Impact of Aging on Copper Isotopic Composition in the Murine Brain

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Abstract

Aging is the main risk factor for Alzheimer's disease (AD). AD is linked to alterations in metal homeostasis and changes in stable metal isotopic composition can occur, possibly allowing the latter to serve as relevant biomarkers for potential AD diagnosis. Copper stable isotopes are used to investigate changes in Cu homeostasis associated with various diseases. Prior work has shown that in AD mouse models, the accumulation of ^{63}Cu in the brain is associated with the disease's progression. However, our understanding of how the normal aging process influences the brain's isotopic composition of copper remains limited. In order to determine the utility and predictive power of Cu isotopes in AD diagnostics; we aim - in this study - to develop a baseline trajectory of Cu isotopic composition in the normally aging mouse brain. We determined the copper concentration and isotopic composition in brains of 30 healthy mice (WT) ranging in age from 6 to 12 months, and further incorporate prior data obtained for 3-month-old healthy mice; this range approximately equates to 20-50 years in human equivalency. A significant ^{65}Cu enrichment has been observed in the 12-month-old mice compared to the youngest group, concomitant with an increase in Cu

concentration with age. Meanwhile, literature data for brains of AD mice display an enrichment in ^{63}Cu isotope compared to WT. It is acutely important that this baseline enrichment in ^{65}Cu is fully constrained and normalized against if any coherent diagnostic observations regarding ^{63}Cu enrichment as a biomarker for AD are to be developed.

1. Introduction

The process of aging provokes wide-ranging effects on the brain, involving various cellular and molecular changes. Mitochondrial dysfunctions in brain cells lead to a decline in energy metabolism efficiency. DNA repair mechanisms become compromised resulting in increased genomic instability. Furthermore, there is an accumulation of soluble (and sometimes neurotoxic) protein precursors [1], misfolded and aggregated proteins, dysfunctional organelles and elevated levels of oxidative stress [2]. Numerous studies have established a direct association between oxidative stress, aging and cognitive decline in humans [3-7]. The progressive increase in oxidative stress during normal aging is attributed to the imbalance between the production of reactive oxygen species and the ability of cellular antioxidant defense systems to counteract them.

Additionally, neuro-inflammation is a hallmark of the aging brain. Senescent glial cells, and the infiltration of immune cells into the brain contribute to a chronic state of neuro-inflammation (see fig1). This process is characterized by elevated levels of pro-inflammatory cytokines and a decline in anti-inflammatory compounds [8, 9]. Chronic neuro-inflammation not only promotes neurodegeneration but also accelerates the overall aging process of the brain [10].

Aging is recognized as the primary risk factor for neurodegenerative diseases, including Alzheimer's disease (AD) [11, 12]. Alzheimer's disease is a global health concern, currently affecting 50 millions of individuals worldwide, with an estimated increase to nearly 113 million by 2050 [12, 13]. It is the fifth leading cause of death in high income countries [14, 108].

At the cellular level, AD is characterized by two major patho-physiological features: the presence of extracellular amyloid plaques (Aβ-plaques), formed by the agglomeration of the Aβ42 peptides, and the formation of intracellular neurofibrillary tangles (NFTs), resulting from abnormal hyperphosphorylation of the tau protein [12, 15-17]. The latter is mainly due to clinical diagnosis *via* positron emission tomography (PET) imaging, however it is noted that this technique is predicated on significant deposition of Aβ-plaques to breach the fidelity threshold of current imaging instrumentation, which are generally unable to detect the soluble Aβ fraction [48-50]. Current *in vivo* detection methods are invasive and onerous, involving imaging techniques such as positron-emission tomography (PET) to visualize amyloid-β deposits [18], or measuring the levels of amyloid-β or tau proteins in cerebro-spinal fluids [19, 20].

During the initial phase of the disease, Aβ-plaques begin to accumulate in the extracellular space, yet clinical symptoms, including loss of memory, difficulties in performing executive functions and solving problems, cognitive decline, apraxia may not manifest for up to 20 years [14, 21]. This asymptomatic/ preclinical stage of the disease holds great significance for the development of early diagnosis techniques and the optimization of AD treatments.

In addition to other neurodegenerative diseases such as Wilson disease, Menkes disease, Amyotrophic Lateral Sclerosis (ALS) [22 – 24, 109, 110], AD has also been associated with a change in the metal homeostasis [25 – 27]. Previous works by Moynier et al., (2019, 2020) have produced evidences that the changes in the metal isotopic composition, of elements like copper serve as relevant biomarkers for the future diagnosis of AD [28, 42], and there is a growing body of literature that more generally suggests viability of stable metal isotopes in disease biomarker development [112].

Copper plays a crucial role in various biological processes in the brain, functioning as a cofactor and structural component of several enzymes involved in essential functions. It participates in redox metabolism, energy metabolism (cytochrome c oxidase), antioxidative defense (Zn,Cu-superoxide dismutases), iron metabolism (ceruloplasmin), neurotransmitter synthesis (dopamine-β-

monoxygenase), regulation of inflammation, and neuropeptide synthesis (peptidylglycine-α-amidating enzyme) (see fig1) [29- 33].

The concentration of Cu in the human brain is typically found to be between 3 and 5 μg/g wet weight [34, 35]. Similarly, a mouse brain contains on average 7 μg/g for a female and 4 μg/g for a male [43]. Copper has two stable isotopes: ^{63}Cu and ^{65}Cu with different relative abundances in nature of 69% and 31%, respectively. Isotopes undergo fractionation due to changes in bonding environments within the body [27, 37 - 44]. Isotope fractionation is a general term referring to the process of creating isotopic variability of a particular element among coexisting species or reservoirs hosting this element [40, 41]. Heavy isotopes tend to accumulate in environments characterized by stiffest bonds [37, 38, 45]. For instance, ^{65}Cu binds preferentially to histidine, glutamate and aspartate [38, 112]. As a result of the fractionation, the isotopic composition of copper differs in organs: ^{65}Cu accumulates in the brain, liver and kidney whilst ^{63}Cu is enriched in red blood cells (RBC) and plasma. Changes in the isotopic composition of copper in body fluids and in different organs have been used as proxies to identify cancers [46 – 49], liver diseases [50 – 54] and neurogenerative diseases [24, 28, 38, 40, 42, 55, 56].

In AD mice models expressing the amyloid-beta precursor protein (APP), the accumulation of ^{63}Cu in the brain has been shown to be related to the progression of the disease [42]. Besides, nuclear magnetic resonance (NMR) studies have established that Cu (II) ions interact directly with Aβ forming a Cu (II)-Aβ complex *in vitro* [32, 57 – 62]. Copper might increase the number of β-sheet and α-helix structures in amyloid peptides, which could be a cause of β-amyloid aggregation [63]. Copper and Zinc might also be, in healthy individuals, linked with the degradation of soluble Aβ [64]. Amyloid-β sequestering copper, the change in bonding environment due to the emergence of protein aggregates, amyloid plaques (Aβ) and neurofibrillary tangles (NFT) is a key to understand this shift in copper isotopic composition in AD brains.

However, prior to any of the above mechanistic pathways for Cu isotope fractionation being identified, it is requisite to first bolster our current understanding regarding the impact of the normal

aging process on the isotopic composition of copper in the brain remains limited, such that if present, this effect can be accounted for in the development of any future biomarker based on stable Cu isotopes. The objective of this study is to establish a baseline for the changes in Cu isotopic composition in the brains of normally aging mice, as a proxy for that in humans. We determined the copper concentration and isotopic composition in a total of 30 mice (15 males and 15 females) aged 6, 9 and 12-months. Our findings reveal that there is no significant difference in the Cu isotopic composition of male and female mice (i.e. no sex-dependence). Therefore, we combined the data for all mice in our analysis. We observed that ^{65}Cu is enriched in the 12-months old mice compared to the younger age groups, which show no distinguishable difference. Additionally, the overall copper concentration in the brain tends to increase with age

2. Materials and Methods

2.1. Mice. *At Hopital Bichat.*

The 30 WT mice used in this study were collected from VTS INSERM 1148, Hopital Bichat, Paris. All the 30 mice had the same genotype: C57BL/6JRj. The mice were housed in 6 different cages, 5 same-sex mice per cage. The dry food given to the mice has a δ^{65}Cu =0.55±0.01 (2SD) [43].

2.2. Sample collection. *At Hopital Bichat.*

Mice aged 6, 9 and 12-months were euthanized by receiving a lethal dose of ketamine and xylazine, followed by exsanguination. Throughout their life cycle, mice were all given the same diet - previously analyzed in Cu isotopic composition- and kept in similar conditions. The brains were collected directly after death using instruments in stainless steel. The brains were then conserved in a -20 degrees Celsius freezer.

The biological standard used was tuna fish ERM-CE464 (TF ERM-CE464).

2.3. Digestion and chemical purification *At Institut de physique du Globe de Paris (IPGP)*

The dissolution, chemical purification and mass-spectrometry analyses are a close modification of the protocols established previously [28, 42].

Dissolution. In polyfluoralkyl (PFA) beakers, whole brain samples and biological standard (TF ERM-CE464), all at masses around 100mg, were dissolved in a few droplets of H_2O_2 and concentrated distilled HNO_3 for a day and a half. Once the dissolution was complete, the beakers were placed opened on a hot-plate at 120°C overnight.

Copper extraction. The Cu purification is conducted using anion- exchange chromatography following a protocol adapted from [65]. The samples are redissolved in 1mL of distilled 7M HCl then loaded on 1.6 mL of AG-MP1 resin. Matrix elements are taken away by passing 8 mL of 7M HCl through the resin. The copper is collected in 16 mL of 7M HCl. This procedure is replicated after evaporation of the acid to ensure pure Cu fractions. The resin is washed following a day-long protocol alternating three series of rinses with MQ water and HNO_3 0.5N in between the two replicates. The copper was extracted from the standard following the same procedure as for the samples. The yield of Cu is >99%.

