

## Zinc and copper modulate Alzheimer A $\beta$ levels in human cerebrospinal fluid

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Received 14 June 2007; received in revised form 15 October 2007; accepted 25 October 2007

Available online 18 December 2007

### Abstract

Abnormal interaction of  $\beta$ -amyloid 42 (A $\beta$ 42) with copper, zinc and iron induce peptide aggregation and oxidation in Alzheimer's disease (AD). However, in health, A $\beta$  degradation is mediated by extracellular metalloproteinases, neprilysin, insulin degrading enzyme (IDE) and matrix metalloproteinases. We investigated the relationship between levels of A $\beta$  and biological metals in CSF. We assayed CSF copper, zinc, other metals and A $\beta$ 42 in ventricular autopsy samples of Japanese American men ( $N=131$ ) from the population-based Honolulu Asia Aging Study. There was a significant inverse correlation of CSF A $\beta$ 42 with copper, zinc, iron, manganese and chromium. The association was particularly strong in the subgroup with high levels of both zinc and copper. Selenium and aluminum levels were not associated to CSF A $\beta$ 42. *In vitro*, the degradation of synthetic A $\beta$  substrate added to CSF was markedly accelerated by low levels (2  $\mu$ M) of exogenous zinc and copper. While excessive interaction with copper and zinc may induce neocortical A $\beta$  precipitation in AD, soluble A $\beta$  degradation is normally promoted by physiological copper and zinc concentrations.

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**Keywords:** Amyloid; Alzheimer's disease; Metalloproteinase; Cerebrospinal fluid; Zinc; Copper; Iron; Manganese; Chromium

### 1. Introduction

The interaction of  $\beta$ -amyloid (A $\beta$ ) with endogenous brain copper, zinc and iron may contribute to the peptide's accumulation and toxicity in the brain in Alzheimer's disease (AD) (Atwood et al., 1998; Bush et al., 1994a,b; Huang et al., 1999a,b; Lee et al., 2002; Opazo et al., 2002). High concentrations of copper (0.4 mM), zinc (1 mM) and iron (1 mM) have been found within amyloid plaques (Lovell et al., 1998), but only zinc and copper have been shown to directly coordinate A $\beta$  (Dong et al., 2003; Opazo et al., 2002). The toxicity of A $\beta$  in cell culture depends upon the peptide binding to copper (Atwood et al., 1998) or iron (Rottkamp et al., 2001) through generating radical chemistry

**Abbreviations:** A $\beta$ 42,  $\beta$ -amyloid ending at residue 42; AD, Alzheimer's disease; APP, amyloid protein precursor; APLP2, amyloid precursor-like protein 2; CAA, congophilic amyloid angiopathy; CERAD, consortium to establish a registry for Alzheimer's disease; CI, confidence interval; CQ, clioquinol; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; GLM, general linear regression model; NFT, neurofibrillary tangle; NP, neuritic plaque; PMI, post-mortem interval; SOD1, superoxide dismutase 1; TPEN,  $N,N,N',N'$ -Tetrakis-(2-pyridylmethyl)-ethylenediamine; MMP, matrix metalloproteinase; SDS, sodium dodecyl sulfate.

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(A $\beta$ 42 > A $\beta$ 40) (Huang et al., 1999a,b; Opazo et al., 2002). Zinc, copper and iron rapidly precipitate A $\beta$  (Atwood et al., 1998; Brown et al., 1997; Cherny et al., 1999; Huang et al., 1997), which then becomes resistant to proteolysis (Bush et al., 1994a,b).

