Comparison of Metal Levels between Postmortem Brain and Ventricular Fluid in Alzheimer's Disease and Nondemented Elderly Controls

Steven T. Szabo,^{*,†,1} G. Jean Harry,[‡] Kathleen M. Hayden,^{§,2} David T. Szabo,[¶] and Linda Birnbaum^{||}

^{*}Veterans Affairs Medical Center, Mental Health Service Line, Durham, North Carolina 27705; [†]Department of Psychiatry and Behavioral Sciences, Duke University Medical Center, Durham, North Carolina 27705; [‡]Neurotoxicology Group, National Toxicology Program Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709; [§]Department of Psychiatry and Behavioral Sciences, Joseph and Kathleen ADRC, Duke University Medical Center, Durham, North Carolina 27705; [¶]RAI Services Company, Winston Salem, North Carolina 27102; and ^{||}National Cancer Institute, National Institutes of Health (NIH), Research Triangle Park, North Carolina 27709

¹To whom correspondence should be addressed. Fax: 919-681-8744. E-mail: steven.szabo@duke.edu.

²Present address: Department of Social Sciences and Health Policy, Wake Forest School of Medicine, Winston-Salem, NC 27157.

ABSTRACT

An essential metal hypothesis for neurodegenerative disease suggests an alteration in metal homeostasis contributing to the onset and progression of disease. Similar associations have been proposed for nonessential metals. To examine the relationship between metal levels in brain tissue and ventricular fluid (VF), postmortem samples of frontal cortex (FC) and VF from Alzheimer's disease (AD) cases and nondemented elderly subjects were analyzed for arsenic (As), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), mercury (Hg), nickel (Ni), tin (Sn), vanadium (V), and zinc (Zn) using inductively coupled plasma sector field mass spectrometry. All metals, with exception of equivalent Pb levels, were lower in the VF, compared to FC. Within-subject comparisons demonstrated that VF levels were not representative of levels within brain tissue. The essential metals Cu, Fe, and Zn were found highest in both compartments. Cd, Hg, and V levels in the VF were below the limit of quantification. In AD cases, FC levels of Fe were higher and As and Cd were lower than levels in controls, while levels of As in the VF were higher. Parameter estimates for FC metal levels indicated an association of Braak stage and higher Fe levels and an association of Braak stage and lower As, Mn, and Zn levels. The data showed no evidence of an accumulation of nonessential metals within the AD brain and, with the exception of As, showed no significant shift in the ratio of FC to VF levels to indicate differential clearance.

Key words: heavy metals; brain; cerebral spinal fluid; amyloid beta; neurodegenerative disease.

Over the years, an interest has emerged in the contribution of essential metals to pathophysiologic processes of neurodegenerative diseases (Bjorklund *et al.*, 2012; James *et al.*, 2012; Squitti, 2012; Zecca *et al.*, 2004). In the normal progression of aging and neurodegenerative diseases, a role for endogenous essential metals such as copper (Cu), iron (Fe), and zinc (Zn) has been

cautiously considered as a factor associated with cognitive decline and pathogenesis (Graham *et al.*, 2014; Squitti *et al.*, 2014; Ventriglia *et al.*, 2012, 2015). An imbalance in metal homeostasis is thought to play an important role in the progression of neurodegenerative disease (Crespo *et al.*, 2014). While a direct association has not been demonstrated between metal exposure and

Alzheimer's disease (AD), a disease-related deficit in clearing metals from the brain is thought to result in an elevated metal burden and yield adverse effects (Ayton et al., 2013; Duce et al., 2010; Greenough et al., 2011). AD plaque-like formations have been associated with the binding of metal ions such as Cu, Fe, and Zn (Greenough et al., 2013; Nabuurs et al., 2013; Suh et al., 2000) and direct interactions of essential metals with AD proteins have been reported (Adlard and Bush, 2006; Fallor, 2009; Savelieff et al., 2013; Xu et al., 2014).