2.4. Mass Spectrometry *At Institut de physique du Globe de Paris (IPGP)*

Copper isotope ratios were determined with multi-collection inductively-coupled-plasma mass-spectrometers (MC-ICP-MS) at the Institut de Physique du Globe de Paris France. Part of the samples were analyzed using a Nu-Instrument Sapphire and the other part using a Thermo-Fisher Neptune. Depending on instrument performance and availability on the day of analyses; no analytical differences are observed between instrument platforms (figS1; Table1B). The samples were analyzed between 1 and 5 times each depending on the amount of copper in each sample (Table1B).

The sample-standard bracketing (SSB) method was applied and the Cu concentration in the samples and standards where match within 10%. This method consists in measuring a standard before and after each sample and using the average of the two standards to normalize the sample ratio [40,44]. During analytical session, the standard utilized was IPGP-Cu, previously determined to have a δ^{65}Cu of +0.271 ±0.006 ‰ (2SE) relative to the NIST SRM 976 [28]. All data herein have been converted to δ^{65}Cu relative to NIST SRM 976 by adding 0.271.

2.5. Statistical analysis

Statistical analysis were performed using GraphPad Prism.

3. Results

3.1. Effect of age on the isotopic composition of Cu in the brain

We incorporated the data from a previous study conducted by Moynier et al., [43] on 3-months old mice that were bred together with the mice studied here. All data are reported in Table 1A and 1B and displayed in fig2. The isotopic composition of the biological standard (TF ERM-CE464) was analyzed every 12 samples on both the Nu-Instrument Sapphire and Thermo-Fischer Neptune instruments. The value obtained (0.09±0.02, 2SD, N=4) was consistent within error with the value reported by [66, 116], (0.11±0.14, 2SD) (Table 2). The Cu isotope compositions of all materials are expressed as the ratio ^{65}Cu to ^{63}Cu relative to that of the internationally recognized standards SRM-976, in per mil (per thousand) notation, or δ^{65}Cu, and have been calculated using the formula:

$$\delta^{65}\mathrm{Cu} = \left(\frac{\left(\frac{^{65}Cu}{^{63}Cu}\right) sample}{\left(\frac{^{65}Cu}{^{63}Cu}\right) NIST\ SRM\ 976} - 1 \right) \times\ 1000$$

Two brain samples were analyzed on both instruments to ensure the consistency of the results. The results obtained were similar within error: brain-1 gave a δ^{65}Cu = 0.86±0.06) on the Neptune and, 0.90±0.05) on the Sapphire. Brain-2 has a δ^{65}Cu of 0.70±0.08 on the Neptune, and of 0.75±0.06) on the Sapphire.

The values of δ^{65}Cu for mice aged 3 to 12 months were analyzed with One-way ANOVA test (p-value < 0.0001; R squared= 0.4934). Our data were analyzed with Mann-Whitney test for each age categories as the data are not normally distributed and the number of data is close to 30. Mann-

Whitney test: (age 9 vs age 6; p-value = 0,0051); (age 6 vs 12; p-value = <0.0001); (age 3 vs age 6; p-value = 0.7915, ns); (age 12 vs age 9; p-value = 0,0008) (see fig2A).

The results (see fig2A, B, C) reveal a notable evolution in the isotopic composition of copper as a function of age. Mice aged between 3 and 6 months exhibit a relatively consistent composition of copper isotopes, with a δ^{65}Cu value of 0.85±0.23 (2SD). However, from the age 9 months onwards (mean: δ^{65}Cu = 0.98±0.22 (2SD)) there is a noticeable shift towards heavier copper content and dispersion of δ^{65}Cu values, particularly evident in the 12 months old mice (mean: δ^{65}Cu = 1.32±0.61 (2SD)) (fig2A and 2B). The simple linear regression (fig2C) plotted using the values displayed in fig2B has a positive slope and shows a linear increase in the values of δ^{65}Cu depending on time. An un-linear curve showing was plotted as well in fig2C and is also displaying a heavier copper isotopic composition as a function of age. Taken as a whole, these data strongly suggest that the isotopic composition of copper undergoes significant changes with age. Specifically, the copper composition becomes progressively heavier with advancing age. Consequently, the aging process is associated with a shift in the isotopic composition of copper, particularly in the brain, which demonstrates an age-dependent increase in the proportion of ^{65}Cu compared to ^{63}Cu.

3.2. Effect of age on copper concentration

Several studies in the literature have reported an age-related increase in copper levels in various organs. In healthy individuals, it has been observed that copper tends to increase with age in human serum [67, 68]. However, in cortical tissue affected by Alzheimer's disease (AD), the level of copper is actually decreased [69]. This decrease is believed to be associated with the binding of copper to senile plaques [70]. Other studies have shown that copper tends to decrease in salivary sediment and hair samples [71]. In rats aged 15 to 49 weeks, Cu level increase in the serum, kidney, liver and five distinct parts of the brain (cortex, corpus striatum, hippocampus, midbrain+medulla and

cerebellum) [72]. These findings further support the notion of age-dependent changes in copper amounts across different tissues and species.

Copper concentrations aren't the main focus in the study. They are determined as a matter of comparison with other studies that determine the concentrations the same way. Our objective was to investigate whether there were any variations in copper concentration within our samples. To accomplish this, we quantified the copper concentration in each brain sample (n=30). For each sample, we performed a factor 100 dilution (10µL of sample in 1mL of 0.5N HNO_3) and measured the signal in volts obtained on the MC-ICP-MS. Given that the concentration and signal were precisely known for the standard (NIST SRM 976), we could compute the concentration of copper in each of the samples. The weight collected for each sample was 100mg±30. The data are in Table 3 and displayed in fig3. The concentrations are given in ppm, equivalent to a concentration given in µg/g. The means were calculated for each age category: mice aged 6 months (mean: C_{copper} = 3.73 ppm, 2sd = 2.50, n=10), mice aged 9 months (mean: C_{copper} = 4.39 ppm, 2sd = 5.58, n=10), mice aged 12 months (C_{copper} = 6.02 ppm, 2sd = 4.56, n=10) (see fig3).

We performed a Mann-Whitney test: age 12 vs 9 (p-value = 0,2475, ns) and age 12 vs age 6 (p-value = 0,0089, **). Ordinary one-way ANOVA and 2way ANOVA were not conclusive. However, we observe a qualitative increasing trend as a function of age. The data obtained for mice aged 9 months are very disperse compared to 6 and 12 months-old mice (see fig3).

4. Discussion

4.1. Effect of sex on copper isotopic compositions and concentrations

Previous studies have demonstrated differences in the isotopic composition of Cu and Fe in function of sex notably in the serum and red blood cells [28, 73, 74]. In archeological human bones, Jouen et al., have shown that the isotopic signature is dependent on sex for Cu and Fe [74].

Furthermore, several studies have indicated sex-related differences in the development of neurodegenerative diseases. For instance, men have a higher disposition for Parkinson's disease [75-77] while women constitute two-thirds of AD patients [78]. Moreover, the progression of the disease progression appears to be accelerated in men [79]. Although these sex-dependent differences are known, their underlying causes remain elusive and require further investigation. To better constrain the sex effect on brain isotopic composition and concentration, we conducted a comparison between the results obtained for males and females, irrespective of age. However, no significant difference was observed between the two groups. Furthermore, we further examined the data by plotting the results for males and females separately in each age category. Even after considering age as a factor, no notable or statistically significant differences between the sexes were found (see fig4; Table4; Table 5).

We conducted a Mann-Whitney test to compare the δ^{65}Cu values between males and females, which revealed no statistical difference (p-value = 0.1276). The mean δ^{65}Cu for males was 0,98±0.48 (2SD), while for females it was 1.13±0.67 (2SD) (see fig4A). Additionally, males and females could not be statistically differentiated based on copper concentrations (see fig4B).

Although there was no significant difference in δ^{65}Cu between males and females, it is worth noting that the data for females exhibited a slightly broader distribution compared to males. Furthermore, there is a tendency in the evolution of δ^{65}Cu values between males and females as a function of time (fig4D), however more samples from future work might drive this further towards statistical significance. The difference in copper isotopic composition between males and females tends to increase as a function of time. As reported in the study by Moynier et al., (2019), at age 12, there was a discernible difference in δ^{65}Cu values between males and females. However, in our study, it did not reach statistical significance (see fig4C)

4.2. δ^{65}Cu decreases after 12 months.

To provide a better understanding of the evolution of δ^{65}Cu with age, we combined our data with literature [43, 107] (fig5). Moynier et al., (2022) measured 4 brains from healthy mice aged 3 months, Morel et al., (2022) measured 29 brains from mice aged 6, 16 and 24 months. All mice have the same genotype. The data show an increase in δ^{65}Cu values before 12 months and a decrease after 12 months. Combining the data from Morel et al., (2022) with our data, we could observe that both data from mice aged 6 months are consistent. Performing Mann-Whitney test for the 6 and 9 months, p-value = 0.0004, ***, which is even lower when the data are combined with Morel et al., (2022) compared to our data alone (p-value = 0.0051). Even if the measurement method and copper extraction vary slightly between the studies, the results are consistent.

4.3. 12 months old WT and AD mice have different brain copper isotopic compositions

Combining our data for 12 months old healthy mice with those from Moynier et al. (2019) [28], we obtained a mean δ^{65}Cu = 1.32±0.61 (2SD) for healthy 12 months-old mice (WT), in our study. For 12 months-old AD mice (AD), the mean was δ^{65}Cu = 0.50±0.29 (2SD). We also took into account the δ^{65}Cu values for healthy mice brains reported in Moynier et al., (2019). The data are statistically differentiable (Mann-Whitney, p-value < 0.01, **). The results (see fig6A; Table 6) show a decrease in the isotopic composition between WT and AD at 12 months. However, we could only have five samples for AD mice ages 12 months. Despite the limited data, this difference is considered robust, as a statistical difference of well over 0.5 per mil is over an order of magnitude larger than typical analytical precision within this and other studies.