The brain and CSF are protected from fluctuations in plasma metal concentrations by the blood–brain barrier. The zinc and copper in plaques is likely to have been released by glutamatergic synapses (Lee et al., 2002; Schlieff et al., 2005). While A $\beta$  interaction with copper and zinc is corrupted in AD, in health a normal amount of interstitial zinc is needed for the degradation of A $\beta$  by zinc-dependent proteinases that are believed to prevent it from accumulating in the interstitium. Such metalloproteinases, which are also present in cerebrospinal fluid (CSF), include neprilysin (Maruyama et al., 2005), insulin degrading enzyme (IDE) (Qiu et al., 1998) and matrix metalloproteinases (MMP2 and 3) (White et al., 2006). Additionally, the normal generation of A $\beta$  may be modulated by zinc since  $\alpha$ -secretases, tumor necrosis factor- $\alpha$ -converting enzyme (TACE or ADAM-17) and ADAM-10 (Buxbaum et al., 1998), are members of the cell surface zinc metalloproteinase family of disintegrin and metalloprotease (ADAM) proteins. Abnormal metal homeostasis in the brain in AD (Religa et al., 2006), may therefore impact upon the processing or catabolism of A $\beta$ .

To test whether there is a biochemical relationship between A $\beta$  and biometal levels in humans, we investigated the association of CSF copper, zinc and other metals to CSF A $\beta$ 42 in a large cohort of ventricular fluid autopsy samples.

## 2. Methods

### 2.1. Human CSF samples

The autopsy sample is from a cohort of Japanese-American men participating in the population-based Honolulu Asia Aging Study (HAAS) (White et al., 1996). The HAAS began in 1991 as a supplement to the Honolulu Heart Program Study to investigate the determinants of dementia. All participants were eligible for the autopsy substudy; cases of dementia were preferentially followed up to ensure adequate sample size for comparison of cases to controls. Autopsied demented decedents were similar to the demented men who were not autopsied, and autopsied non-demented men were similar to the non-demented decedents who were not autopsied. The institutional review board of Kuakini Medical Center approved the study, and informed consent for autopsy was obtained from the appropriate surviving person.

### 2.2. Neuropathology

Neuropathologic methods in the population-based Honolulu Asia Aging Study (HAAS) have been detailed

elsewhere (Petrovitch et al., 2001). Briefly, 8- $\mu$ m tissue sections from multiple brain regions were analyzed with several stains. Analyses presented here utilize counts of neurofibrillary tangles, and neuritic plaques, from the left side of the brain for two regions of the hippocampus (CA1 and subiculum) and four areas of the neocortex (middle frontal gyrus, middle temporal gyrus, inferior parietal lobule and occipital lobe). All plaque and neurofibrillary tangle (NFT) counts were based on modified Bielschowski stains. Neuritic plaques (NPs) were defined as plaques containing silver-positive neurites. Five fields standardized to 1 mm<sup>2</sup> were examined for each of the four neocortical and two hippocampal areas. The field with highest count was taken to represent the brain area. Counts for NP and NFT were calculated by averaging across the four neocortical or two hippocampal areas. Results were recorded as NP per square millimeter and were truncated at 17/mm<sup>2</sup> (Petrovitch et al., 1997). There was no upper limit for NFT counts. Congophilic amyloid angiopathy (CAA) was quantified by A $\beta$  immunohistochemistry of parenchymal arteries and arterioles, from all four neocortical areas (CAA absent = all vessels non-reactive).

Samples were evaluated by one of three neuropathologists who participated in a training session aimed to standardize reading techniques. CERAD criteria were used for pathologic diagnosis of AD (Mirra et al., 1991). These criteria are based on semiquantitative assessment of NP. Three illustrations representing sparse, moderate, and frequent plaque densities per square millimeter are used as guides for comparison with the microscopic case being assessed. A match with the frequent NP illustration confers neuropathologic diagnosis of definite AD, and the diagnosis of probable AD is made when the case matches the moderate frequency illustration. A maximum NP count of  $\geq 17$ /mm<sup>2</sup> was used to meet CERAD requirements for definite AD, and a count of at least 4/mm<sup>2</sup> was used to meet CERAD requirements for probable AD.