Recent findings indicated an age-related accumulation of Fe complexes in various brain regions associated with motor and cognitive impairments (Hagemeier et al., 2012; Ramos et al., 2014; Raven et al., 2013). However, an earlier study only found Zn elevations in AD brains with no difference in Cu or Fe levels (Lovell et al., 1998). A meta-analysis of the existing literature for metal levels in the brain and AD (Schrage et al., 2011) identified a significant reporting bias for Fe. No changes in Zn were observed in AD brains, while a depletion of Cu was found to be associated with AD. Other meta-analyses reported an AD-related elevation in Cu and reduction in Zn levels in the brain, with no change in cerebral spinal fluid (CSF) levels (Bucossi et al., 2011; Ventriglia et al., 2012, 2015). Continued studies have failed to clarify the association between essential metal burden and AD (Akatsu et al., 2012; Exley et al., 2012; Graham et al., 2014; House et al., 2012; James et al., 2012). Yet, 1 study on a limited sample size of 5 suggested that differences reported in the literature were related to the sub-region of the brain examined (Andrasi et al., 2000). Age- and disease-related shifts in essential metal homeostasis have led to speculations of similar associations with nonessential metals. Thus far, the majority of studies have relied on serum levels with few studies expanding into examining the CSF or brain tissue. Higher plasma levels of various metals including cadmium (Cd), aluminum (Al), arsenic (As), and selenium (Se) have been reported in AD patients, compared with healthy volunteers (Basun et al., 1991). Elevated plasma levels of manganese (Mn) and mercury (Hg) were reported in AD subjects that were not reflected in CSF (Gerhardsson et al., 2008, 2009). An isolated report on AD patients in Hong Kong indicated a decrease in serum levels of Zn and an association between elevated serum levels of As and clinical presentation (Baum et al., 2010). In a recent study, no association was found between serum levels of lead (Pb), Cd, Hg, or As and cognitive deficits in AD patients (Park et al., 2014). However, in an analysis of brain levels, AD patients displayed higher levels of tin (Sn), Al, and Mn in the parietal cortex (Srivastava and Jain, 2002).

To examine an association between environmental and essential metals with AD and to compare relative associations between brain and ventricular fluid (VF) levels, postmortem samples of each were collected from individual AD patients and nondemented elderly controls. Levels of As, Cd, Cr, cobalt (Co), Cu, Fe, Pb, Mn, Hg, nickel (Ni), Sn, vanadium (V), and Zn were determined by inductively coupled plasma sector field mass spectrometry, and the relationship between substrates and association of levels and disease state were determined.

MATERIALS AND METHODS

Samples. Postmortem samples from patients were obtained from the brain and VF repository of the Kathleen Price Bryan Brain Bank at the Joseph and Kathleen Bryan Alzheimer Disease Research Center (Bryan ADRC) and Department of Psychiatry and Behavioral Sciences, Duke University Medical Center in Durham, NC. Patient enrolment, sample autopsy, and tissue collections were conducted as previously reported (Hulette et al., 1997) under a protocol approved by the Duke University Medical Institution

Review Board. All cases had a minimum set of background data that included age, sex, diagnoses, and postmortem interval. No significant difference was observed in the distribution of males and females in each group ($p = .363$). The average age of controls was 88 years, while in the AD cases, the average age was 78 years ($p < .0001$). APOE genotypes were determined by polymerase chain reaction and mini sequencing for all subjects. Of the control subjects, 14 showed no $\epsilon 4$ allele and 1 displayed 1 $\epsilon 4$ allele. For AD patients, 2 displayed no $\epsilon 4$ allele, 11 patients showed 1 $\epsilon 4$ allele, and 1 patient showed 2 $\epsilon 4$ alleles. Evidence of atherosclerosis, infarcts, and postmortem delay (mean = 10 h) were similar between groups ($p = .17$, $.584$, and $.4$, respectively). All AD patients were diagnosed and followed in the Bryan ADRC. Classifications of dementia were based primarily on Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV). Control subjects enrolled for brain tissue donation provided detailed medical information, family history of dementia, and neurological disorders and underwent neuropsychological evaluations. Donors were scheduled for annual evaluations; however, 100% compliance was not always observed. With autopsy, efforts to confirm nondemented status were taken to validate use of tissue as control. Brains were examined neuropathologically and classified as AD or normal according to NIA-Reagan Institute criteria (Hyman and Trojanowski, 1997). Neurofibrillary changes were staged according to Braak stages I–VI (Braak and Braak, 1991), and plaque frequency across several regions of the neocortex was estimated according to criteria specified by the Consortium to Establish a Registry for AD (CERAD) (Mirra et al., 1991). All controls were classified as normal by CERAD and all AD cases were confirmed by CERAD. Braak stage for controls (6 in Stage 1, 5 in Stage 2, and 4 in Stage 3) and AD cases (4 in stage 3, 4 in stage 4, 3 in stage 5, and 5 in stage 6) were significantly different ($p < .0001$). The normal subjects with Braak stage III displayed no clinical symptoms or neurofibrillary neuropathology of AD.

