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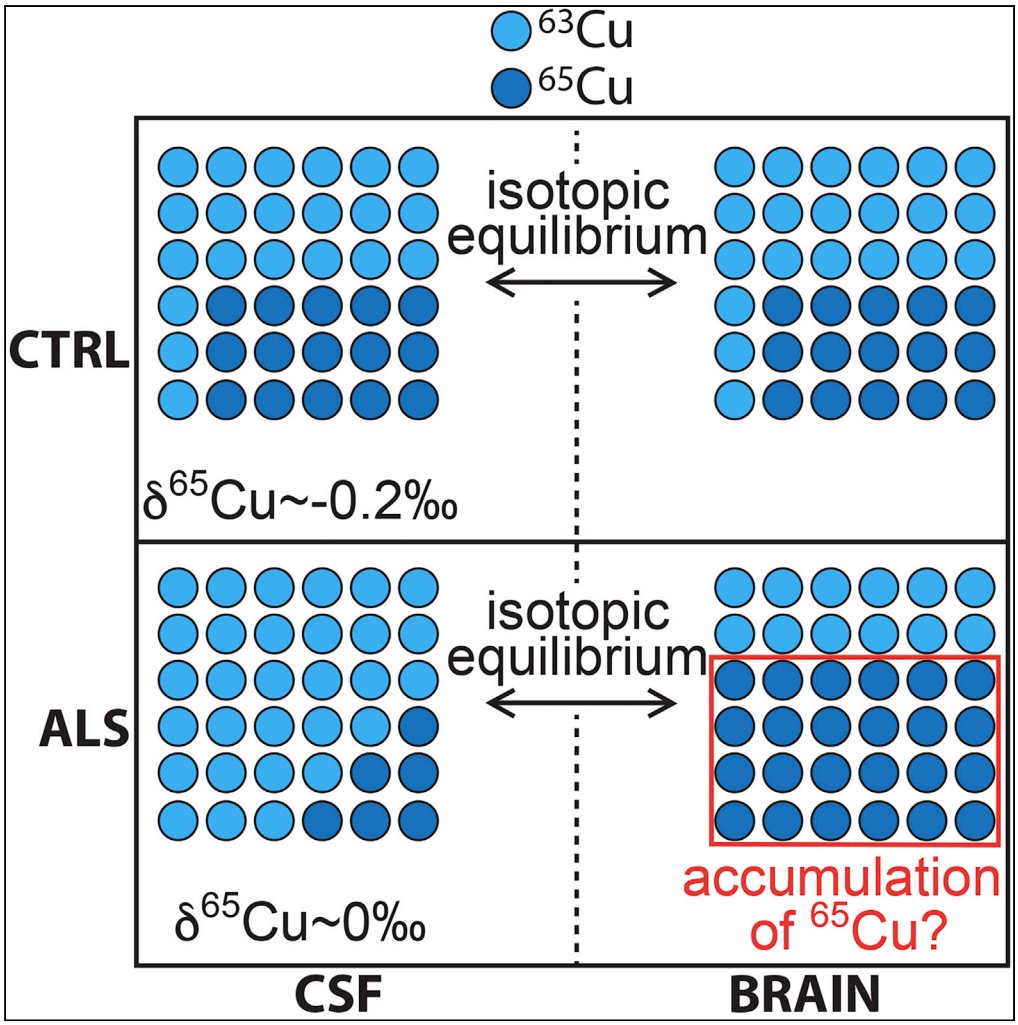

Lucie Sauzéat, Emilien Bernard, Armand Perret-Liaudet, ..., Emmanuel Broussolle, Pascal Leblanc, Vincent Balter

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HIGHLIGHTS

Redox-active metals are implicated in ALS through oxidative stress

Concentrations of these metals in CSFs of patients with ALS are non-specific

Copper stable isotope composition in CSFs of patients with ALS are specific

Isotopic balance between CSFs and brain is probably the mechanism

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Isotopic Evidence for Disrupted Copper Metabolism in Amyotrophic Lateral Sclerosis

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SUMMARY

Redox-active metals are thought to be implicated in neurodegenerative diseases including amyotrophic lateral sclerosis (ALS). To address this point, we measured the concentrations of 12 elements and, for the first time, the stable isotope compositions of copper (redox-active) and zinc (redox-inactive) in human cerebrospinal fluids of 31 patients with ALS, 11 age-matched controls (CTRL), and 14 patients with Alzheimer disease. We first show that metal concentrations weakly discriminate patients with ALS from the two other groups. We then report that zinc isotopic compositions are similar in the three groups, but that patients with ALS have significantly 65copper-enriched isotopic compositions relative to CTRL and patients with AD. This result unambiguously demonstrates that copper is implicated in ALS. We suggest that this copper isotopic signature may result from abnormal protein aggregation in the brain parenchyma, and propose that isotopic analysis is a potential tool that may help unraveling the molecular mechanisms at work in ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is one of the most harmful neurodegenerative diseases characterized by the progressive deterioration of the upper and lower motor neurons, leading to muscle weakness and atrophy, severe paralysis, and finally death typically within 3–5 years after symptom onset (Narasimhan, 2015). Currently, although genetic factors have been identified to play a role in familial ALS, which represents only 10% of the reported cases (Taylor et al., 2016), the leading mechanisms accounting for motor neuron degeneration in sporadic ALS (i.e., 90% of ALS cases) remains unknown. In addition, because of the complexity of the pathology, reliable markers (Savage, 2017) and consequently efficient treatments are still missing (Scott, 2017). Recently, biomarkers in cerebrospinal fluid (CSF) including concentrations of neurofilament light chain (Lu et al., 2015) and chemokines (Lind et al., 2016) have been identified. However, concentrations of neurofilament light chain in CSF have also been reported to be elevated in other neurological disorders such as Alzheimer disease (AD) (Zetterberg et al., 2016) and Parkinson disease (Bäckström et al., 2015). Robust and specific ALS markers are still scarce.

As for other neurodegenerative diseases, the progression of ALS is associated with the production of free radicals such as reactive oxygen species (Barnham and Bush, 2014), the production of which can be favored by the presence of free, redox-active metals like copper (Cu) (Barnham and Bush, 2014). Conversely, these metals can also be catalytic cofactors of several enzymes, like Cu/Zn superoxide dismutase (SOD1), involved in free radical detoxification by catalyzing highly toxic products (i.e., superoxide) to less dangerous species such as dioxygen and hydrogen peroxide (Valentine et al., 2005). Redox-active metals thus play a pivotal role in both the pro- and anti-oxidant homeostasis, and it is expected that dysregulation affecting these pathways will be characterized by significant elemental impairment. In mice models of familial ALS caused by mutations in SOD1, Cu has been observed to accumulate in the spinal cord (Tokuda et al., 2013). Metal dysregulations have also been observed for other redox-active metals in CSFs of patients with ALS including iron and manganese (Barnham and Bush, 2014; Roos et al., 2013). Zinc (Zn), which is not redox active, but binds to SOD1, has also been reported to be dysregulated in CSFs of patients with ALS (Hozumi et al., 2011). Comparing published results generally leads to equivocal conclusions about the usefulness of metal concentrations in CSFs to diagnose and study ALS (Figure S1).