### 2.3. CSF Studies

As the first step in the autopsy, post-mortem CSF was obtained from the cisterna magna by needle aspiration. CSF samples were then chilled in ice and transferred to  $-70^{\circ}\text{C}$  for long-term storage. CSF A $\beta$  42 was measured using a special high-sensitivity version of sandwich ELISA [INNOTEST  $\beta$ -amyloid (1–42), Innogenetics, Ghent, Belgium] (Andreasen et al., 1999). The lowest detectable level was 7 ng/L. Levels ranged from 7 to 858 ng/L with a median of 57 ng/L (interquartile range 12–142).

CSF metals were measured by inductively coupled plasma mass spectrometry as previously described (Cherny et al., 2001). Levels of CSF selenium below 0.04  $\mu\text{M}$  ( $n=3$ ) and CSF aluminum below 0.008  $\mu\text{M}$  ( $n=74$ ), which are below the standard calibration range of the spectrometer, were set to the lowest detectable value.

## 2.4. Analytical sample

At the time of this analysis, 231 men were included in the autopsy study and had CSF data available. Visible blood contamination significantly increased iron, zinc and copper levels (data not shown). Therefore, CSF samples were excluded when visual inspection indicated blood contamination (hemolysis) after centrifugation, leaving 152 individuals with clear CSF considered for this analysis.

The interval between time of CSF collection and death was inversely correlated with A $\beta$ 42 levels (Spearman's correlation coefficient =  $-0.5$ ;  $p$ -value  $<0.001$ ), and positively correlated with CSF metal levels (copper Spearman's correlation coefficient =  $0.6$ ;  $p$ -value  $<0.001$ ; zinc Spearman's  $\rho = 0.5$ ;  $p$ -value  $0.001$ ). Therefore, we excluded 21 samples with a collection interval  $>24$  h, and controlled for the effects of post-mortem interval as a confounder in the final analytical sample of  $N = 131$ .

Clinical diagnoses were established ante-mortem. The subjects included 82 clinically non-demented individuals (62.1 %). Clinical ante-mortem dementia diagnoses used in this analysis include: 25 cases of Alzheimer's disease (AD), 18 cases of vascular dementia (VD) and 6 cases of other dementias (including Parkinson's disease and Lewy Body dementia, trauma plus dementia and dementia of undetermined cause). Clinical dementia was diagnosed according to DSM IIIR criteria, AD following NINCDS-ADRDA criteria, and VD following criteria of the California Alzheimer's Disease Diagnostic and Treatment Centers guidelines. Among all clinical AD cases, 21 cases met probable and 4 cases met definite pathological CERAD criteria.

## 2.5. Synthetic A $\beta$ stability in CSF

Human cerebrospinal fluid from neuropathologically normal individuals was obtained from the Victorian Brain Bank Network. A $\beta$ 40 (230  $\mu$ L, 1 ng/ $\mu$ L in doubly-distilled deionized water) was added to CSF (30  $\mu$ L)  $\pm$  CuCl $_2$  or ZnCl $_2$ , TPEN or water (10  $\mu$ L) and mixed vigorously at 37 °C for 4 h. SDS-sample buffer was then added. The samples were heated (90 °C, 5 min) and separated on a 26-well criterion 10–20% Tris–tricine gel. Blocking was performed for 1 h in 5% skim milk in TBS-T followed by overnight incubation with primary antibody WO2 (detecting residues 5–8 of A $\beta$ ; 1:25 dilution in TBS-T). Following washing the blot was incubated in anti-mouse HRP secondary antibody for one hour, washed in TBS-T and developed with ECL reagent. Imaging was performed using a LAS-3000 system (FujiFilm) and the amounts of A $\beta$  monomer quantified using MultiGauge V3.0 software. All analysis was performed using JMP V.5.01 statistical discovery software (SAS) for Macintosh.

