAXgene tube	-80°C	Lady Davis Institute
Extra samples	Eppendorfs : « All serum », “Serum+EDTA”, « Xtra CPT serum”, “CuEXC”	-80°C	Lady Davis Institute

J- Approval of laboratory guide – version 6, 12 February 2022

Dre Koren Mann _____ Signature _____ Date

Dre Geneviève Matte _____ Signature _____ Date

Prof. Susan Gaskin _____ Signature _____ Date