Contrary to concentrations, stable isotope compositions may offer a more comprehensive view on biological reactions and neurological disease progression. This is due to two main reasons. First, stable isotope

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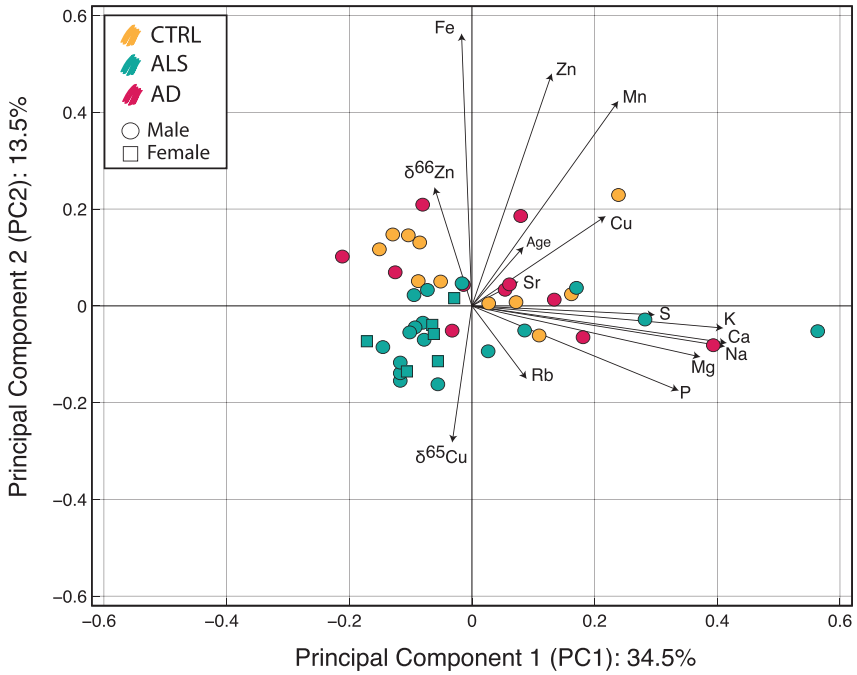

Figure 1. Principal Component Analysis of the Results

The PCA allows the distinction of patients with ALS (green points) from CTRL subjects (yellow points) and subjects with AD (pink points). Concentrations and isotopic compositions are normalized to their SD. The two principal components (PC1 and PC2) are represented and explain ~50% of the total variance in chemical composition. For each component, black straight lines show the loading factors (i.e., the weight of each variable on the principal components). Circles and squares points stand for male and female subjects, respectively. No significant variation is observed between the male and the female subjects within the ALS group.

compositions are measured with a precision of about two orders of magnitude better than concentrations. Second, the intensity of enrichment or depletion of a metal in a given compartment is hardly predictable, whereas isotopic fractionation, i.e., the variation of the natural abundances of stable isotope ratios between coexisting compartments, can usually be quantitatively predicted by *ab initio* calculations (Albarède et al., 2016). In blood, Cu isotope compositions (δ^{65}Cu) exhibit, for example, significant differences between men and women (Jaouen et al., 2012) as well as during aging (Jaouen et al., 2013). In addition, the δ^{65}Cu value varies in pathological conditions such as cancer (Balter et al., 2015), Wilson disease, or liver cirrhosis (Costas-Rodriguez et al., 2016). Regarding neurodegenerative disorders, although Cu isotope compositions seem to be insensitive to the SOD1^{G93A} mutation in the brain of mouse model (Enge et al., 2017), they are highly responsive in models of prion protein knockout (PrP-KO) mice (Büchl et al., 2008; Miller et al., 2016). Zinc isotope compositions (δ^{66}Zn) were also scrutinized in mouse models of neurodegenerative diseases. In PrP-KO mice, the brain δ^{66}Zn values were heavier than in wild-type controls (Büchl et al., 2008), a pattern also observed in mutant mouse (APPswe/PSEN1dE9) developing Alzheimer-like disease (Moynier et al., 2017).

In the present work, to further explore the potential of elemental concentrations and isotopic compositions for clinical studies of ALS, we measured the concentration of 12 major and trace elements and the Cu and Zn isotopic compositions in the CSFs of patients with ALS (n = 31), age-matched controls (CTRL, n = 11), and patients with AD (n = 14).

RESULTS

Major and trace element concentrations as well as Cu and Zn isotopic compositions measured in CSFs of subjects with ALS, CTRL, and subjects with AD are all reported in Data S1.

ALS-Related Dysregulations of Major and Trace Element Concentrations

To evaluate the whole pattern of variations, we used a principal component analysis (PCA) (Figure 1). The most noticeable feature is the chemical distinction between CSFs of patients with ALS and CTRL as

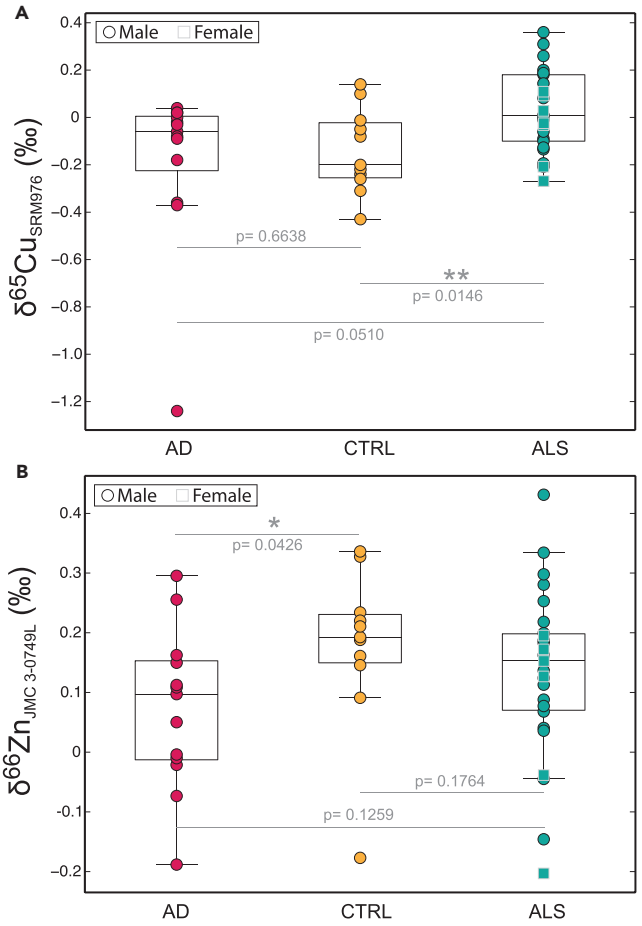

Figure 2. Copper (δ^{65}Cu) and Zinc (δ^{66}Zn) Isotopic Variability in Cerebrospinal Fluids