## 2.6. Statistical analysis

We considered as possible confounders: age at death, education in years, time interval in hours from death to collection

of cerebrospinal fluid, and apolipoprotein E4 allele (genotyped with restriction isotyping using a polymerase chain reaction). As the severity of dementia might have changed in the time period from diagnosis to death, we also adjusted for the time interval from diagnosis or last clinical evaluation to death (mean 3.9 years (range 0.3–8.7)).

Stata 8.0 (Statacorp, College Station, TX) software was used for analyses. General linear regression was employed to describe CSF A $\beta$ 42 level differences among tertiles of metals. Since the distribution of the CSF A $\beta$ 42 concentration was skewed, we fit a general linear regression model (GLM) with a log-link and a gamma distribution (McCullagh and Nelder, 1989). Compared to the commonly used ordinary least square regression model with log transformed values, this GLM model preserves the original scale of the variable and hence yields readily interpretable regression coefficients.

To examine the combined effect of both CSF copper and zinc we divided the sample based on their median CSF copper and zinc levels. This gave us four groups: low for both metals ( $-Zn, -Cu$ ) for participants with CSF  $-$  copper and CSF  $-$  zinc levels below their median level ( $n = 53$ ); high for both metals ( $+Zn, +Cu$ ) for participants with both metal levels above their median level ( $n = 52$ ); high for zinc ( $+Zn, -Cu$ ) for participants with high levels of zinc and low levels of copper ( $n = 13$ ), and low for zinc ( $-Zn, +Cu$ ) for participants with low zinc and high copper ( $n = 13$ ). The low group ( $-Zn, -Cu$ ) served as the reference group for the analyses.

Multiple regression analyses based on the over dispersed Poisson model were used to examine the association of NPs and NFTs to tertiles of metal levels. This statistical model yields a ratio of counts; for example a ratio of 0.8, means that the exposed group has 20% fewer maximum counts of NP per mm $^2$  than the reference group.

## 3. Results

### 3.1. Inverse correlation between CSF A $\beta$ 42 and biometal levels

The relative abundances of elements we surveyed in the CSF were  $Fe > Zn > Cu > Se > Al > Mn > Cr$ . Iron had the highest concentration, at a median level of 9.94  $\mu$ mol/L (range 0.5–31.7), followed by zinc at 1.84  $\mu$ mol/L (range 0.21–12.45) and copper at 0.52  $\mu$ mol/L (range 0.01–5.7). Selenium had a mean level of 0.43  $\mu$ mol/L (range 0.04–0.92), aluminum of 0.25  $\mu$ mol/L (range 0.008–5.78), manganese of 0.22  $\mu$ mol/L (range 0.03–0.52), and chromium of 0.20  $\mu$ mol/L (range 0.03–0.43). Baseline characteristics of the sample by copper and zinc tertiles are presented in Table 1.

Increasing biometal concentrations were associated with a decrease in CSF A $\beta$ 42 concentration in the postmortem CSF after adjusting for all covariates (Table 2, Fig. 1). The association was strongest for copper ( $\beta$  coefficient =  $-1.3$ ; 95% CI [ $-1.78, -0.8$ ];  $p$ -trend =  $<0.0001$ ) and zinc in the

Table 1

Baseline characteristics of the sample by copper and zinc tertiles: the Honolulu-Asia Aging Autopsy Study