VF and Brain Sample Collection. At autopsy, the bony calvarium was cut prior to removal of the skull plate. VF was removed from the lateral ventricle by low-pressure syringe aspiration. Samples underwent low-speed centrifugation for 10 min at 2000 rpm to remove any blood, skin, or bone fragments that may have been inadvertently aspirated into the sample through the 50-gauge needle while pulling fluid from the ventricle. Supernatants were removed and 1 ml aliquots were stored in Nalgene cryovials polypropylene tubes at -80°C . The brain was excised, rinsed with sterile deionized water, meninges removed, and the right hemisphere sectioned along the coronal plane. Sections of frontal cortex (FC) approximately 1 cm in thickness (1 cm^3) were dissected, bagged, and stored in -80°C freezer for long-term storage. Brain samples were removed from frozen storage kept on a solid platform on dry ice and from each an approximate 100 mg sample of the grey matter was collected using a Teflon-coated blade. Sample were weighed and placed in a 10 ml Pyrex microwave digestion tube.

Metal Analysis. Prior to study sample analysis, method performance was demonstrated for the determination of the metals suite in both FC and VF matrices following microwave digestion in the presence of high-purity acids and oxidants. Linearity was demonstrated across a range of concentrations for each metal. All metal standards were traceable to the National Institute of Standards and Technology and were purchased from High Purity Standards (Charleston, SC). Spiked matrix samples were used to assess analyte recovery, and method blanks were used to assess analyte background from the reagents and the

procedure. Sample containers were assessed for analyte background and were confirmed to be free from contamination. Sample preparation was conducted in a cleanroom environment limiting false-positive values from contamination.

Prior to the start of the study, all tubes were rinsed in 10% HNO₃ for a minimum of 12 h, rinsed with DI H₂O, and allowed to dry under HEPA-filtered air. To each 100 mg FC sample or 0.100 ml VF sample, 0.48 ml of Ultrex Grade (J.T. Baker, Center Valley, PA) HNO₃, 0.02 ml of Ultrex Grade HCl, 0.100 ml of Ultrex Grade, non-stabilized hydrogen peroxide (H₂O₂), 0.25 ml of 10 µg/ml gold (Au; for stabilization of mercury), and 1.4 ml of high-purity (~18 MΩ quality; Pure Water Solutions, Hillsborough, NC) deionized (DI) H₂O were added. Identical solutions were added to empty microwave digestion tubes to serve as blank controls. Capped tubes were placed in a CEM (Matthews, NC) DiscoverSPD microwave and run in the following sequence: temperature, 200°C; ramp, 8 min; hold, 10 min; pressure, 300 PSI; power, 200 W; stir, medium. Samples were allowed to return to room temperature, and under a Class 100 clean hood, contents were transferred to 15 ml centrifuge tubes with multiple rinses of DI H₂O. Each tube was spiked to contain a final nominal concentration of 1 ng/ml of internal standard elements indium, praseodymium, and scandium. Samples were analyzed for trace metal content using a Thermo Element2 inductively coupled plasma sector field mass spectrometry (Bremen, Germany). Measured samples concentrations were converted to ng/g and ng/ml for FC and VF samples, respectively. Level of quantitation (LOQ) was conservatively defined based on the lowest calibration standard included in the regression equation for each analyte, expressed as equivalent concentration in tissue/fluid. This provided a high degree of confidence in data reported that exceeded this level.

Statistics. For subject characteristics, categorical variables were compared with chi-square tests and continuous variables were compared with t-tests. Significant differences in analyte levels in the FC and in the VF between normal controls and AD cases were determined by Student's t-test. The relative change in analyte

levels between the VF and the FC was analyzed for differences between normal controls and AD cases using Student's t-test. Correlations between individual subject FC and VF levels were calculated by Spearman's rank-order coefficient. Regression analyses were carried out with the FC as the dependent variable, with factors determined *a priori* and designated as covariates including age (years), sex post mortem delay (h), Braak Stage, and presence or absence of amyloid angiopathy and atherosclerosis. Metal levels were log transformed for regression analyses. Statistical significance was set at $p < .05$.