This suggests that AD brains are isotopically lighter than healthy ones in terms of copper isotopes (see fig6B). This is also what is found in literature in the brains of AD and WT humans [42]. This can be explained by the change in bonding environment caused by the appearance of amyloid plaques and neurofibrillary tangles, as copper directly interacts with Aβ [42,57,58, 70]. The new environment created by the presence of amyloid plaques in the brain might encourage the binding of

lighter isotopes and thus generating a shift in the natural isotopic fractionation of copper. Copper being a redox active metal [25] and Cu (II) ions binding preferentially to Aβ plaques this also could be a source of explanation for this shift [42,57,58, 70]. Conversely, the presence of Cu (0) has been detected in AD human brains [80].

Normal aging and AD have different signatures in terms of copper concentration as well. In the literature, it is reported that AD is accompanied by a deficiency in copper in the brain [69,81, 82]. Interestingly, the amount of Cu in aged human brains has been shown to have a negative correlation with the advance of Aβ plaques [83]. Normally aging individuals tend to have copper accumulating in the brain as a function of age. Thus, AD and healthy individuals appear to display different copper signatures regarding copper isotopic composition and copper concentration.

4.4. Copper related oxidative stress might be a key to understand normal aging in the brain

Oxidative stress is thought to be one of the main causes of aging in mammals [3-6]. In the brain, the levels of antioxidants are low: it makes it susceptible to oxidative stress induced by the redox-active nature of Cu [84]. Superoxide dismutases (SODs) are a category of ubiquitous enzymes representing one of the main antioxidant defense systems against reactive oxygen species (ROS). SODs catalyze the dismutation reaction of $O_2^{\bullet -}$ to H_2O_2 which is then reduced to H_2O by the action of catalase [85]. The reaction requires alternate reduction and reoxidation of a redox active transition metal (Cu or Mn). In mammals, there are three isoforms that are all bound to catalytic metals: intracellular SOD1 or CuZn-SOD, mitochondrial SOD2 or Mn-SOD and extracellular SOD3 [86]. SOD1 is intracellular [87, 88].

Warner et al., (1994), have shown, through genetic manipulations increasing CuZn-SOD activity, a resistance to most forms of oxidative stress [89]. CuZn-SOD deficiency increases the vulnerability to oxidative stress and thus potentially normal aging. Copper is bound to several proteins, frequently enzymes, including metallothionein. It has been shown that SOD1 was enriched

306 in ^{65}Cu relative to the metallothionein (MT) in human cortex [90]. Thus, probably due to specific
307 catalytic micro-environments, SOD1 seems to be a source of Cu fractionation.
308 The distributions of Cu isotopes between MT and SOD1 in AD patients is also different
309 compared to healthy patients [90]. CuZn-SOD activity is negatively correlated with age [91] which
310 might partly explain the increase of ROS in cells and tissues. However, while the activity of the
311 enzyme seems to decrease with age, its concentration appears to follow the opposite tendency. It was
312 shown that in the cerebro-spinal fluid (CSF), the concentration of SOD1 tends to increase with age
313 [111]. As we mentioned, SOD1 binding preferentially ^{65}Cu, which might explain the increase in
314 $^{65}Cu/^{63}Cu$ ratio in CSF compared to the decrease in the $^{65}Cu/^{63}Cu$ ratio observed in the brain [90]
315 (fig2). The variations observed in this study and in other studies (fig6) [107] for both $\delta^{65}Cu$ values
316 and Cu concentrations (ppm) as a function of age might be related to SOD1 concentration and activity
317 during aging and -more generally - to enzymes binding copper in the brain. Further investigations
318 regarding this aspect would be interesting to understand precisely the mechanisms involved, notably
319 including the parallel-operating influence of MT and the influence this may have on Cu isotope
320 composition.
321
322 **4.5. Effect of glial cells and neuroinflammation on copper contents in the brain**
323
324 Astrocytes are a specific type of glial cells in the central nervous system (CNS) that are
325 reported to have - amongst others - a role in the maintenance of extracellular ion homeostasis [92,
326 93]. They are key regulators of Cu and Fe in the brain [94 – 96]. Astrocytes express copper
327 transporter 1 (Ctr1) [97] as well as MT [98] and glutathione (GSH) [99]. Ctr1 is the main copper
328 transporter in eukaryotic cells and is responsible for copper entrance in the cell [100]. When the brain
329 ages, astrocytes encounter senescence and their functions become dysregulated [101]. This is thought
330 to have an impact on the accumulation of debris and especially Aβ plaques [102, 103]. Furthermore,
331 copper has been shown to activate the secretion of inflammatory products such as IL- 6, NO and

TNF-α by interacting with microglia [104]. Microglia are as well as astrocytes a type of glial cell and the main type of immune cell in the brain [105]. Inflammation in the brain promotes the appearance and development of neurodegenerative diseases [10,106]. Ashraf et al., (2019), demonstrated that glial senescence may induce differential regional content of metals (Cu, Zn, Fe) [106]. In chronic and acute inflammatory conditions, copper is reported to be at higher concentrations in the brain. In the same conditions, labile copper was found to be also at higher concentrations in microglia. It has been shown in copper-deficient rats that a lack of copper is linked with a sensitivity to acute inflammatory agents [113]. Copper seems to be implied in the regulation of inflammation in the brain. Moreover, the copper complex copper*bis(*thiosemicarbazones) (Cu^{II} (atsm)) was shown to be a potential therapeutic candidate for strokes and acute brain injuries [114]. This same copper complex has also been shown to have protective effects in animal models of neurodegenerative diseases such as ALS and AD [114, 115]. Cu^{II} (atsm) was reported to have an anti-inflammatory action by potentially restoring the brain copper homeostasis [114]. We propose that the senescence of glial cells, as well as neuro-inflammation and its peripheral processes during ageing, could be key contributing factors to understanding the accumulation of copper, and potentially other metals, in the ageing brain, and therefore hold relevance in understanding the evolution of Cu isotope compositions in the brain as a function of age as well as potential therapeutic candidates.

5. **Conclusions**

This study has provided evidence that the isotopic composition of copper in the brain undergoes changes with normal ageing, manifesting an increase in the $^{65}Cu/^{63}Cu$ ratio with age. These results suggest that the copper isotopic signature becomes progressively heavier as individual ages, accompanied by a tendency towards Cu accumulation in the aging brain. Notably, we observed a distinct shift in $^{65}Cu/^{63}Cu$ ratio between healthy and AD model mice at 12 months. While the ultimate utility of Cu isotopes in AD diagnostics remains to be clear, the present work provides an invaluable baseline trajectory of Cu isotope compositions in the brain with age. This baseline progression must

be well-understood so that it can be deconvolved from other alterations to Cu isotope compositions, namely the observed light Cu isotope excursion in the AD brain; this is paramount to mechanistically and statistically isolating a potential biomarker signal induced by the disease. These findings greatly contribute to the development of a baseline Cu isotope signature associated with normal aging, enhancing our ability to understand Cu isotope changes induced by neurodegenerative diseases such as Alzheimer's.

6. Acknowledgments

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7. Disclosure statement

The authors have no actual or potential conflicts of interest.

8. Data availability

The data underlying this article are available in the article and in its online supplementary material.

Figure captions

Fig1. The roles of copper in a healthy brain. Made with BioRender.

Fig 2. A. δ^{65}Cu in healthy mouse brain in function of age. Boxes extend from the 25th and 75th percentile, the line inside the box represents the median, the whiskers show the minimum and

maximum of the values. p-value < 0.001; **, p-value < 0,0001; ****. B. δ^{65}Cu in healthy mouse brain in function of age. Each point corresponds to the mean for one mouse, n corresponds to the number of brains measured per age category. C. Simple linear regression for the data displayed in figure 2B (black full line) with 95% confidence intervals (dotted line), R^2= 0.3946 and quadratic second order polynome (red full line), R^2= 0.5126. GraphPad Prism.

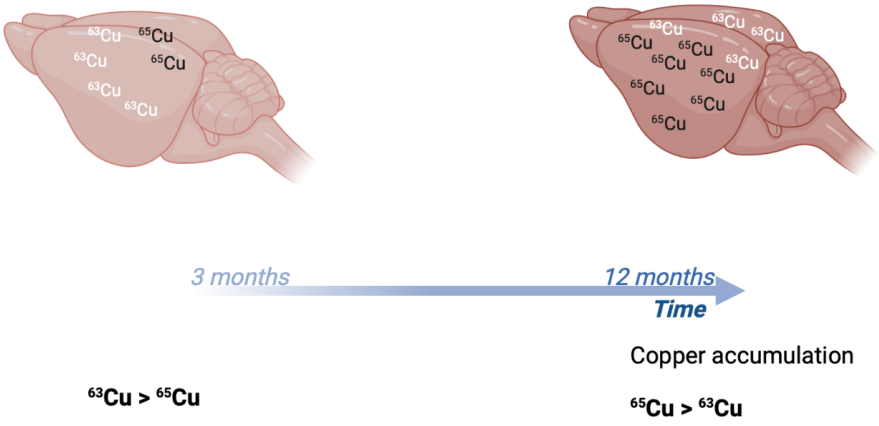
Fig 3. Concentration of copper (ppm) in function of age. Boxes extend from the 25th and 75th percentile, the line inside the box represents the median, the whiskers show the minimum and maximum of the values. p-value < 0.02, *. GraphPad Prism.

Fig 4. A, B, C. δ^{65}Cu in function of sex (A), copper concentration in function of sex (B), δ^{65}Cu in function of sex and age (C). (D) is the evolution of the p-values for unpaired-t tests between males and females as a function of age, R^2 = 0.9988. Boxes extend from the 25th and 75th percentile, the line inside the box represents the median, the whiskers show the minimum and maximum of the values.

Fig5. δ^{65}Cu in healthy mouse brain in function of age. Data from (Moynier et al., 2022) and (Morel et al., 2022) were incorporated to the data from this study. Boxes extend from the 25th and 75th percentile, the line inside the box represents the median, the whiskers show the minimum and maximum of the values. p-value < 0.001, ***. GraphPad Prism.