(A and B) (A) Copper (δ^{65}Cu) and (B) zinc (δ^{66}Zn) isotopic compositions measured in CSFs of CTRL (yellow dots), patients with AD (red dots), and patients with ALS (green dots). Circle and square points are for male and female subjects, respectively. For each boxplot, the central mark is the median; the edges of the box are the first (i.e., 25th percentiles) and the third quartiles (i.e., 75th percentiles), respectively; and the points outside the boxes extend to the most extreme data points (i.e., not considered outliers). Significance level (i.e., p value) was determined using non-parametric "two-sided," Wilcoxon-Mann-Whitney U tests. **p < 0.005, *p < 0.05.

illustrated by the y principal component axis (Figure 1). Subjects with ALS have, for example, significant lower Fe concentrations (Wilcoxon-Mann-Whitney, p value = 2.426×10^{-3}) and Mn concentrations (Wilcoxon-Mann-Whitney, p value = 2.206×10^{-3}) (Figure 1) but higher Rb concentrations (Wilcoxon-Mann-Whitney, p value = 1.003×10^{-2}) (Figure 1 and Data S1). Conversely, Zn concentrations (Wilcoxon-Mann-Whitney, p value = 0.0715) and Cu concentrations (Wilcoxon-Mann-Whitney, p value = 0.1610) do not exhibit any systematics (Figure S1), an observation that also holds for other trace and major elements such as Sr, S, P, or Na (Data S1). Although these results are in line with a couple of studies, e.g., Ostachowicz et al. (2006), Kanias and Kapaki (1997), and Kapaki et al. (1997), opposite or equivocal results have also been reported (Figure S1 and references therein).

ALS-Related Dysregulations of Cu-Zn Isotopic Compositions

Regarding the Cu and Zn isotopic compositions, Cu isotopic ratios in ALS (median $\delta^{65}Cu_{ALS}$ = 0.01‰, 25th percentile = −0.10‰; 75th percentile = 0.18‰) are significantly heavier (Wilcoxon-Mann-Whitney, p value = 0.0146) than CTRL (median $\delta^{65}Cu_{CTRL}$ = −0.20‰, 25th percentile = −0.26‰; 75th percentile = −0.02‰) (Figure 2A). By contrast, no significant difference is observed between Zn isotopic compositions of ALS (median $\delta^{66}Zn_{ALS}$ = 0.15‰, 25th percentile = 0.07‰; 75th percentile = 0.20‰) and CTRL (median $\delta^{66}Zn_{CTRL}$ = 0.19‰, 25th percentile = 0.15‰; 75th percentile = 0.23‰) (Wilcoxon-Mann-Whitney, p value = 0.1764) (Figure 2B).

Similar observations can be made by comparing the $\delta^{65}Cu$ value of patients with ALS and AD (median $\delta^{65}Cu_{AD}$ = −0.06‰, 25th percentile = −0.23‰; 75th percentile = 0.01‰), patients with AD being chemically indistinguishable from CTRL subjects (Wilcoxon-Mann-Whitney, p value = 0.6638) (Figure 2A). However, subjects with AD tend to have a slightly lower $\delta^{66}Zn$ value (median $\delta^{66}Zn$ = 0.10‰, 25th percentile = −0.01‰; 75th percentile = 0.15‰) than CTRL subjects (Wilcoxon-Mann-Whitney, p value = 0.0426).

Effect of Biological and Clinical Parameters on Elemental Concentrations and Isotopic Compositions

Biological (gender and age at sample collection) and clinical (localization of first symptoms, Awaji criteria, ALSFRS-R score, delay between sampling date and first visible symptoms) parameters are given in Table S1. Focusing on the ALS and CTRL groups, neither clinical parameters nor biological parameters show significant association with elemental concentrations and Cu-Zn isotopic compositions. An illustration is the similar $\delta^{65}Cu$ values observed between male and female subjects in the ALS group (median $\delta^{65}Cu_{ALS\text{-}female}$ = −0.02‰, 25th percentile = −0.16‰, 75th percentile = 0.07‰; median $\delta^{65}Cu_{ALS\text{-}male}$ = +0.01‰, 25th percentile = −0.10‰, 75th percentile = 0.20‰) (Wilcoxon-Mann-Whitney, p value = 0.2721) as well as between patients with ALS characterized by different site at onset (i.e., lower limbs, ML; upper limbs, MS; bulbar, bulb) (median $\delta^{65}Cu_{ALS\text{-}MI}$ = 0.08‰, 25th percentile = 0.01‰, 75th percentile = 0.14‰; median $\delta^{65}Cu_{ALS\text{-}MS}$ = −0.06‰, 25th percentile = −0.15‰, 75th percentile = 0.07‰; and median $\delta^{65}Cu_{ALS\text{-}bulb}$ = −0.03‰, 25th percentile = −0.13‰, 75th percentile = 0.18‰) (Table S1).

DISCUSSION

Disruption of the homeostasis of metals such as Cu and Zn is a key feature of neurodegenerative diseases, including ALS, leading to multiple abnormalities in the CSF, brain, and spinal cord, where they are inappropriately redistributed (Barnham and Bush, 2014). The changes in the distribution of metals seem to be controlled by their sequestration within misfolded protein aggregates as shown, for example, in amyloid-β (Aβ) plaques within the AD brain (Pithadia and Lim, 2012). In ALS, misfolded protein aggregates including TAR DNA-binding protein 43 (TDP-43), fused in sarcoma (FUS) (Pokrishevsky et al., 2012), and SOD1 (Valentine et al., 2005) have been reported to be present in the brain. Described as the main pathological hallmark of ALS, these proteins, like Aβ for AD, may concentrate metals. The local accumulation is likely to deplete the surrounding environment, ultimately scavenging the CSF burden, leading to decreased CSF concentrations. However, no change in elemental concentrations in patients with ALS, or AD, compared with CTRL, is observed in the present study, and more generally in the literature (Figure S1 and reference therein). The absence of any systematics may result from the natural wide range of metal concentrations in human fluids (e.g., Iyengar and Wolttiez, 1988). A more robust pattern of variations of metal concentrations is probably specific to each patient and would be more accessible through analysis of cohorts. The absence of any systematics may also result from exogenous contamination. Collection, storage, and preparation of CSFs can be sensitive to trace element contamination (Garçon et al., 2017). CSFs are made of 99% water and have very low metal contents; hence the risk of contamination is high. In this study, we ensure the absence of external contamination by quantifying the chemical content that may be released by sampling procedures and storage in tubes as well as dropper-type pipettes used to collect and conserve CSF. A complete description of the procedure is given in Transparent Methods. We also took care of low-acid blanks before and after each column chemistry, and we only used vinyl gloves as suggested by Garçon et al. (2017).