RESULTS

Metal Levels in Brain Tissue. All metals analyzed could be detected in the FC of all subjects; however, Co levels were below the level of quantification (LOQ) for approximately 90% of samples and were not reported. Hg and V levels were below their respective LOQ for a number of the samples (Table 1). Levels of the essential metals (Fe, Zn, Cu) were highest for all samples. In the FC, levels of As ($p = .033$) and Cd ($p = .031$) were significantly lower and Fe levels significantly higher ($p = .018$) in the AD cases as compared to controls (Table 1). No significant difference was observed for the remaining analytes. When FC levels (>LOQ) were compared to specific features of the disease in the AD subjects, a marginal correlation was observed between amyloid for Fe ($r = 0.73$) and As ($r = 0.70$), with no association demonstrated for Braak stage or with other analytes. Given the nature of tissue collection from human patients, contribution of metals within the vasculature could not be excluded. However, this would be consistent across all human brain tissue studies.

Metal Levels in VF. For each respective metal, VF levels were lower as compared to levels within the FC (Table 1). Levels of the essential metals (Fe, Zn, Cu) were highest of those examined. Levels of Co were below LOQ. Hg levels exceeded LOQ in 2 control samples (13 and 50 ng/g); Cd in only 3 samples (3.9–

TABLE 1. Metal Levels in Frontal Cortex (FC) and Ventricular Fluid (VF) of AD Cases and Nondemented Elderly Controls

Elements	FC					VF					% VF/FC				
	Control		AD Cases		Statistics	Control		AD Cases		Statistics	Control		AD Cases		Statistics
	Mean	SD	Mean	SD		Mean	SD	Mean	SD		Mean	SD	Mean	SD	
As	159	11.8	145	24	.033	13	5.7	18	8.6	.044	0.95	0.04	0.88	0.09	.021
Cd	30	12	20	12	.031	< LOQ		< LOQ							
Cr	91	66	154	332	.478	14.4	0.8	17.5	1.7	.109	0.79	0.12	0.66	0.26	.102
Cu	2398	733	2112	768	.54	104.2	8.7	117.5	21	.556	0.95	0.02	0.93	0.1	.412
Fe	34 140	9027	42 571	11 323	.018	1035.6	87	6994.9	5923.7	.332	0.97	0.01	0.79	0.72	.348
Hg	9	6.5	10	5	.329	< LOQ		< LOQ							
Mn	182	45	151	37	.061	23.5	0.9	25.1	2.6	.574	0.86	0.04	0.8	0.16	.188
Ni	36	23	52	25	.097	12	1	19.6	4	.083	0.61	0.2	0.49	0.48	.361
Pb	27	10.8	27	9.5	.772	27.5	6.8	34	6.9	.508	−0.2	1.2	−0.36	0.81	.668
Sn	32	27	26	15	.357	5	1.6	7	5.5	.73	0.81	0.08	0.8	0.12	.827
V	3	0.8	5	3.6	.211	< LOQ		< LOQ							
Zn	12 073	2072	12 099	2741	.993	732.4	66.8	845.2	97.9	.357	0.94	0.02	0.92	0.06	.269

Data represent mean and standard deviation (SD) of analyte levels in FC (ng/g) and VF (ng/ml) of Alzheimer's disease (AD) cases and nondemented elderly controls. Statistics represent comparisons between control and AD cases for each analyte as determined by Student t-test. Statistical significance was set at $p < .5$ for a two-tailed test. Abbreviations and levels of quantitation (LOQ; ng/g and ng/ml): As = arsenic (10); Cd = cadmium (3.75); Cr = chromium (10); Co = cobalt (10); Cu = copper (500); Fe = iron (1250); Pb = lead (5); Mn = manganese (2.5); Hg = mercury (3.75); Ni = nickel (10); Sn = tin (2.5); V = vanadium (2.5); and Zn = zinc (1250). Levels for Co were below LOQ for 95% of the samples and not presented. FC levels for Hg exceeded LOQ in 9 control samples and 8 AD samples; V for 10 control samples and 14 AD samples. In the VF, Hg and V failed to reach LOQ; Cd levels exceeded LOQ in 3 samples (3.9–5.6 ng/g) and Sn levels exceeded LOQ in 10 control and 6 AD samples. All other analytes exceeded LOQ for all samples.