Fig 6. A, B. Isotopic composition of copper in the brain of healthy and AD mice. p-value < 0.001, ** (A), copper isotopic composition of a healthy (green) and Alzheimer brain (red) (B). Boxes extend from the 25th and 75th percentile, the line inside the box represents the median, the whiskers show the minimum and maximum of the values. GraphPad Prism, Bio Render.

Figures



Graphical abstract.

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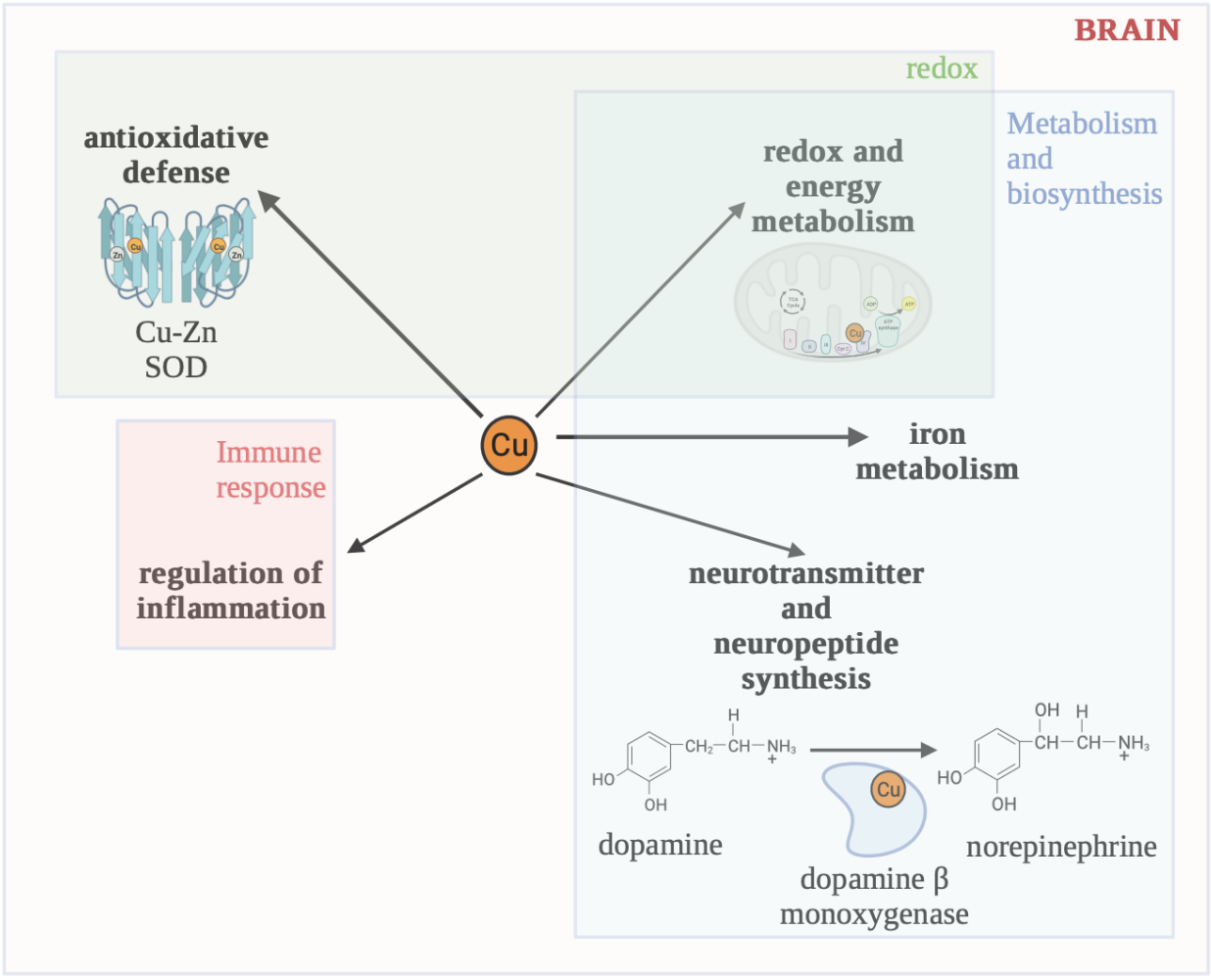
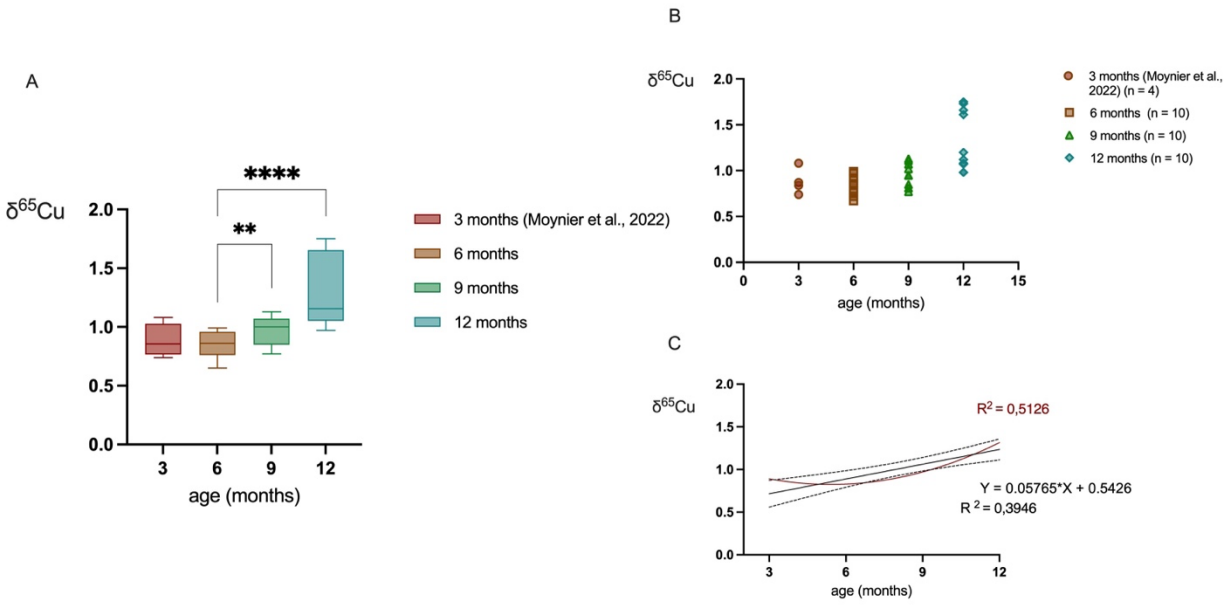


Fig1. The roles of copper in a healthy brain. Made with BioRender.



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448 Fig 2. A. δ^{65}Cu in healthy mouse brain in function of age. Boxes extend from the 25th and 75th

449 percentile, the line inside the box represents the median, the whiskers show the minimum and

450 maximum of the values. p-value < 0.001; **, p-value < 0,0001; ****. B. δ^{65}Cu in healthy mouse

451 brain in function of age. Each point corresponds to the mean for one mouse, n corresponds to the

452 number of brains measured per age category. C. Simple linear regression for the data displayed in

453 figure 2B (black full line) with 95% confidence intervals (dotted line), R^2= 0.3946 and quadratic

454 second order polynome (red full line), R^2= 0.5126. GraphPad Prism.

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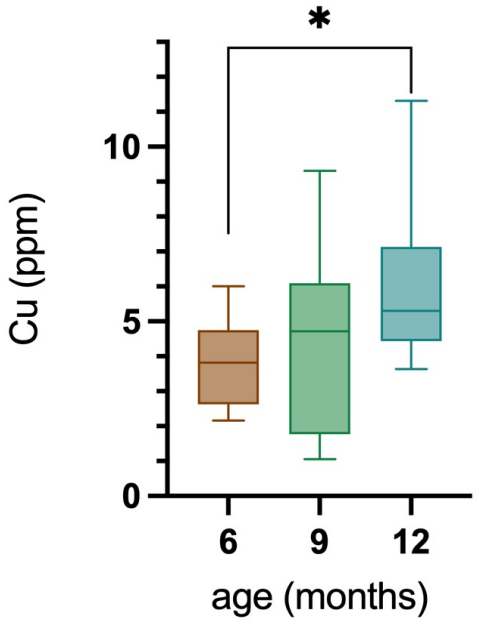


Fig 3. Total concentration of copper (ppm) in healthy mouse brain in function of age. Boxes extend from the 25th and 75th percentile, the line inside the box represents the median, the whiskers show the minimum and maximum of the values. p-value < 0.02, *. GraphPad Prism.

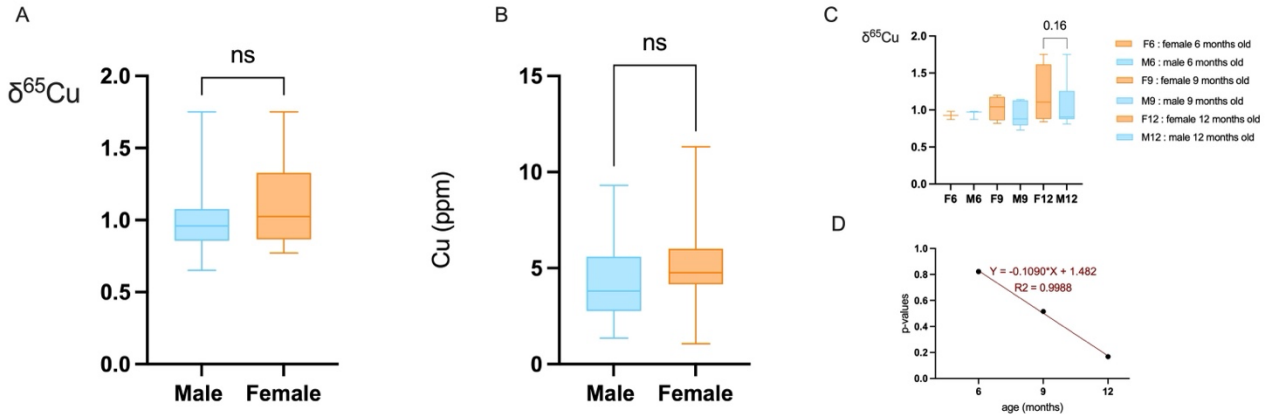


Fig 4. A, B, C, D. δ^{65}Cu in healthy mouse brain in function of sex (A), copper concentration in healthy mouse brain in function of sex (B), δ^{65}Cu in healthy mouse brain in function of sex and age, p-value = 0.16, non-significative (C). (D) is the evolution of the p-values for unpaired-t tests between males and females as a function of age, $R^2 = 0.9988$. Boxes extend from the 25th and 75th percentile, the line inside the box represents the median, the whiskers show the minimum and maximum of the values. ns: not significative.