One advantage of using isotopic composition over concentration for a given element is that isotopic fractionation is not dependent on the amount of the element. Isotopic compositions are thus theoretically more reliable biomarkers than concentrations. Here, we found that CSFs of patients with ALS have significantly heavier Cu isotopic composition compared with age-matched CTRL (Wilcoxon-Mann-Whitney, p value = 0.0146) and also tend to be different from those of patients with AD by having slightly heavier $\delta^{65}Cu$ (Wilcoxon-Mann-Whitney, p value = 0.0510) (Figure 2A), whereas again no distinction is observed for Cu concentration (Figure S1). Using receiver operating characteristic (ROC) analysis, we determined a $\delta^{65}Cu$ cutoff value of −0.05‰ with a sensitivity and a specificity of 73% and 65% respectively, and an accuracy of 76% (Figure S2). This is a modest score compared, for instance, with the results obtained by Pasinetti et al. (2006), with a sensitivity, specificity, and accuracy of 91%, 97%, and 95%, respectively, using three protein concentrations in CSF. Noteworthy is the specificity of the CSF $\delta^{65}Cu$ values of ALS as illustrated by the absence of significant $\delta^{65}Cu$ difference between subjects with AD and CTRL subjects (Wilcoxon-Mann-Whitney, p value = 0.6638) (Figure 2A). Further additional studies

and evidence of the mechanisms at work are undoubtedly needed to improve the present results achieved by the ROC test.

Recently, Moynier et al. (2017) showed that brains of mice with AD have higher $\delta^{66}Zn$ value than wild-type mice and suggested that this isotopic enrichment may result from the formation of Aβ plaques in the brain parenchyma binding preferentially heavier Zn isotopes. Following this assumption, if the binding of a metal in the protein aggregates of the brain is associated with an isotopic fractionation, this must be balanced in the CSF, the brain and CSF being two complementary reservoirs in a closed system, the CNS. At first glance, our results on patients with AD support the hypothesis of an isotopic equilibrium between brain and CSF, because patients with AD exhibit CSF depleted in heavier Zn isotopes relative to CTRL subjects. However, this requires further analysis of CSF of patients with AD and CTRL subjects, which is beyond the scope of the present work. Focusing on ALS, a similar reasoning can be proposed with aggregation of SOD1 in the brain associated with a Cu isotopic fractionation. Direct evidence could be obtained by measuring the Cu isotope composition of aggregates, but here we can test if the assumption of an isotopic equilibrium between brain and CSF resists mass conservation laws. The concentration of Cu in brain varies between 3 and 5 μg/g (Scheiber et al., 2014). The brain volume ranges from 1,300 to 1,500 mL leading to a brain Cu burden (M_B) of 3.9–7.5 mg. Regarding CSF, the concentration of Cu ranges from 0.02 to 0.2 μg/mL (Table S1; Iyengar and Wolttiez, 1988) and, with a volume of 150–250 mL, this gives a total Cu mass (M_{CSF}) of 3–5 μg. As a first approximation, the CNS can be considered as a closed system at steady state and, $\delta^{65}Cu_B$ and $\delta^{65}Cu_{CSF}$ being the Cu isotopic composition in brain and CSF, respectively, one can write that:

$$M_B \,.\, \delta^{65}Cu_B \Leftrightarrow M_{CSF} \,.\, \delta^{65}Cu_{CSF}$$

If the isotopic composition of Cu varies in one compartment by a factor Δ, this must be balanced in the other one such that:

$$M_B \,.\, \Delta\delta^{65}Cu_B = M_{CSF} \,.\, \Delta\delta^{65}Cu_{CSF}$$

It is thus possible to calculate the Cu isotopic offset in the CSF ($\Delta\delta^{65}Cu_{CSF}$) that would result from the incorporation of Cu in the brain with an isotopic fractionation $\Delta\delta^{65}Cu_B$. The assumption that the CNS is a closed system is made for the sake of simplicity for purposes of mass balance calculations. Physiologically, metals and other molecules are supplied in the CNS by blood, but classically stay there and accumulate with time. The inward flux of Cu in the CNS by time unit is unknown but should be very small compared with the mass of the CNS, considering that the Cu amount in blood (~5 μg) is three orders of magnitude less than that in CNS. Results of the calculations are illustrated by Figure 3. Intuitively, because there are between 78 and 2,500 more Cu in the brain than in the CSF, the $\Delta\delta^{65}Cu_B$ value must be very small. Indeed, when the highest M_B/M_{CSF} ratio is considered (2,500), a contribution of only 0.01% of pathological Cu associated with a $\Delta\delta^{65}Cu_B$ value of 0.5‰ can trigger a $\Delta\delta^{65}Cu_{CSF}$ offset of almost 0.2‰, which is the observed Cu isotopic difference between ALS and CTRL. An identical $\Delta\delta^{65}Cu_{CSF}$ offset is obtained with a contribution of pathological Cu of 0.5% when the lowest M_B/M_{CSF} ratio of 78 is considered. Altogether, these results show that minute proportions of aggregates formation associated with relevant Cu isotopic fractionation can likely explain the observed differences between the CSFs $\delta^{65}Cu$ values of CTRL and patients with ALS. Whatever the proportion of formed aggregates, the increase of the ALS $\delta^{65}Cu$ values implies for mass balance requirement that ^{63}Cu preferentially binds in aggregates. Measuring the Cu isotopic composition of protein aggregates and normal adjacent areas in autopsies of brains of patients with ALS, and/or in brains of ALS animal model like the $SOD1^{G93A}$ mice, can be the aim of future experiments to challenge this hypothesis.

Another way to explain the specific Cu isotopic composition of ALS would involve a differential Cu isotope fractionation between detergent-soluble SOD1 and aggregated, insoluble, SOD1. In mutant (G37R and G93A) SOD1 proteins from SOD1-ALS transgenic mice spinal cords, Lelie et al. (2011) found that aggregated, insoluble, SOD1 is metal depleted, whereas soluble SOD1 is highly Cu metallated. Exchange of Cu between soluble and aggregated SOD1 is unlikely because soluble SOD1 is thought to be highly stable. Lelie et al. (2011) also argued that aggregation occurs when SOD1 is in its immature, unmodified, apo form (Shaw et al., 2008). All these results suggest that the aggregation of SOD1 leads to the over-metallation of soluble SOD1. The abnormal Cu metallation of soluble SOD1 can be accompanied by different isotopic fractionation from normal conditions, which can explain the present results. Measurement of the Cu