TABLE 2. Parameter Estimates for Frontal Cortex (FC) Metals Levels

Variable	df	Arsenic				Cadmium				Iron				Manganese				Zinc			
		Parameter Estimate	SE	t Value	Pr > t	Parameter Estimate	SE	t Value	Pr > t	Parameter Estimate	SE	t Value	Pr > t	Parameter Estimate	SE	t Value	Pr > t	Parameter Estimate	SE	t Value	Pr > t
Intercept	1	2.33	0.65	3.55	0.0019	-2.51	2.279	-1.1	0.284	8.81	1.551	5.68	0.0001	1.641	1.41	1.16	0.258	5.195	1.541	3.37	0.003
VF	1	-0.016	0.026	-0.61	0.548	0.27	0.189	1.44	0.165	-0.053	0.065	-0.82	0.42	-0.216	0.195	-1.11	0.28	0.067	0.112	0.6	0.554
Age	1	0.026	0.006	4.18	0.0004	0.06	0.02	2.8	0.01	0.016	0.013	1.02	0.243	0.041	0.011	3.65	0.002	0.036	0.011	3.36	0.003
SEX	1	0.096	0.058	1.65	0.113	-0.462	0.189	-2.45	0.02	0.153	0.12	1.27	0.217	0.095	0.104	0.92	0.37	0.137	0.095	1.44	0.166
Postmortem delay	1	0.02	0.005	3.79	0.001	0.0272	0.018	1.49	0.15	0.01	0.011	0.95	0.353	0.029	0.01	3.03	0.006	0.02	0.009	2.21	0.384
Atherosclerosis	1	-0.009	0.052	-0.18	0.86	-0.074	0.172	-0.43	0.669	0.011	0.107	0.11	0.917	-0.059	0.092	-0.64	0.526	0.013	0.083	0.61	0.877
Amyloid Angiopathy	1	0.017	0.057	0.3	0.77	0.126	0.186	0.67	0.508	-0.009	0.118	-0.07	0.942	-0.134	0.102	-1.31	0.204	-0.088	0.096	-0.92	0.368
Braak Stage	1	0.102	0.034	2.99	0.007	0.152	0.124	1.22	0.235	0.182	0.07	2.56	0.018	0.172	0.061	2.81	0.01	0.183	0.059	3.08	0.006

5.63 ng/g), and for Sn 10 control and 6 AD samples exceeded LOQ. Levels of As were significantly elevated in the AD cases compared to controls ($p = .044$). No significant differences between AD cases and normal control levels were observed in the VF for the remaining analytes. When VF was matched to FC for individual samples, analyte levels within the VF did not track with levels in the FC regardless of disease state. The ratio of each analyte level within the VF to that within the FC was calculated for each case. The relationship between the 2 compartments, calculated as a percent change, was similar between normal controls and AD cases for all analytes except As ($p = .021$) (Table 1).

Regression Analysis of Sample Characteristics with Analyte Levels in FC. Regression analyses were carried out with the FC as the dependent variable and VF levels, age (years), sex postmortem delay (h), atherosclerosis, Braak Stage, and amyloid angiopathy as covariates. Postmortem delay is often considered a factor of concern for tissue analysis. All samples were collected between 0.83 h and 30 h with no significant difference observed between groups. As a parameter, postmortem delay was associated with As ($p = .0011$), Mn ($p = .0064$), and Zn ($p = .0384$) levels with a suggestion of lower levels as the time interval increased (Table 2). Samples in the elderly control group were obtained from subjects generally older than the AD cases limiting the ability to appropriately assess age as a parameter for FC levels independent from disease state (Fig. 1). Clinical diagnosis of AD, as reflected in Braak Stage, was found to be a significant parameter estimating FC levels of As ($p = .007$), Fe ($p = .0183$), Mn ($p = .0104$), and Zn ($p = .0057$) (Table 2). However, age at death was also a significant parameter estimating FC levels of As ($p = .0004$), Mn ($p = .0015$), Zn ($p = .003$), and Cd ($p = .0106$). Age was not a significant parameter for Fe ($p = .24$) (Table 2).

DISCUSSION

In the current investigation, we found that essential metals, Fe, Zn, and Cu, showed the highest levels within the FC, followed by a cluster of Mn, As, and Cr and a lower level cluster of Ni, Sn, Cd, and Pb. Given the nature of the association between the VF of the CNS and brain tissue, tests of fluid samples were thought to approximate associations found in brain tissue. In a direct comparison between samples, we now report that levels of analytes in the VF did not accurately reflect levels observed within the brain. Comparisons between samples obtained from elderly controls and AD cases showed an elevation in the essential metal Fe in the FC and for nonessential metals, As was the only metal that showed significantly lower levels in the FC of AD cases.