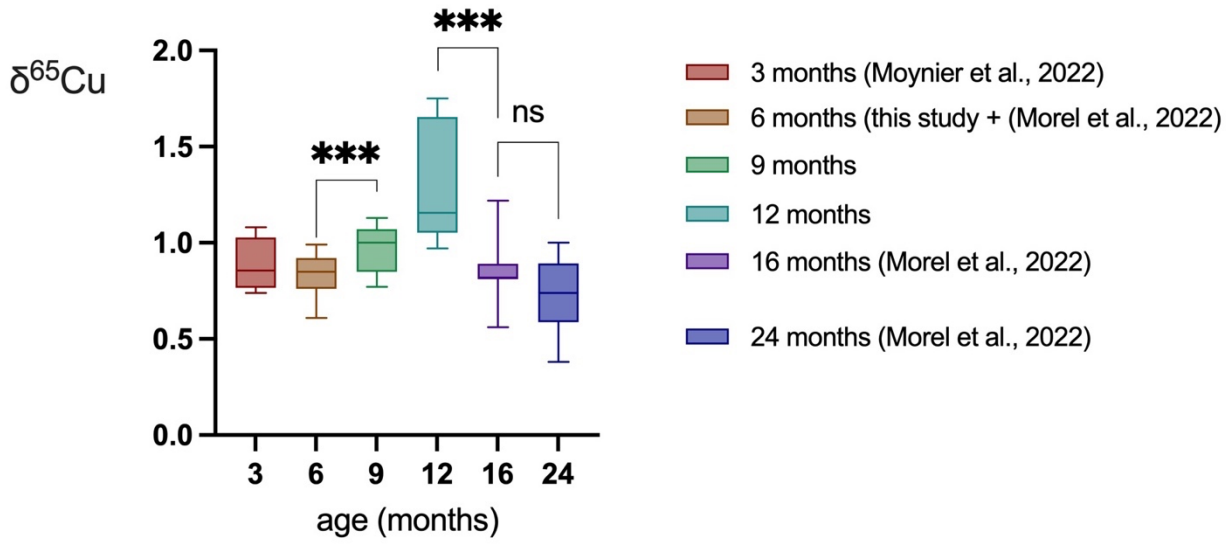


Fig5. δ^{65}Cu in healthy mouse brain in function of age. Data from (Moynier et al., 2022) and (Morel et al., 2022) were incorporated to the data from this study. Boxes extend from the 25th and 75th percentile, the line inside the box represents the median, the whiskers show the minimum and maximum of the values. p-value < 0.001, ***. GraphPad Prism.

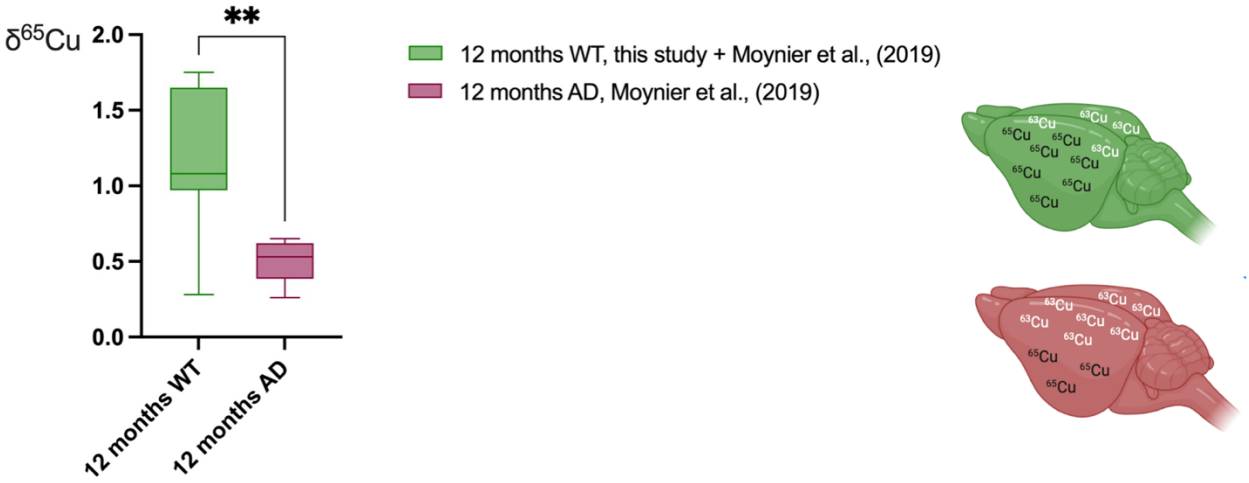
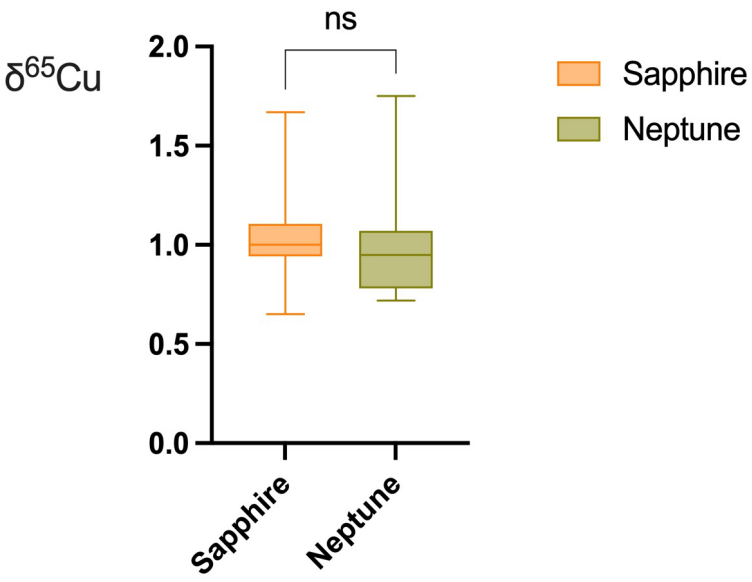


Fig 6. A, B. Isotopic composition of copper in the brain of healthy and AD mice. p-value < 0.001, ** (A), copper isotopic composition of a healthy (green) and Alzheimer brain (red) (B). Boxes extend from the 25th and 75th percentile, the line inside the box represents the median, the whiskers show the minimum and maximum of the values. GraphPad Prism, Bio Render.



FigS1. Copper icotopic composition of healthy mice brains depending on the MC-ICP-MS used for the measure. ns = not significative. GraphPad Prism.

References

1. McLean, C. A., Cherny, R. A., Fraser, F. W., Fuller, S. J., Smith, M. J., Beyreuther, K., Bush, A. I., Masters, C. L. Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. Ann. Neurol, 1999, 46, 860–866. doi: 10.1002/1531-8249(199912)46:6<860::aid-ana8>3.0.co;2-m

2. Mattson, MP., Arumugam, TV. Hallmarks of brain aging: adaptive and pathological modification by metabolic states. Cell. Metab, 2018, 27, 1176-1199. doi: 10.1016/j.cmet.2018.05.011.

3. Junqueira VB, Barros SB, Chan SS, Rodrigues L, Giavarotti L, Abud RL, Deucher GP. Aging and oxidative stress. Mol. Aspects. Med., 2004, 5-16. doi: 10.1016/j.mam.2004.02.003.

4. Harman, D. Protein oxidation in aging and age-related diseases. Gerontology, 1956, 11, 298-300. doi:10.1093/geronj/11.3.298.

5. Harman, D. The biologic clock: the mitochondria? J. Am. Geriatr. Soc., 1972, 4,145-7. doi:10.1111/j.1532-5415.1972.tb00787.x.

6. Beckman KB, Ames BN. The free radical theory of aging matures. Physiol Rev, 1998, 78, 547-81. doi: 10.1152/physrev.1998.78.2.547.

7. Berr, C., Balansard, B., Arnaud, J., Roussel, AM., Alpérovitch, A. Cognitive Decline Is Associated with Systemic Oxidative Stress: The EVA Study. J Am Geriatr Soc, 2000, 48, 1285-1291. doi: 10.1111/j.1532-5415.2000.tb02603.x.

8. Gemechu, JM., Bentivoglio, M., T cell recruitment in the brain during normal aging. Front. Cell. Neurosci., 2012, 6, 38. doi: 10.3389/fncel.2012.00038.

9. Deleidi, M., Jäggle, M., Rubino, G. Immune aging, dysmetabolism, and inflammation in neurological diseases. Front. Neurosci., 2015, 9, 172. doi: 10.3389/fnins.2015.00172.