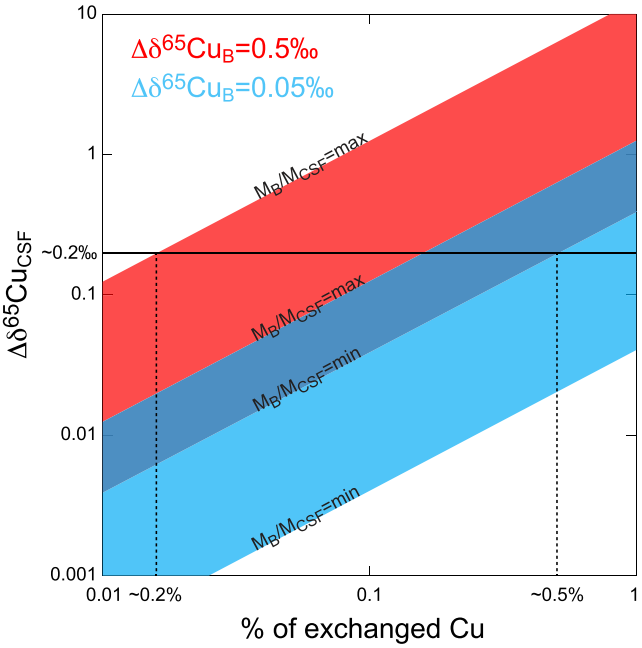

Figure 3. Cu Isotope Mass Balance between CSF and Brain

Calculation of the Cu isotopic offset in the CSF ($\Delta\delta^{65}Cu_{CSF}$) that results from the incorporation of Cu in the brain (% of exchanged Cu) associated with an isotopic fractionation $\Delta\delta^{65}Cu_B$. The calculations are made with two values of $\Delta\delta^{65}Cu_B$ (0.5‰ in red and 0.05‰ in blue) and two extreme values of the M_B/M_{CSF} ratio. The results thus define two areas in red and blue that overlap giving the dark blue area. The results discussed in the text corresponding to some proportions of exchanged Cu for a $\Delta\delta^{65}Cu_{CSF}$ value of 0.2‰, which represents the difference between CTRL and ALS $\delta^{65}Cu$ mean values, are illustrated.

isotope composition of the soluble and insoluble fractions of SOD1-ALS transgenic mice spinal cords would help to confirm this hypothesis.

To conclude, our results, i.e., the first to report Cu and Zn isotopic compositions in CSFs of patients with ALS and AD and CTRL subjects, demonstrate that Cu isotopic measurements in CSF may offer a more comprehensive view of the ALS metallome than elemental concentrations and may potentially reinforce any diagnosis. Similarly, the lighter $\delta^{66}Zn$ value observed in the CSF of patients with AD compared with that of CTRL subjects may also offer promising information regarding Zn dyshomeostasis in AD. Increasing the number of Cu and Zn isotopic measurements in CSFs of ALS and AD, respectively, as well as in other neurodegenerative pathologies, such as Parkinson or Huntington diseases, would undoubtedly challenge the proposition that $\delta^{65}Cu$ and $\delta^{66}Zn$ values in CSF are probably future candidate biomarkers of neurodegenerative diseases.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Transparent Methods, three figures, one table, and two data files and can be found with this article online at https://doi.org/10.1016/j.isci.2018.07.023.

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AUTHOR CONTRIBUTIONS

Conceptualization, E. Bernard, E. Broussolle, P.L., and V.B.; Methodology, L.S. and V.B.; Investigation, L.S., E. Bernard, A.P.-L. I.Q. A.V., P.K.-S., and E. Broussolle; Resources, E. Bernard, A.P.-L., I.Q., A.V., P.K.-S., E. Broussolle, and V.B.; Writing – Original Draft, L.S., E. Bernard, E. Broussolle, P.L., and V.B.; Writing – Reviews and Editing, L.S., E. Bernard, E. Broussolle, P.L., and V.B.; Funding Acquisition, V.B.

DECLARATION OF INTERESTS

The authors have no financial interests to declare.

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SUPPORTING CITATIONS

Costa et al., 2012; del Campo et al., 2012; Ihara et al., 2013; Jaouen et al., 2016; Kapaki et al., 1989; Maréchal et al., 1999; McKhann et al., 2011.

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Supplemental Information

Isotopic Evidence for Disrupted Copper Metabolism in Amyotrophic Lateral Sclerosis