	Copper (μmol/L)			<i>p</i> trend <sup>b</sup>	Zinc (μmol/L)			<i>p</i> trend <sup>b</sup>
	Low (<0.4)	Mid (0.4–0.75)	High (>0.75)		Low (<1.3)	Mid (1.3–2.5)	High (>2.5)	
Demographic factors								
Age at death (year ± S.D.)	85.8 (5.4)	85.0 (5.1)	86.6 (5.6)	0.5	85.5 (5.6)	84.8 (4.8)	87.0 (5.6)	0.2
Post-mortem interval (h ± S.D.)	9.1 (5.3)	12.4 (5.8)	16.0 (4.5)	<0.001	8.8 (5.2)	12.4 (5.4)	16.2 (4.7)	<0.001
APOE ε4 allele (%)	24.4	20.9	16.3	0.7	20.5	23.3	18.2	0.7
Clinical dementia (%)	44.4	30.2	37.2	0.4	34.1	44.2	34.1	0.8
Alzheimer's disease ( <i>n</i> = 25)	17.8	18.6	20.9	0.2 <sup>c</sup>	18.2	20.9	18.2	0.2 <sup>c</sup>
Vascular dementia ( <i>n</i> = 18)	17.8	9.3	14.0	0.7 <sup>c</sup>	11.4	16.3	13.6	0.9 <sup>c</sup>
Other dementia <sup>a</sup> ( <i>n</i> = 6)	8.9	2.3	2.3	0.2 <sup>c</sup>	4.6	7.0	2.3	0.2 <sup>c</sup>
Time from last clinical exam until death (year ± S.D.)	3.7 (1.6)	4.2 (1.4)	4.2 (2.1)	0.33	3.6 (1.6)	3.9 (1.7)	4.0 (2.0)	0.06
Clinico-pathologic groups (%)								
Prob/def CERAD <sup>d</sup>	22.2	11.6	23.3	0.8	18.2	20.9	18.2	0.6
Cerebrovascular lesions (% with any)								
Infarct ≥ 1 cm	17.8	27.9	39.5	0.1	18.2	30.2	36.4	0.3
Lacune < 1 cm	48.9	44.2	44.2	0.4	50	46.5	40.9	0.1
Microinfarct	69.4	62.5	54	0.1	68.6	63.2	55	0.1
CAA	53.3	51.2	46.5	0.9	52.3	51.2	47.7	0.9

<sup>a</sup> Other dementias included Parkinson's disease and Lewy body disease, trauma plus dementia and dementia of undetermined cause.<sup>b</sup> Adjusted for age at death and post-mortem interval using a logistic regression analysis for dichotomous variables or general linear regression analysis for linear variables.<sup>c</sup> Adjusted for age at death and post-mortem interval using metals as linear independent variables.<sup>d</sup> CERAD cases (*n* = 25) compared to rest of the autopsied samples (*n* = 106).

Table 2

Analysis of the association of A $\beta$ 42 and metal levels in cerebrospinal fluid

Metals (μM)	Mean Aβ42 (ng/L)	Mean difference (95% CI)	p-trend
Copper (range 0.01–5.7)			
Low (<0.4)	114.9	0 (reference)	<0.0001
Mid (0.4–0.75)	94.7	−0.19 (−0.62, 0.2)	
High (>0.75)	32.6	−1.26 (−1.7, −0.8)	
Zinc (range 0.21–12.45)			
Low (<1.3)	116.7	0 (reference)	<0.0001
Mid (1.3–2.5)	95.2	−0.2 (−0.6, 0.2)	
High (>2.5)	33.8	−1.2 (−1.7, −0.76)	
Iron (range 0.53–31.7)			
Low (<7.2)	118.6	0 (reference)	0.001
Mid (7.2–12.0)	70.6	−0.52 (−0.99, −0.05)	
High (>12.0)	52.2	−0.83 (−1.4, −0.34)	
Manganese (range 0.32–0.52)			
Low (<0.16)	110.5	0 (reference)	0.003
Mid (0.16–0.24)	75.1	−0.39 (−0.9, 0.1)	
High (>0.24)	53.3	−0.73 (−1.2, −0.2)	
Chromium (range 0.03–0.43)			
Low (<0.157)	94.3	0 (reference)	0.01
Mid (0.157–0.23)	102	0.08 (−0.4, 0.5)	
High (>0.23)	43.8	−0.77 (−1.23, −0.3)	
Selenium (range 0.04–0.92)			
Low (<0.32)	84.2	0 (reference)	0.3
Mid (0.32–0.53)	89.1	0.06 (−0.4, 0.6)	
High (>0.53)	65.6	−0.25 (−0.7, 0.2)	
Aluminum (range 0.008–5.78)			
Low (≤0.008)	86.3	0 (reference)	0.5
Mid (0.0081–0.096)	58.9	−0.38 (−1.05, 0.3)	
High (>0.096)	74.4	−0.15 (−0.56, 0.37)	

Statistical analysis using a general linear regression model with a log-link, adjusted for age at death, education, APOE  $\epsilon 4$ , post-mortem interval until CSF collection, and time from last clinical exam until death.