The majority of studies that have examined an association between metals and AD have focused on essential metals, with only a few that measured levels within the brain and even fewer examining environmental metals. In FC tissue, we observed limited AD-related differences in metal levels with an elevation in Fe and a decrease in As and Cd. Exactly how to compare this to the existing literature is not clear given the significant differences across reported studies. These differences may be attributed to a number of factors including the substrate examined (serum, CSF, brain tissue), the brain region sampled (Andrasi et al., 2000; Ramos et al., 2014), and the sensitivity and accuracy of metal determination. For example, elevated levels of Zn and Fe have been reported in the hippocampus of AD patients (Danscher et al., 1997; Deibel et al., 1996); however, Corrigan et al. (1993) reported a reduction in Zn levels and no change in Sn

levels. A comparison between the hippocampus and cortex showed an elevation in Zn in the cortex of AD cases (Andrasi *et al.*, 2000). In contrast, Panayi *et al.* (2002) showed similar levels of Cd and Zn across various cortical regions, hippocampus, and thalamus with a decrease observed in AD cases. Srivastava and Jain (2002) reported higher levels of Sn, Al, and Mn in the parietal cortex of AD cases in the absence of differences observed in cerebellar tissue. Thus, while the hippocampus is a prominent region of interest for AD, an association with metals appears to be more consistent when the cortex is examined.

Public health concerns of environmental exposure and neurodegenerative disease are compounded by the absence of an adequate biomarker for brain tissue analyte levels. Sampling for exposure has relied heavily on blood and urine and direct sampling of the nervous system has been limited. Based upon identified metal exchange mechanisms between the brain and CSF (Scheiber *et al.*, 2014), the CSF has been considered as a potential surrogate for brain tissue in estimating central nervous system metal exposure. Most environmental metals can be transported across the protective blood-brain barrier; however, the level of efficiency and extent of such transport normally results in levels within the brain that are significantly lower than those in the blood and peripheral tissues. However, if the blood-CSF barrier is compromised, this could alter clearance of metals from the CNS. Previous work by Gerhardsson *et al.* (2011) determining quantitative levels of essential and nonessential metals in the CSF reported no difference in 17 metals for AD patients as compared to controls. Mn and Pb levels were reported as significantly lower in AD patients; however, the range of values for both metals indicated higher individual levels in the AD group as compared to controls. CSF samples from late-onset AD patients as compared to controls showed higher median levels of Cu and Zn with a high level of variability (4-fold). No differences were observed for Fe, Mg, or Mn (Hozumi *et al.*, 2011). In our comparative analysis, one of the primary issues identified was the relatively low level of nonessential metals in the VF. Sufficient levels of the essential metals, Fe, Cu, and Zn, were detected and, while they ranked highest in both the FC and the VF, we found that the ability to predict brain levels from VF levels was marginal. Ratios between VF and FC levels of As were significantly lower in AD cases as compared to controls, but no differences were observed for the other analytes. Earlier work suggested that the mean CSF/serum ratios of 29 subjects with various neurological complaints were similar for Mn and Mg but were at approximate 0.02 for Fe, Cu, and Zn. The ratio of Mn, Pb, and Hg levels in the CSF, relative to levels in the plasma, were significantly lower in subjects with AD, compared to healthy controls (Gerhardsson *et al.*, 2011). We observed a similar ratio between VF and FC for Mn; however, for the other analytes, the ratio was significantly greater than that observed between CSF and serum by Nischwitz *et al.* (2008). Overall, the data suggested that sampling of VF does not appear to adequately reflect levels of metals within the FC samples, and caution is recommended in any metals/neurodegenerative disease association studies utilizing VF.

The transport of essential metals across the blood-brain barrier is mediated by mechanisms that sequester metals at the apical surface and export them at the basolateral surface for parenchyma distribution. While such transporters have been identified for essential metals (McCarthy and Kosman, 2015; Zheng and Monnot, 2012), they likely regulate the transport of nonessential metals across the blood-brain barrier (Bressler *et al.*, 2007; Yokel, 2006). The metal hypothesis of neurodegeneration puts forth the concept that the inability of the brain to regulate essential metal levels via clearance through the ventricular and