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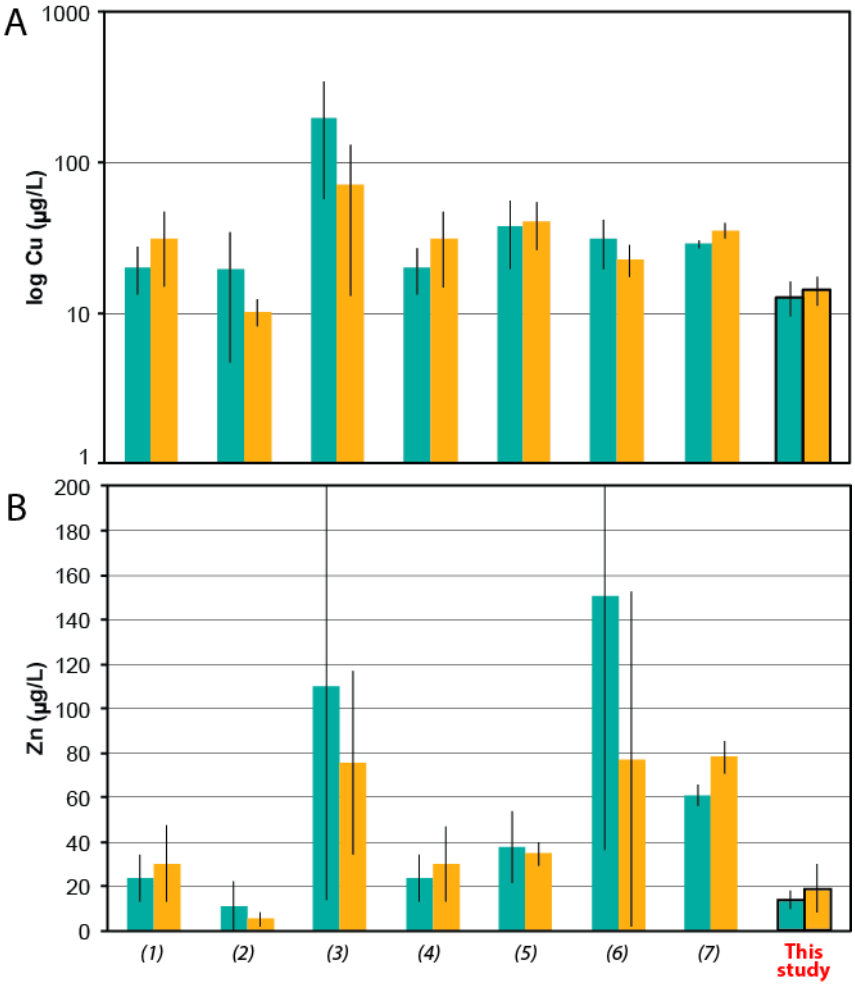
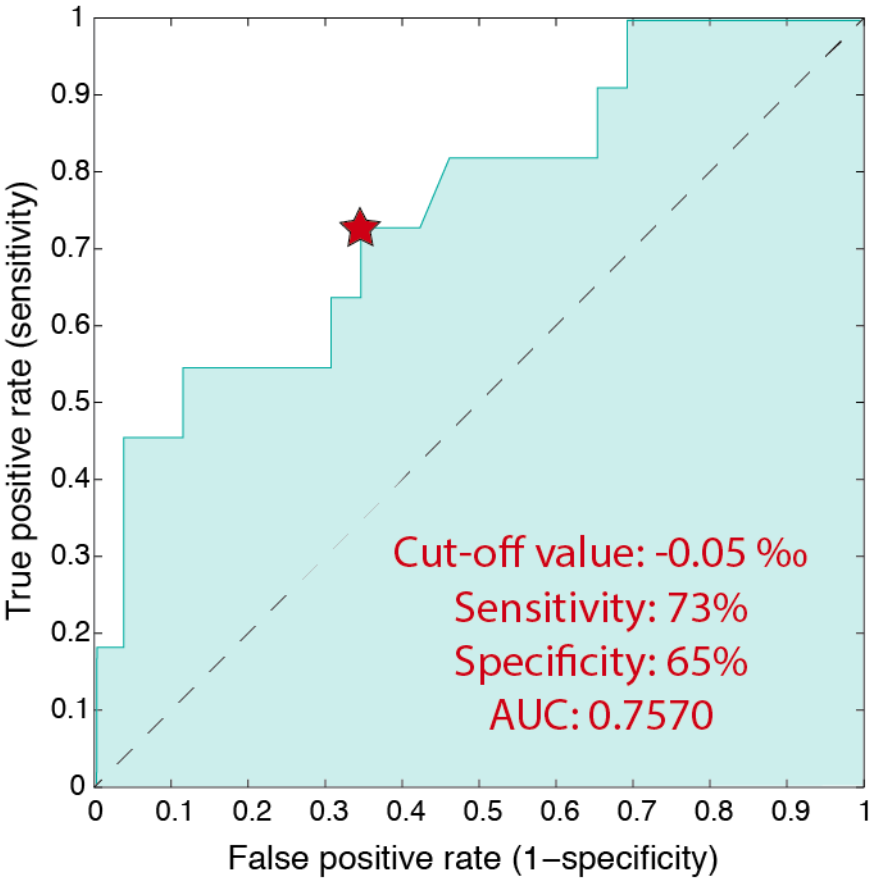
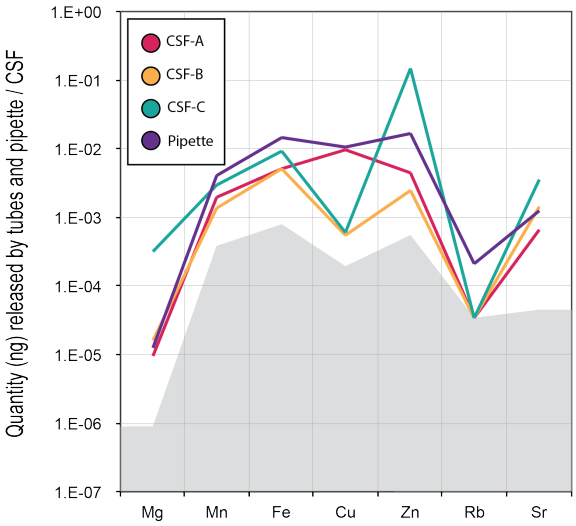


Supplementary Figure S1: Elemental concentrations variability in CSF of ALS and CTRL subjects, related to Figure 1. Compilation of a) Cu and b) Zn concentrations of cerebrospinal fluids (CSFs) in both control (yellow) and amyotrophic lateral sclerosis subjects (green), from the literature and the present study. Error bars represent two standard deviations of the mean. Cu diagram is represented with a logarithm scale. Data from the present study are labeled in red, and literature data, in italic from 1 to 7, are for Kapaki et al. (1997), Hozumi et al. (2011), Ihara et al. (2013), Kanias et al. (1997), Kapaki et al. (1989), Roos et al. (2013), Ostachowicz et al. (2006), respectively.



Supplementary Figure S2: Receiver Operating Curve (ROC), related to Figure 2. ROC analysis of the δ^{65}Cu values in CSF of ALS patients compared to that of CTRL subjects.



Supplementary Figure S3: Estimation of blank levels, related to Figure 2. Amount of trace element released by sampling and storage tubes as well as dropper-like pipette normalized to the amount of elements initially present in 2mL of cerebrospinal fluids (CSFs) (i.e. the average volume analyzed in this study). The grey field represents values below the limit of detection (DL) defined following IUPAC guideline (i.e. $DL_i = xb_i + k*sb_i$ where k=3, xb_i and sb_i are respectively the mean and the standard deviation of the number of counts measured in blanks). When the amount detected in the solution was lower the limit of detection, data were represented as equal to the detection limit.

Supplementary Table S1: General information and patients' clinical records, related to Figure 1

Sample Name	Gender	Age (years) at sample collection	Localization of first symptoms *	Awaji criteria	ALSFRS-R **	Time (months) between sampling date and first symptoms
ALS1	Male	45	MI	definite	44	7
ALS2	Male	62	MS	definite	39	6
ALS4	Male	81	MS	definite	40	6
ALS5	Male	46	bulb	definite	40	12
ALS6	Male	34	MS	definite	42	12
ALS7	Male	65	bulb	definite	44	12
ALS8	Male	86	MI	possible	37	6
ALS9	Male	68	MI	definite	45	12
ALS10	Male	73	MS	definite	37	6
ALS11	Male	64	MS	probable	28	6
ALS12	Male	60	MS	definite	33	6
ALS13	Male	60	bulb	definite	34	6
ALS14	Male	57	MI	definite	32	24
ALS 15	Male	49	bulb	definite	41	6
ALS 16	Male	50	MS	probable	39	12
ALS 17	Male	69	MI	probable	45	36
ALS 18	Male	60	bulb	definite	39	24
ALS 19	Male	49	bulb	definite	39	12
ALS 20	Male	75	MI	probable	35	12
ALS 21	Male	70	MS	definite	23	24
ALS 22	Male	76	bulb	definite	42	12
ALS 23	Male	54	bulb	definite	43	7
ALS 24	Male	44	MI	probable	46	24
ALS 25	Female	71	MS	probable	43	4
ALS 26	Female	64	bulb	definite	40	6
ALS 27	Female	63	bulb	definite	40	24
ALS 28	Female	67	MI	definite	40	5
ALS 29	Female	69	MI	probable	34	6
ALS 30	Female	64	MI	probable	40	9
ALS 31	Female	45	MS	definite	41	24
ALS 32	Female	43	MI	probable	42	6
CTRL2	Male	49				
CTRL3	Male	62				
CTRL4	Male	69				
CTRL5	Male	48				
CTRL6	Male	46				
CTRL7	Male	67			n/a	
CTRL8	Male	60				
CTRL9	Male	44				
CTRL10	Male	70				
CTRL11	Male	58				
CTRL 12	Male	79				
AD4	Male	75				
AD5	Male	78				
AD6	Male	68				
AD7	Male	68				
AD8	Male	78				
AD9	Male	63				
AD10	Male	70			n/a	
AD11	Male	59				
AD12	Male	63				
AD13	Male	71				
AD14	Male	73				
AD15	Male	73				
AD16	Male	79				
AD17	Male	78				