554 10. Kempuraj, D., Thangavel, R., Natteru, PA., Selvakumar, G. P., Saeed, D., Zahoor, H., Zaheer,
555 A. Neuroinflammation induces neurodegeneration. J. Neurosurg.: Spine., 2016, 1.
556
557
558 11. Roberts, BR., Ryan, TM., Bush, AI., Masters, CL., Duce, JA. The role of metallobiology and
559 amyloid - β peptides in Alzheimer's disease. J. Neurochem., 2012, 120, 149-166. doi:
560 10.1111/j.1471-4159.2011.07500.x.
561
562 12. Knopman, DS., Amieva, H., Petersen, RC. Alzheimer disease. Nat. Rev. Dis. Primers., 2021,
563 7, 33 2021. doi: 10.1038/s41572-021-00269-y
564
565 13. Prince, MJ., Wimo, A., Guerchet, MM., Ali, GC., Wu, YT., Prina, M. World Alzheimer Report
566 2015-The Global Impact of Dementia: An analysis of prevalence, incidence, cost and trends.
567 2015.
568 14. Hampel, H., Hardy, J., Blennow, K. The Amyloid-β Pathway in Alzheimer's Disease. Mol.
569 Psychiatry., 2021, 26, 5481–5503. doi: 10.1038/s41380-021-01249-0
570
571 15. Mucke, L., Selkoe, DJ. Neurotoxicity of amyloid β-protein: synaptic and network dysfunction.
572 Cold Spring Harb. Perspect. Med. , 2012, 2, a006338. doi: 10.1101/cshperspect.a006338.
573
574 16. Hardy, JA., Higgins, GA. Alzheimer's disease: the amyloid cascade hypothesis. Science, 1992,
575 256, 184-185. doi: 10.1126/science.1566067
576
577
578 17. Zempel, H., Mandelkow, E. Lost after translation: missorting of Tau protein and consequences
579 for Alzheimer disease. Trends. Neurosci., 2014, 37, 721-732. doi: 10.1016/j.tins.2014.08.004.
580

18. Gordon, BA., Blazey, TM., Su, Y., Hari-Raj, A., Dincer, A., Flores, S., Benzinger, TL. Spatial patterns of neuroimaging biomarker change in individuals from families with autosomal dominant Alzheimer's disease: a longitudinal study. Lancet. Neurol., 2018, 17, 241-250. doi:10.1016/S1474-4422(18)30028-0.

19. Blennow, K., Zetterberg, H. Biomarkers for Alzheimer's disease: current status and prospects for the future. J. Intern. Med., 2018, 284, 643-663. doi: 10.1111/joim.12816.

20. Olsson, B., Lautner, R., Andreasson, U., Öhrfelt, A., Portelius, E., Bjerke, M., Zetterberg, H. CSF and blood biomarkers for the diagnosis of Alzheimer's disease: a systematic review and meta-analysis. Lancet Neurol., 2016, 15, 673-684. doi: 10.1016/S1474-4422(16)00070-3.

21. Dubois, B., Feldman, HH., Jacova, C., Hampel, H., Molinuevo, JL., Blennow, K. Advancing research diagnostic criteria for Alzheimer’s disease: the IWG-2 criteria. Lancet. Neurol., 2014, 13, 614–29. doi: 10.1016/S1474-4422(14)70155-3.

22. Faa, G., Lisci, M., Caria, M. P., Ambu, R., Sciot, R., Nurchi, V. M., Crisponi, G. Brain copper, iron, magnesium, zinc, calcium, sulfur and phosphorus storage in Wilson's disease. J. Trace Elem Med. Biol., 2001, 15, 155-160. doi: 10.1016/S0946-672X(01)80060-2.

23. Nooijen, JL., De Groot, CJ., Van Den Hamer, CJ., Monnens, LA., Willemse, J., Niermeijer, MF. Trace element studies in three patients and a fetus with Menkes' disease. Effect of copper therapy. Pediatr. Res., 1981, 15, 284-289. doi: 10.1203/00006450-198103000-00017.

607 24. Sauzéat L, Bernard E, Perret-Liaudet A, Quadrio I, Vighetto A, Krolak-Salmon P, Broussolle
608 E, Leblanc P, Balter V. Isotopic Evidence for Disrupted Copper Metabolism in Amyotrophic
609 Lateral Sclerosis. iScience, 2018, 6, 264-271. doi: 10.1016/j.isci.2018.07.023.
610
611
612 25. Barnham KJ., Bush AI. Biological metals and metal-targeting compounds in major
613 neurodegenerative diseases. Chem. Soc. Rev, 2014, 43, 6727–6749. doi : 10.1039/C4CS00138A
614
615 26. Pithadia, AS., Lim, MH. Metal-associated amyloid-β species in Alzheimer's disease. Curr. Opin.
616 Chem. Biol., 2012, 16, 67-73. doi: 10.1016/j.cbpa.2012.01.016.
617
618 27. Moynier, F., Vance, D., Fujii, T., Savage, P. The isotope geochemistry of zinc and copper. Rev.
619 Mineral. Geochem., 2017, 82, 543-600. doi:10.2138/rmg.2017.82.13.
620
621 28. Moynier, F., Creech, J., Dallas, J., Le Borgne, M. Serum and brain natural copper stable isotopes
622 in a mouse model of Alzheimer's disease. Sci. Rep., 2019, 9, 11894. doi : 10.1038/s41598-019-
623 47790-5.
624
625 29. Scheiber, IF., Dringen, R. Astrocyte functions in the copper homeostasis of the brain.
626 Neurochem. Int., 2013, 62, 556-565. doi: 10.1016/j.neuint.2012.08.017.
627
628
629 30. Kaim, W., Rall, J. Copper—a "modern" bioelement. Angewandte Chemie International Edition
630 in English, 1996, 35, 43-60. doi: 10.1002/anie.199600431.
631

632 31. Rubino, JT., Franz, KJ. Coordination chemistry of copper proteins: how nature handles a toxic
633 cargo for essential function. J. Inorg. Biochem., 2012, 107, 129-143. doi:
634 10.1016/j.jinorgbio.2011.11.024.
635
636 32. Atwood, CS., Scarpa, RC., Huang, X., Moir, RD., Jones, WD., Fairlie, DP., Bush, AI.
637 Characterization of Copper Interactions with Alzheimer Amyloid β Peptides: Identification of
638 an Attomolar-Affinity Copper Binding Site on Amyloid β1-42. J. Neurochem, 2000, 75, 1219-
639 1233. doi: 10.1046/j.1471-4159.2000.0751219.
640
641 33. Conforti, A., Franco, L., Milanino, R., Totorizzo, A., Velo, GP. Copper metabolism during acute
642 inflammation: studies on liver and serum copper concentrations in normal and inflamed rats. Br.
643 J. Pharmacol., 1983, 79, 45. doi: 10.1111/j.1476-5381.1983.tb10493.x.
644
645 34. Lech, T., Sadlik, JK. Copper concentration in body tissues and fluids in normal subjects of
646 southern Poland. Biol. Trace. Elem. Res., 2007, 118, 10-15. doi:10.1007/s12011-007-0014-z.
647
648
649 35. Rahil-Khazen, R., Bolann, BJ., Myking, A., Ulvik, RJ. Multi-element analysis of trace element
650 levels in human autopsy tissues by using inductively coupled atomic emission spectrometry
651 technique (ICP-AES). J. Trace Elem. Med. Biol., 2002, 16, 15-25. doi: 10.1016/S0946-
652 672X(02)80004-9.
653
654 36. Wong, PC., Waggoner, D., Subramaniam, JR., Tessarollo, L., Bartnikas, TB., Culotta, VC.,
655 Gitlin, JD. Copper chaperone for superoxide dismutase is essential to activate mammalian
656 Cu/Zn superoxide dismutase. Proc. Natl. Acad. Sci. U.S.A., 2000, 97, 2886-2891. doi:
657 org/10.1073/pnas.040461197

37. Mahan, B., Moynier, F., Jørgensen, AL., Habekost, M., Siebert, J. Examining the homeostatic distribution of metals and Zn isotopes in Göttingen minipigs. Metallomics, 2018, 10, 1264-1281. doi : 10.1039/c8mt00179k.

38. Fujii, T., Moynier, F., Abe, M., Nemoto, K., Albarède, F. Copper isotope fractionation between aqueous compounds relevant to low temperature geochemistry and biology. Geochim. Cosmochim. Acta., 2013, 110, 29-44. doi: 10.1016/j.gca.2013.02.007.

39. Paquet, M., Fujii, T., Moynier, F. Copper isotope composition of hemocyanin. J. Trace Elem. Med. Biol., 2022, 71, 126967. doi: 10.1016/j.jtemb.2022.126967.

40. Mahan, B., Antonelli, M. A., Burckel, P., Turner, S., Chung, R., Habekost, M., Moynier, F. Longitudinal biometal accumulation and Ca isotope composition of the Göttingen minipig brain. Metallomics, 2020, 12, 1585-1598.

41. Albarede, F., Télouk, P., Balter, V., Bondanese, VP., Albalat, E., Oger, P., Fujii, T. Medical applications of Cu, Zn, and S isotope effects. Metallomics, 2016, 8, 1056-1070. doi: 10.1039/c5mt00316d

42. Moynier, F., Borgne, M. L., Lahoud, E., Mahan, B., Mouton-Liger, F., Hugon, J., Paquet, C. Copper and zinc isotopic excursions in the human brain affected by Alzheimer's disease. Alzheimer's. Dement.: Diagn. Assess. Dis., 2020, 12, e12112. doi:10.1002/dad2.12112.