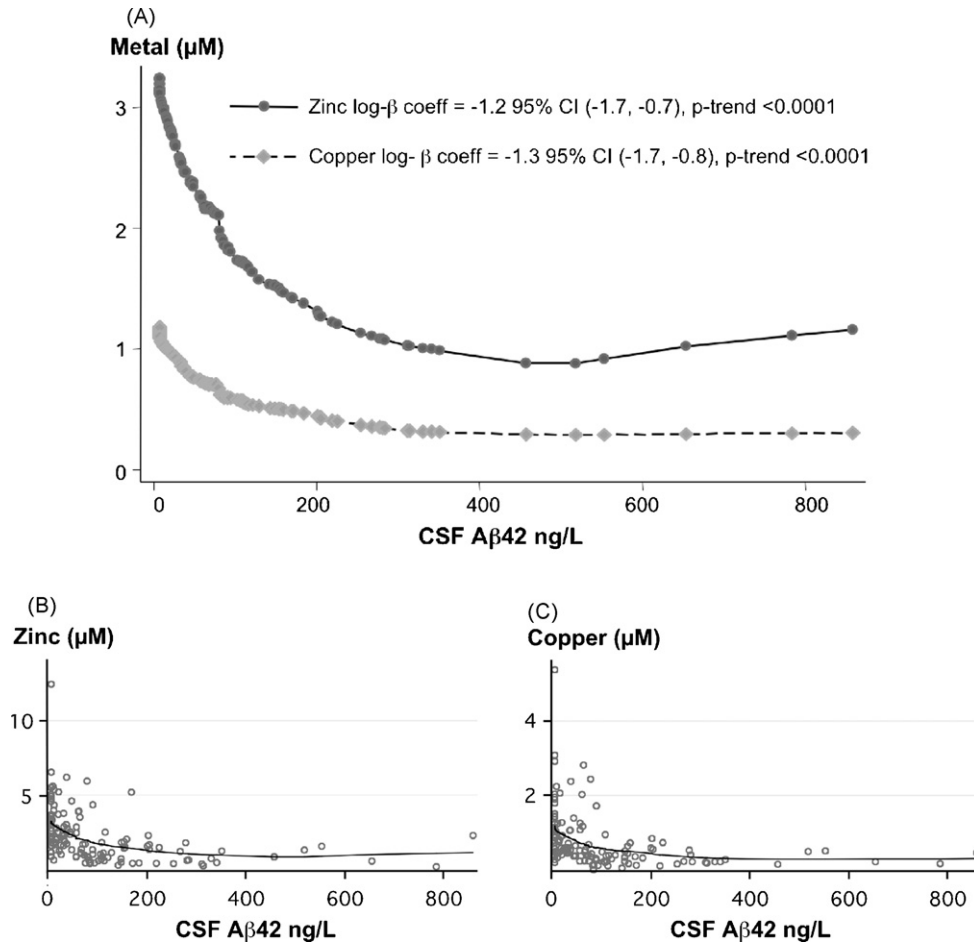


Fig. 1. Relationship of CSF zinc and copper to CSF Aβ42: (A) plot of mean adjusted smoothed values achieved by locally weighted scatterplot smoother, bandwidth = 0.8 (Stata 8.0), (B) raw data for zinc levels with mean adjusted smoothed values used for (A) and (C) raw data for copper levels with mean adjusted smoothed values used for (A).