Footnote:
"n/a" stands for unspecified value
CTRL stands for control subjects, ALS and AD are for Amyotrophic lateral sclerosis and Alzheimer patients respectively

*Site at onset: - MI=lower limbs
- MS=upper limbs
- bulb=bulbar

**ALSFRS-R stands for the revised version of the Amyotrophic Lateral Sclerosis Rating scale

Transparent methods

Subjects and samples

In this study, all subjects were hospitalized in the Department of Neurology for diagnosis purposes, which included cerebrospinal fluid (CSF) analyses among other tests. All patients signed informed consent about the potential use of their CSF for further research purposes and an ethical approval was also delivered by the local Ethics committee of the Hospices Civils de Lyon (date of delivery: 7th October, 2016). We investigated the CSF of 31 ALS patients diagnosed using the Awaji criteria (Costa et al., 2012). They were compared to a group of 25 patients suffering from neurocognitive complaint referred to expert memory clinic linked to Lyon Center for Memory Resources and Research. These 25 patients underwent lumbar puncture using a standard procedure (del Campo et al., 2012) in the context of clinical diagnosis of neurodegenerative diseases. Cerebrospinal fluid biomarker for AD pathology (*i.e* total and phosphorylated TAU proteins and Amyloid beta 1-42 peptide) were performed blind to the clinical diagnosis in the Neurochemistry Unit of Lyon University Hospital using commercially available enzyme-linked immunosorbent assays (INNOTEST hTau-Ag, INNOTEST phosphorylated-Tau181, and INNOTEST Aβ1-42; Fujirebio Europe) according to the manufacturer's instructions. The Neurochemistry Unit participated in the Alzheimer Association Quality Control Program for these biomarkers. Diagnoses were finally proposed in the framework of multidisciplinary consultation taking into account medical history, caregivers interviews, neurologic examination, neuropsychological evaluation, brain imaging and results of cerebrospinal fluid biomarker for AD pathology. AD diagnostic was excluded in 11 of these 25 patients who were finally classified as suffering mainly from psychiatric conditions and were then considered as controls for this study. The remaining 14 patients met the diagnostic criteria of Alzheimer 's disease according to McKhann et al. (2011). Both male and female from different ages ranging from 34 to 86 years old were studied. To avoid any sample bias, we also considered diverse ALS cases *i.e.* characterized by different onset brain location (bulbar, lower and upper limbs), distinct value of Awaji criteria and ALSFRS as well as variable time of symptom onset (6 to 36 months). All the details are summarized in Supplementary Table S1.

Major and trace element concentrations

All chemical analyses were carried out in clean laminar flow hoods using double-distilled acids to avoid any exogenous contaminations. Samples were first weighted and then dissolved in a mixture of 15M HNO_3 and H_2O_2 (30%) in Savillex® beakers at 120°C for about 72h. When dissolved, major and trace element concentrations were measured in a small aliquot on an ICP-AES (iCAP 6000 Radial) and a quadrupole ICP-MS Thermo iCap-Q respectively at the Ecole Normale Supérieure (ENS) of Lyon following the method described in Garçon et al. (2017). Trace and major element concentrations are reported in ng/mL and µg/mL respectively in Supplementary Dataset 1. Briefly, the concentrations were calculated using calibration curves based on multi-elemental solutions. These solutions were also used to monitor and correct the instrumental drift over the analytical session. Oxide interference and analytical drift were also corrected using indium (In) and scandium (Sc) addition as internal standards for trace and major elements, respectively. The precision of the results was assessed by complete duplicate and re-run analyses (referred as "dup" and "bis" samples respectively in Supplementary Dataset 1) and accuracy and reproducibility were monitored by replication of certified reference materials (1577c, DORM2) and an in-house standards (OEP, FBS) measured as unknown samples (Supplementary Dataset 2). The results are generally reproducible and consistent within 10% (2sd) of previous published data (see Supplementary Dataset 2). Based on the analysis of reference standards and duplicates analyses, we therefore estimate that the measurement precision is, on average, better than 10% for both major and trace elements.

Copper and zinc isotopic compositions

Samples were purified by ion-exchange chromatography using quartz columns filled with 1.8mL of Bio-Rad AGMP-1 (100-200 mesh) anion-exchange resin. Copper and zinc were successively eluted with 20mL of HCl 7M + 0.001% H_2O_2 and 10mL of HNO_3 0.5M respectively following the procedure described by Maréchal et al. (1999). The total procedural blanks were on average 0.4 ng for Cu (n=7) and 3.0 ng for Zn (n=7), which is below the amount of element isolated from the sample and available for isotopic measurement (*i.e.* average Cu_{CSFs} and Zn_{CSFs} of 20 ng). Blank contribution in the samples is therefore higher for Zn than for Cu. To

ensure the quality of the Zn isotopic data, we quantified the impact of this exogenous contamination using the mixing equation provided by Garçon et al. (2017). Tubes and pipettes used to collect and store the CSFs cannot release significant amount that may account for exogenous contamination (see below *Effect of exogenous contamination induced by tubes and pipettes on major and trace element concentrations measured in CSFs*). Conversely, gloves contain high Zn content that may be easily mobilized during sample preparation (Garçon et al., 2017). Gloves are probably the main source of contamination. With a $\delta^{66}Zn_{gloves}$ of 0.10 ± 0.32 ‰ (2sd), we show that for Zn = 20 ng (*i.e.* the content available for isotopic measurement in the samples), no significant shift can however be induced beyond the measurement uncertainties (*i.e.* ±0.07 ‰, 2sd) (Supplementary figure 3) ensuring the reliability of our isotopic data measurements.