683 43. Moynier, F., Merland, A., Rigoussen, D., Moureau, J., Paquet, M., Mahan, B., Le Borgne, M.
684 Baseline distribution of stable copper isotope compositions of the brain and other organs in mice.
685 Metallomics, 2022, 14. doi:10.1093/mtomcs/mfac017.
686
687 44. Moynier, F., Fujii, T. Theoretical isotopic fractionation of magnesium between chlorophylls.
688 Sci. Rep., 2017, 7, 6973. doi: 10.1038/s41598-017-07305-6.
689
690 45. Schauble, EA. Applying stable isotope fractionation theory to new systems. Rev. Mineral.
691 Geochem., 2004, 55, 65-111. doi: 10.2138/gsrmg.55.1.65.
692
693 46. Balter, V., Nogueira da Costa, A., Bondanese, V. P., Jaouen, K., Lamboux, A., Sangrajrang, S.,
694 Hainaut, P. Natural variations of copper and sulfur stable isotopes in blood of hepatocellular
695 carcinoma patients. Proc. Natl. Acad. Sci, 2015, U.S.A, 112, 982-985. doi:
696 10.1073/pnas.1415151112
697
698 47. Télouk P, Puisieux A, Fujii T, Balter V, Bondanese VP, Morel AP, Clapisson G, Lamboux A,
699 Albarede F. Copper isotope effect in serum of cancer patients. A pilot study. Metallomics, 2015,
700 299-308. doi: 10.1039/c4mt00269e.
701
702
703 48. Schilling, K., Larner, F., Saad, A., Roberts, R., Kocher, H. M., Blyuss, O., Crnogorac-Jurcevic,
704 T. Urine metallomics signature as an indicator of pancreatic cancer. Metallomics, 2020, 12, 752-
705 757 doi: 10.1039/d0mt00061b
706
707 49. Schilling, K., Moore, R. E., Capper, M. S., Rehkämper, M., Goddard, K., Ion, C., Larner, F.
708 Zinc stable isotopes in urine as diagnostic for cancer of secretory organs. Metallomics, 2021,
709 13, mfab020. doi: 10.1093/mtomcs/mfab020

50. Costa-Rodriguez, M., Anoushkina, Y., Lauwens, S., Van Vlierberghe, H., Delanghe, J., Vanhaecke, F. Isotopic analysis of Cu in blood serum by multi-collector ICP-mass spectrometry: A new approach for the diagnosis and prognosis of liver cirrhosis? Metallomics, 2015, 7, 491–498. doi:10.1039/c4mt00319e.

51. Costas-Rodríguez, M., Van Campenhout, S., Hastuti, AA., Devisscher, L., Van Vlierberghe, H., Vanhaecke, F. Body distribution of stable copper isotopes during the progression of cholestatic liver disease induced by common bile duct ligation in mice. Metallomics, 2019, 11, 1093-1103. doi:10.1039/c8mt00362a.

52. Lamboux, A., Couchonnal-Bedoya, E., Guillaud, O., Laurencin, C., Lion-François, L., Belmalih, A., Balter, V. The blood copper isotopic composition is a prognostic indicator of the hepatic injury in Wilson disease. Metallomics, 2020, 12, 1781-1790. doi:10.1039/d0mt00167h.

53. Lauwens, S., Costas-Rodríguez, M., Van Vlierberghe, H., Vanhaecke, F. Cu isotopic signature in blood serum of liver transplant patients: a follow-up study. Sci. Rep., 2016, 6, 30683. doi: 10.1038/srep30683.

54. Van Campenhout, S., Hastuti, AA., Lefere, S., Van Vlierberghe, H., Vanhaecke, F., Costas-Rodríguez, M., Devisscher, L. Lighter serum copper isotopic composition in patients with early non-alcoholic fatty liver disease. BMC Research Notes, 2020, 13, 225. doi: 10.1186/s13104-020-05069-3.

736 55. Larner, AJ. MACE for diagnosis of dementia and MCI: examining cut-offs and predictive
737 values. Diagnostics, 2019, 9, 51. doi: 10.3390/diagnostics9020051.
738
739 56. Solovyev, N., El-Khatib, A. H., Costas-Rodríguez, M., Schwab, K., Griffin, E., Raab, A.,
740 Vanhaecke, F. Cu, Fe, and Zn isotope ratios in murine Alzheimer's disease models suggest
741 specific signatures of amyloidogenesis and tauopathy. J. Biol. Chem., 2021, 296. doi :
742 10.1016/j.jbc.2021.100292.
743
744 57. Tõugu, V, Tiiman, A, Palumaa, P. Interactions of Zn(ii) and Cu(ii) ions with Alzheimer's
745 amyloid-beta peptide. Metal ion binding, contribution to fibrillization and toxicity. Metallomics,
746 2011, 3, 250–. doi:10.1039/c0mt00073f
747
748
749 58. Zhao, J., Shi, Q., Tian, H., Li, Y., Liu, Y., Xu, Z., Meunier, B. TDMQ20, a specific copper
750 chelator, reduces memory impairments in Alzheimer's disease mouse models. ACS Chem.
751 Neurosci., 2020, 12, 140-149. doi: 10.1021/acschemneuro.0c00621.
752
753 59. Sarell, CJ., Syme, CD., Rigby, SE., Viles, JH. Copper (II) binding to amyloid-β fibrils of
754 Alzheimer's disease reveals a picomolar affinity: stoichiometry and coordination geometry are
755 independent of Aβ oligomeric form. Biochemistry, 2009, 48, 4388-4402. doi:
756 10.1021/bi900254n.
757
758 60. Barritt, JD., Viles, JH. Truncated amyloid-β (11–40/42) from Alzheimer disease binds Cu2+
759 with a femtomolar affinity and influences fiber assembly. J. Biol. Chem, 2015, 290, 27791-
760 27802. doi: 10.1074/jbc.M115.684084
761
762

763 61. Drew, SC. The case for abandoning therapeutic chelation of copper ions in Alzheimer's Disease.
764 Front. Neurosci., 2017, 11, 317. doi: 10.3389/fnins.2017.00317
765
766 62. Mital, M., Wezynfeld, NE., Frączyk, T., Wiloch, MZ., Wawrzyniak, UE., Bonna, A., Drew, SC.
767 A functional role for Aβ in metal homeostasis? N-truncation and high-affinity copper binding.
768 Angew. Chem., 2015, 127, 10606-10610. doi: 10.1002/ange.201502644.
769
770
771 63. Dai, B., Sargent, CJ., Gui, X., Liu, C., Zhang, F. Fibril self-assembly of amyloid–spider silk
772 block polypeptides. Biomacromolecules, 2019, 20, 2015-2023. doi:
773 10.1021/acs.biomac.9b00218.
774
775 64. Strozyk, D., Launer, LJ., Adlard, PA., Cherny, RA., Tsatsanis, A., Volitakis, I., Bush, AI. 2009.
776 Zinc and copper modulate Alzheimer Aβ levels in human cerebrospinal fluid. Neurobiol.
777 Aging., 30, 1069-1077. doi:10.1016/j.neurobiolaging.2007.10.012.
778
779
780 65. Maréchal, CN., Télouk, P., Albarède, F. Precise analysis of copper and zinc isotopic
781 compositions by plasma-source mass spectrometry. Chem. Geol., 1999, 156, 251-273. doi:
782 10.1016/S0009-2541(98)00191-0
783
784 66. Sauzéat, Marta Costas-Rodríguez, Emmanuelle Albalat, Nadine Mattielli, Frank
785 Vanhaecke,Vincent Balter, Inter-comparison of stable iron, copper and zinc isotopic
786 compositions in six reference materials of biological origin, Talanta, 2021, 221, 121-576. doi:
787 10.1016/j.talanta.2020.121576.
788

789 67. Maynard, CJ., Cappai, R., Volitakis, I., Cherny, RA., White, AR., Beyreuther, K., Li, QX.
790 Overexpression of Alzheimer's disease amyloid-β opposes the age-dependent elevations of brain
791 copper and iron. J. Biol. Chem., 2002, 277, 44670-44676. doi: 10.1074/jbc.M204379200.
792
793 68. Madaric, A., Ginter, E., Kadrabova, J. Serum copper, zinc and copper/zinc ratio in males:
794 influence of aging. Physiol. Res., 1994, 43, 107-107.
795
796 69. Deibel, MA., Ehmann, WD., Markesbery, WR. Copper, iron, and zinc imbalances in severely
797 degenerated brain regions in Alzheimer's disease: possible relation to oxidative stress. J. Neurol.
798 Sci., 1996, 143, 137-142. doi: 10.1016/S0022-510X(96)00203-1.
799
800 70. Miller, LM., Wang, Q., Telivala, TP., Smith, RJ., Lanzirotti, A., Miklossy, J. Synchrotron-based
801 infrared and X-ray imaging shows focalized accumulation of Cu and Zn co-localized with β-
802 amyloid deposits in Alzheimer's disease. J. Sruct. Biol., 2006, 155, 30-37. doi:
803 10.1016/j.jsb.2005.09.004.
804
805
806 71. Bales, CW., Freeland-Graves, JH., Askey, S., Behmardi, F., Pobocik, RS., Fickel, JJ., Greenlee,
807 P. Zinc, magnesium, copper, and protein concentrations in human saliva: age-and sex-related
808 differences. Am. J. Clin. Nutr., 1990, 51, 462-469. doi: 10.1093/ajcn/51.3.46.
809
810 72. Palm R, Wahlström G, Hallmans G. Age related changes in weight and the concentrations of
811 zinc and copper in the brain of the adult rat. Lab. Anim., 1990, 24, 240-245.
812 doi:10.1258/002367790780866128
813
814 73. Albarède, F. Metal stable isotopes in the human body: a tribute of geochemistry to medicine.
815 Elements, 2015, 11, 265-269. doi: 10.2113/gselements.11.4.265

74. Jaouen K., Balter V., Herrscher E., Lamboux A., Télouk P., Albarède F. Fe and Cu stable isotopes in archeological human bones and their relationship to sex. Am. J. Phys. Anthropol., 2012, 148:334–340. doi: 10.1002/ajpa.22053.

75. Van Den Eeden SK, Tanner CM, Bernstein AL, Fross RD, Leimpeter A, Bloch DA, Nelson LM. Incidence of Parkinson's disease: variation by age, gender, and race/ethnicity. Am. J. Epidemiol. 2003, 157, 1015-22. doi: 10.1093/aje/kwg068.

76. Wooten GF, Currie LJ, Bovbjerg VE, Lee JK, Patrie J. Are men at greater risk for Parkinson's disease than women? J. Neurol. Neurosurg. Psychiatry., 2004, 637-9. doi: 10.1136/jnnp.2003.020982.

77. Shulman, A., Goldstein, B., Strashun, A. M. Central nervous system neurodegeneration and tinnitus: a clinical experience. Int Tinnitus J, 2007, 13, 118-31.

78. Hebert LE, Weuve J, Scherr PA, Evans DA. Alzheimer disease in the United States (2010-2050) estimated using the 2010 census. Neurology, 2013, 80, 1778-83. doi: 10.1212/WNL.0b013e31828726f5.