blood-CSF barrier (Miller *et al.*, 2005) results in an aberrant accumulation and increased metal burden resulting in a shift in the dynamics of AD pathology and clinical progression (Bush and Tanzi, 2008). Essential metals such as Fe, Cu, and Zn have been reported to have the potential to aggregate amyloid beta (A β) (Atwood *et al.*, 2000; Ha *et al.*, 2007), thus offering an association between metal burden and amyloid angiopathy. In a study by Baum *et al.* (2010), 12 metals were examined in the serum of AD and control subjects ($n > 40$) and the decrease in serum Zn levels was postulated to be the result of brain amyloid sequestration of Zn. Lower CSF levels of Fe and Zn were found correlated with memory and cognitive functions in AD patients. In a study by Religa *et al.* (2006), an association between cortical Zn levels and increased tissue amyloid levels were observed in AD cases. There was no observed difference in cortical Cu levels (Religa *et al.*, 2006). This is in contrast to the earlier work by Basun *et al.* (1991) reporting elevated levels of Cu in the CSF of patients with dementia, Alzheimer type. In this study, we observed no differences in Zn or Cu levels in the VF or FC of AD patients. For VF levels of Fe, the levels were highly variable across subjects and the individual ratios between the 2 substrates were not significantly different between groups. This suggests caution for any interpretation of altered Fe clearance in the AD cases but does indicate an increased tissue burden of Fe. Upon examination of a range of metals, Baum *et al.* (2010) found no AD-associated changes; however, with individual comparisons, a correlation was found between As serum levels and performance deficit on the MMSE. Similarly, in our study, As levels were found to be elevated in the VF of AD patients accompanied by a decrease in FC levels. Whether this indicates an increased clearance of As from the brain in AD, a distribution related to age of the subject or low levels found in the VF is not clear; however, the ratio of As levels in the VF relative to FC was the only ratio found to be statistically significant. Basun *et al.* (1991) reported lower levels of Cd in the CSF of patients with dementia, Alzheimer type. While levels of Cd within the VF could be detected in the study samples, they failed to reach the LOQ and thus, we were unable to evaluate an association with AD. We did, however, observe lower levels within the FC. It is likely that much of the contradictory data that exists in the literature on metal levels and AD is the result of varying accuracy and sensitivity of detection for quantitation as well as sample selection.

In general, with the exception of Fe, our data do not support an elevation of metals in the FC or VF of AD patients. We found that Braak stage, but not amyloid angiopathy, was an estimate parameter for As, Fe, Mn, and Zn levels in the FC; however, the covariate of age hindered the ability to draw a clear conclusion of this association for all analytes except Fe. This raises questions about the applicability of the hypothesis of essential metal overload for neurodegenerative disease progression and the relationships with nonessential metals. However, we, as well as others, have only measured levels of the total metal and, while no association between brain metal levels and AD has been clearly demonstrated, detection of metals within human brain tissue raises the issue of whether speciation of the metal would uncover an association. These findings do not rule out the possibility of effects occurring within specific brain regions or cell populations as imaging techniques now allow for more refined detection. For example, microglia are considered a prominent source of Fe for other neural-specific cells (Zhang *et al.*, 2006) and an increase in microglia Fe has been implicated in microglia dystrophy observed with aging and AD (Lopes *et al.*, 2008). In addition, Fe has a tendency to accumulate in the vicinity of amyloid plaques and can be found within oligodendrocytes and

FIG. 1. Levels of metals (ng/g tissue weight) in the frontal cortex (FC) of normal elderly control subjects and Alzheimer's disease cases at age of death. All samples reported exceeded the level of quantitation.

astrocytes under various neurodegenerative conditions (Quintana et al., 2006; Weigel et al., 2014; Zhang et al., 2006). Thus, future studies refined on the basis of speciation of the chemical and cellular localization may provide critical information to evaluate the association between metals, metal homeostasis, and neurodegenerative disease.

In this study, we confirmed previous work suggesting an increase in Fe within brain tissue with AD and identified As as a metal of interest for further evaluation. In addition, by directly comparing FC and VF samples from the same subject, we demonstrated that future studies attempting to define or characterize an association between metal levels and neurodegenerative disease clearly require samples obtained from the brain as a target organ and cannot solely rely on CSF fluid samples for an accurate estimate. An expanded study comparing biological fluids (blood, urine, CSF) with tissue samples (central nervous system, bone) and exposure history would significantly advance our understanding of the tissue distribution with applicability of body fluids as surrogate matrices for long-term exposure and allow for a more comprehensive evaluation of the potential association between metals and neurodegenerative disease.

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