The isotopic compositions of Cu and Zn are measured by multi-collector inductively coupled plasma mass spectrometry (MC-ICP-MS, Nu500) in wet plasma conditions following the procedure described by Maréchal et al. (1999). On the day of analyses, Cu and Zn purified solutions are diluted in a Zn-doped solution (Zn JMC 3-0749L, Johnson Matthey Royston, UK) and a Cu-doped solution (Cu SRM 976, National Institute of Standards and Technology, Gaithersburg, MD, USA), respectively, to match the concentration of the standard mixture run between the samples (between 75 and 300 µg.L^{-1} depending on the sample). The delta values (expressed in ‰) are reported relative to the isotopic solution reference material NIST SRM 976 for Cu and JMC 3-0749L for Zn and are referred as:

$$\delta^{65}Cu = \left[\frac{\left(^{65}Cu/^{63}Cu\right)_{sample}}{\left(^{65}Cu/^{63}Cu\right)_{standard}} - 1\right] \times 1000 \text{ and } \quad \delta^{66}Zn = \left[\frac{\left(^{66}Zn/^{64}Zn\right)_{sample}}{\left(^{66}Zn/^{64}Zn\right)_{standard}} - 1\right] \times 1000$$

Instrumental mass fractionation and temporal drift is corrected with an exponential law using the elemental-doping method and standard-sample bracketing respectively as recommended by Maréchal et al. (1999). The precision and the accuracy of Cu and Zn isotopic ratios were assessed by repeated measurements of re-run and duplicate samples and by reference materials (1577c, bovine liver) and in-house standard solutions (OEP, sheep plasma), respectively. The average $\delta^{66}Zn$ of reference materials 1577c and OEP were -0.19 ± 0.04 (2sd, n=4) and +0.64 ± 0.06 (2sd, n=2), respectively, which is in good agreement with our in-house previous average values: $\delta^{66}Zn_{1577c}$ = -0.19 ± 0.05 (2sd, n=13) and $\delta^{66}Zn_{OEP}$ = +0.72 ± 0.08 (2sd, n=15) as well as with previous published results ($\delta^{66}Zn_{1577c}$ = -0.13 ± 0.02 (2sd,

n=4), *37*) (Jaouen et al., 2016) (Supplementary Dataset 2). For $\delta^{65}Cu$, we measured +0.35 ± 0.07 (2sd, n=4) for 1577c and -1.16 ± 0.03 (2sd, n=3) for OEP which is also in agreement with our in-house reference values: $\delta^{65}Cu_{1577c}$ = +0.37 ± 0.12 (2sd, n=10) and $\delta^{65}Cu_{OEP}$ = -1.14 ± 0.09 (2sd, n=20) (Supplementary Dataset 2). Based on these results, we estimate the 2sd analytical uncertainty of our isotopic measurements at ± 0.07. Note that the long-term precision based on the repeated measurements of the pure Zn JMC 3-0749L and Cu SRM 976 solutions run every two samples are very similar to these values (± 0.05 ‰ (2s, n = 140)).

Principal component analysis

In this study, we used a correlation-based principal component analysis (PCA) to discriminate ALS patients from AD patients and age-matched CTRL as well as to quantify the effect of the ALS disease on elemental concentrations and Cu-Zn isotopic compositions. The method consists in identifying new variables called principal components (PCs), which are linear combination of the original variables and along which data variation is maximal. The variables include the chemical concentrations of 12 major and trace elements measured in cerebrospinal fluids of ALS, AD and control subjects, as well as $\delta^{65}Cu$ and $\delta^{66}Zn$ values. All data were normalized, and samples with incomplete data were excluded. PCA was implemented in MATLAB™.

Boxplot

In the present study, all the boxplot diagrams were implemented in MATLAB™ and significance level was determined using a non-parametric 'two-sided', Wilcoxon-Mann-Whitney U-test. For each boxplot, the central mark is the median, the edges of the box are the first (*i.e.* 25th percentiles) and third quartiles (*i.e.* 75th percentiles) respectively and the whiskers extend to the most extreme data points (*i.e.* not considered outliers).

ROC

Receiver Operating Curve (ROC) was used to evaluate the reliability of $\delta^{65}Cu$ values as a potential ALS diagnostic test with the aim to confirm the presence of ALS disease but also to rule out the presence of this pathology in healthy subjects. In this study, the ROC test was implemented in MATLAB™ and four distinctive

parameters were obtained: 1) the cut-off value *i.e.* the threshold value discriminating ALS form CTRL subjects; 2) the true positive rate (*i.e.* sensitivity) corresponding to the probability that a result will be higher than the cut-off value when the ALS disease is present; 3) the false positive rate (*i.e.* 1-specificity) defined as the probability that a result will be lower than the cut-off value when the ALS disease is not present; 4) The area under the curve (AUC). Optimal performance test correspond to a sensitivity and specificity of 100% and an AUC of 1.

Effect of exogenous contamination induced by tubes and pipettes on major and trace element concentrations measured in CSFs.

Cerebrospinal fluids (CSFs) are made of 99% water and have low amount of major and trace elements. To ensure the absence of external contamination in CSFs during sample collection and/or storage, we quantify the content of the most contaminable trace elements (*i.e.* Mn, Fe, Cu, Zn, Rb and Sr as well as Mg) that may be released by tubes and pipettes being directly in contact with the samples. This includes sampling (CSF-A: polypropylene, Sarstedt, 10mL, 92x15.3mm and CSF-B: polypropylene, Sarstedt, 5mL, 57x15.3mm) and storage tubes (CSF-C: polypropylene, Sarstedt, 1.5mL) as well as dropper-type pipette.

The detailed procedure consist of putting 2mL of HNO_3 0.5M + 2ppb indium (In) in the tubes, before storing them 3 weeks in a fridge. The volume of 2mL corresponds to the average volume of CSFs analyzed in this study. The duration of the test correspond to the approximate period for which CSF samples were stored in the tubes. To assess the amount of trace elements released by dropper-type pipette, we used a slightly different technique. For this test, we pour 2mL of HNO_3 0.5M + 2ppb In in clean savillex® beaker and washed the pipette three times with the solution. An aliquot of each solution is then analyzed on the Thermo iCap-Q mass spectrometer following the method described in the *Major and trace element concentrations* section.

For all the elements measured in this study, the amount released by tubes and pipette that have been in contact with low concentrated HNO_3 acid is always lower (<0.01) than the quantity present in the CSF samples (Supplementary figure S3).

Only one exception is noted for the amount of Zn released by the dropper-type pipette, but this amount remains relatively low (<0.1) compared to the amount initially present in the samples. Although we used low concentrated acid solutions, it

is important to note that chemical elements are more easily released in acidic solution than when they are in contact with non-acidic CSF samples. Therefore, our study likely provides maximized results being ten times higher that the real amounts released by tubes and pipettes when they are only filled with CSFs.

Altogether, these results show that storage and sampling tubes and dropper-type pipettes cannot induce significant exogenous contamination and bias the Mg and trace element concentrations including Fe, Cu, Zn, Mn, Rb and Sr measured in CSFs. Similar conclusion can also be drawn for Ca, K, Na, P and S, the latters being initially more concentrated in CSFs compared to trace elements.