79. Lapane, KL., Gambassi, G., Landi, F., Sgadari, A., Mor, V., Bernabei, R. Gender differences in predictors of mortality in nursing home residents with AD. Neurology, 2001, 56, 650-654. doi:10.1212/WNL.56.5.650.

80. Everett, J., Lermyte, F., Brooks, J., Tjendana-Tjhin, V., Plascencia-Villa, G., Hands-Portman, I, Telling, N. D. Biogenic metallic elements in the human brain? Sci. Adv., 2021, 7, eabf6707. doi: 10.1126/sciadv.abf6707.

81. Giacoppo, S., Mandolino, G., Galuppo, M., Bramanti, P., Mazzon, E. Cannabinoids: new promising agents in the treatment of neurological diseases. Molecules, 2014, 19, 18781-18816. doi : 10.3390/molecules191118781.

82. Klevay, LM. Alzheimer's disease as copper deficiency. Med. Hypotheses., 2008, 70, 802-807. doi: 10.1016/j.mehy.2007.04.051

83. Exley, C., House, E., Polwart, A., Esiri, MM. Brain burdens of aluminum, iron, and copper and their relationships with amyloid-β pathology in 60 human brains. J. Alzheimer's Dis., 2012, 31, 725-730. doi: 10.3233/JAD-2012-120766.

84. Hung YH, Bush AI, La Fontaine S. Links between copper and cholesterol in Alzheimer's disease. Front. Physiol., 2013, 4-111. doi: 10.3389/fphys.2013.00111

85. Abreu IA, Cabelli DE. Superoxide dismutases-a review of the metal-associated mechanistic variations. Biochim Biophys Acta. 2010, 263-74. doi: 10.1016/j.bbapap.2009.11.005. Epub 2009 Nov 13. PMID: 19914406.

86. Fukai, T., Ushio-Fukai, M. Superoxide dismutases: role in redox signaling, vascular function, and diseases. Antioxid Redox Signal., 2011, 15, 1583-606. doi: 10.1089/ars.2011.3999.

87. Crapo, JD., Oury, T., Rabouille, C., Slot, JW., Chang, LY. Copper, zinc superoxide dismutase is primarily a cytosolic protein in human cells. Proc. Natl. Acad. Sci. U S A, 1992, 89, 10405-9. doi: 10.1073/pnas.89.21.10405.

88. Okado-Matsumoto A., Fridovich I. Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu,Zn-SOD in mitochondria. J. Biol. Chem., 2001, 276, 38388-93. doi: 10.1074/jbc.M105395200.

89. Warner, HR. Superoxide dismutase, aging, and degenerative disease. Free. Radic. Biol. Med., 1994, 17, 249-258. doi: 10.1016/0891-5849(94)90080-9

90. Larner, F., McLean, CA., Halliday, A., Blaine R. Copper Isotope Compositions of Superoxide Dismutase and Metallothionein from Post-Mortem Human Frontal Cortex. Inorganics, 2019, 7, 86–. doi:10.3390/inorganics7070086

91. Rybka J, Kupczyk D, Kędziora-Kornatowska K, et al. Glutathione-related antioxidant defense system in elderly patients treated for hypertension. Cardiovasc. Toxicol., 2011, 1-9. doi: 10.1007/s12012-010-9096-5.

92. Parpura V., Heneka M. T., Montana V., Oliet S. H., Schousboe A., Haydon PG., Glial cells in (patho)physiology. J. Neurochem., 2012, 121, 4–27. doi: 10.1111/j.1471-4159.2012.07664.x

93. Schmidt, MM., Dringen, R. Glutathione (GSH) synthesis and metabolism. Neur. Met. in vivo, 2012, 1029-1050. doi: 10.1007/978-1-4614-1788-0_36.

94. Dringen, R., Bishop, GM., Koeppe, M., Dang, TN., Robinson, SR. The pivotal role of astrocytes in the metabolism of iron in the brain. Neurochem. Res, 2007, 32, 1884–1890. doi: 10.1007/s11064-007-9375-0

95. Tiffany-Castiglioni, E., Hong, S., Qian, Y. Copper handling by astrocytes: insights into neurodegenerative diseases. Int. J. Dev. Neurosci., 2011, 29, 811-818. doi: 10.1016/j.ijdevneu.2011.09.004

96. Dringen, R., Scheiber, IF., Mercer, JF. Copper metabolism of astrocytes. Front. Aging. Neurosci., 2013, 5, 9. doi: 10.3389/fnagi.2013.00009.

97. Scheiber IF., Mercer JF., Dringen R. 2010. Copper accumulation by cultured astrocytes. Neurochem. Int, 451-60. doi: 10.1016/j.neuint.2009.12.002.

98. Aschner M., Astrocyte metallothioneins (MTs) and their neuroprotective role. Ann.N. Y.Acad.Sci. 1997, 825, 334-347. doi: 10.1111/j.1749-6632.1997.tb48445.x

99. Dringen, R., Hamprecht, B. Glutathione restoration as indicator for cellular metabolism of astroglial cells. Dev. Neurosci, 1998, 20, 401-7. doi: 10.1159/000017337.

100. Lee, J., Pena, M. M. O., Nose, Y., Thiele, D. J. Biochemical characterization of the human copper transporter Ctr1. J. Biol. Chem., 2002, 277, 4380-4387. doi: 10.1074/jbc.M104728200

101. Sikora, E., Bielak-Zmijewska, A., Dudkowska, M., Krzystyniak, A., Mosieniak, G., Wesierska, M., Wlodarczyk, J. Cellular senescence in brain aging. Front. Aging. Neurosci., 2021, 13, 646924. doi: 10.3389/fnagi.2021.646924.

926 102. Bhat R., Crowe EP., Bitto A., Moh M., Katsetos CD., Garcia FU., Astrocyte Senescence as a
927 Component of Alzheimer's Disease. PLoS ONE 7, 2012, e45069. doi :
928 10.1371/journal.pone.0045069
929
930
931 103. Han, X., Zhang, T., Liu, H., Mi, Y., Gou, X. Astrocyte senescence and Alzheimer's disease:
932 A review. Front. Aging. Neurosci., 2020, 12, 148. doi:10.3389/fnagi.2020.00148.
933
934 104. Zhou, Q., Zhang, Y., Lu, L., Zhang, H., Zhao, C., Pu, Y., Yin, L. Copper induces microglia-
935 mediated neuroinflammation through ROS/NF-κB pathway and mitophagy disorder. Food.
936 Chem. Toxicol., 2022, 168, 113-369. doi : 10.1016/j.fct.2022.113369.
937
938 105. Nayak, D., Roth, TL., McGavern, DB. Microglia development and function. Annu. Rev.
939 Immunol., 2014, 32, 367–402. doi:10.1146/annurev-immunol-032713-120240
940
941 106. Ashraf, A., Michaelides, C., Walker, TA., Ekonomou, A., Suessmilch, M., Sriskanthanathan,
942 A., So, PW. Regional distributions of iron, copper and zinc and their relationships with glia in a
943 normal aging mouse model. Front. Aging. Neurosci, 2019, 351. doi: 10.3389/fnagi.2019.00351
944
945 107. Morel, JD., Sauzéat, L., Goeminne, L.J.E. et al. The mouse metallomic landscape of aging
946 and metabolism. Nat Commun, 2022, 607. https://doi.org/10.1038/s41467-022-28060-x
947
948 108. World Health Organization, Dementia, Key facts, 2015, https://www.who.int/news-
949 room/fact-sheets/detail/dementia.
950
951 109. Lamboux, A., Couchonnal, E., Guillaud, O., Laurencin, C., Lion-François, L., Belmalih, A.,
952 Mintz, E., Brun, V., Bost, M., Lachaux, A., Balter, V., The blood copper isotopic composition

is a prognostic indicator of the hepatic injury in Wilson disease, Metallomics, 2020, 12. 10.1039/D0MT00167H.

110. Aramendía, M., Rello V., Luis Resano, M., Vanhaecke, F. Isotopic analysis of Cu in serum samples for diagnosis of Wilson's disease: A pilot study 2013. J. Anal. At. Spectrom.. 675-681. 10.1039/C3JA30349G.

111. Frutiger, K., Lukas, T., J., Gorrie, G.,, et al. Gender difference in levels of Cu/Zn superoxide dismutase (SOD1) in cerebrospinal fluid of patients with amyotrophic lateral sclerosis. 2008 Amyotrophic Lateral Sclerosis, 184-187. 10.1080/17482960801984358

112. Mahan, B., et al. Isotope metallomics approaches for medical research. Cell Mol Life Sci, 77, 2020. doi:10.1007/s00018-020-03484-0

113. An, Y., Li, S., Huang, X., Chen, X., Shan, H., Zhang M. The Role of Copper Homeostasis in Brain Disease. Int J Mol Sci. 2022, 23. doi:10.3390/ijms232213850

114. Huuskonen, MT., Tuo, QZ., Loppi, S, et al. The Copper bis(thiosemicarbazone) Complex Cu^{II}(atsm) Is Protective Against Cerebral Ischemia Through Modulation of the Inflammatory Milieu. Neurotherapeutics. 2017, 14. doi:10.1007/s13311-016-0504-9

115. Soon, CPW., Donnelly, PS., Turner, BJ., et al. Diacetylbis(N(4)-methylthiosemicarbazonato) copper(II) (CuII(atsm)) protects against peroxynitrite-induced nitrosative damage and prolongs survival in amyotrophic lateral sclerosis mouse model. J Biol Chem. 2011, 286. doi:10.1074/jbc.M111.274407

116. Luu TH, Peters D, Lahoud E, Gérard Y, Moynier F. Copper Isotope Compositions Measured Using a Sapphire Dual Path MC-ICPMS with a Collision/Reaction Cell. Anal Chem. 2024 Jan 5. doi: 10.1021/acs.analchem.3c05192. Epub ahead of print. PMID: 38179926.

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