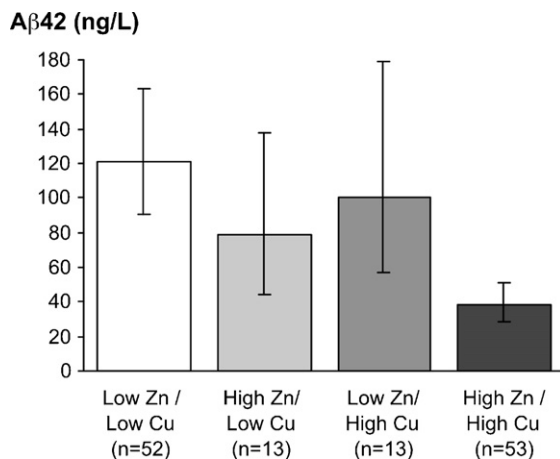


Fig. 2. Combined association of CSF zinc and copper levels to Aβ42 levels. Results represent mean levels adjusted for age at death, post-mortem interval, education, APOE ε4 allele, and time from last clinical assessment until death. Error bars are 95% confidence intervals.

highest tertile ( $\beta$  coefficient =  $-1.26$ ; 95% CI  $[-1.7, -0.8]$ ;  $p$ -trend  $\leq 0.0001$ ), when compared to the lowest tertile. These associations remained significant with a post-mortem interval of less than twelve hours, and equally significant ( $p$ -trend  $\leq 0.0001$ ) after removing outliers (the six highest Aβ42 and the one highest metal readings) (data not shown).

Iron, manganese and chromium were also significantly inversely associated with CSF-Aβ42. There was no relationship of CSF Aβ42 to aluminum and selenium (Table 2).

Copper and zinc were synergistically associated with CSF-Aβ42 levels. Combined high zinc and high copper levels (+Zn, +Cu) were associated with lower CSF-Aβ42 levels (log  $\beta$  coefficient  $-1.16$   $[-1.6, -0.7]$ ), when compared to low levels of both CSF metals (−Zn, −Cu) (Fig. 2). There was no association with CSF-Aβ42 if only one metal was elevated, i.e. high zinc and low copper (+Zn, −Cu) or low zinc and high copper (−Zn, +Cu).



Table 3

Analysis of the association of pathologic hallmarks of AD and CSF metals in cerebrospinal fluid

	Neocortex		Hippocampus	
	Neuritic plaques IRR (95% CI)	Neurofibrillary tangles IRR (95% CI)	Neuritic plaques IRR (95% CI)	Neurofibrillary tangles IRR (95% CI)
Copper				
Low (<0.4 $\mu\text{M}$ )	1	1	1	1
Mid (0.4–0.75 $\mu\text{M}$ )	0.99 (0.5–1.9)	1.26 (0.5–3.2)	1.0 (0.5–2.1)	1.11 (0.6–1.9)
High (>0.75 $\mu\text{M}$ )	0.95 (0.5–2.0)	2.23 (0.8–5.9)	0.85 (0.4–2.0)	1.08 (0.6–2.0)
Zinc				
Low (<1.3 $\mu\text{M}$ )	1	1	1	1
Mid (1.3–2.5 $\mu\text{M}$ )	0.95 (0.48–1.87)	2.09 (0.8–5.2)	1.11 (0.5–2.6)	<b>2.16 (1.2–3.8)*</b>
High (>2.5 $\mu\text{M}$ )	0.82 (0.39–1.74)	1.43 (0.5–4.2)	1.14 (0.5–2.8)	1.38 (0.7–2.7)

\*  $p < 0.05$ .

### 3.2. CSF metals, clinical dementia and neuropathology

Prevalence of ante-mortem dementia diagnosis did not vary between postmortem CSF copper, CSF zinc tertiles (Table 1) or other metals. There was a trend of higher CSF copper and zinc levels with fewer neuritic plaque counts in either the neocortex or the hippocampus (Table 3). There was no relationship with other metal levels and neuropathology (data not shown). There were 25 cases of pathologically confirmed AD cases (CERAD). Those cases did not differ by metal levels from other cases (Table 1).

### 3.3. Degradation of synthetic A $\beta$ by CSF metalloproteinases

To explore whether the inverse correlation between CSF A $\beta$  and Cu or Zn could reflect the modulation of proteolytic activities by the metal ions, we studied the effects of modulating Cu or Zn, the only metal ions shown to directly coordinate A $\beta$  *in vivo* (Dong et al., 2003; Opazo et al., 2002), upon the stability of synthetic A $\beta$  added to samples of normal CSF (Fig. 3). We incubated synthetic A $\beta$ 1-40 added to CSF with the presence of additional Cu or Zn at a physiological concentration (2  $\mu\text{M}$ ) or TPEN (150  $\mu\text{M}$ ), a zinc/copper-selective chelator. The presence of added Cu or Zn induced the A $\beta$  to degrade markedly after 4 h incubation, whereas there was only slight loss of A $\beta$  in the absence of the metals or in the presence of TPEN (Fig. 3). The loss of A $\beta$  was not due to precipitation because previous studies have shown that the SDS buffer, which was added to the samples to recover the A $\beta$ , completely dissolves metal-induced A $\beta$  precipitates (Bush et al., 1995).

## 4. Discussion

In this general population autopsy study, we found a strong inverse correlation of CSF-A $\beta$ 42 with CSF metals copper, zinc, iron, manganese and chromium. There was no association with selenium or aluminum. There was also a synergistic interaction of elevated copper and zinc being associated with

lower CSF A $\beta$ 42 levels. These associations were controlled for several confounding variables, including post-mortem interval. Therefore, we conclude that levels of CSF A $\beta$ 42 may have a relationship with levels of CSF biometals. This was supported by *in vitro* data that low concentrations of exogenous Zn and Cu promoted the degradation of synthetic A $\beta$  added to CSF samples (Fig. 3).

A $\beta$  is produced in health where it is a normal component of brain and CSF, and is turned over in CSF rapidly as it exits the CNS into the blood (Brody and Holtzman, 2006). The steady state levels of A $\beta$  in CSF reflect factors that influence amyloid protein precursor (APP) processing, and factors that clear and degrade A $\beta$ . The small amount of A $\beta$  degradation by CSF activities *in vitro* in the presence of a chelator (Fig. 3) indicates that non-metalloproteinase activities may degrade A $\beta$  in CSF. However, the rapid degradation of A $\beta$  by CSF activities upon the addition of 2  $\mu\text{M}$  Cu or Zn (Fig. 3), is consistent with a major role for metalloproteinases in soluble A $\beta$  degradation. Since the addition of TPEN did not alter A $\beta$  degradation in CSF, we conclude metalloproteinases may be

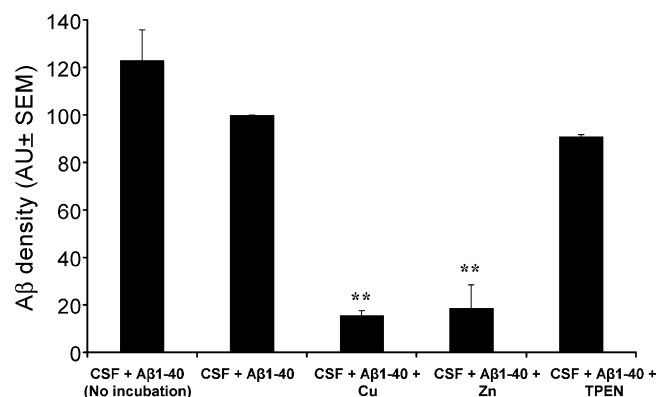


Fig. 3. Zinc and copper promote the degradation of A $\beta$  in CSF. Synthetic A $\beta$ 1-40 was added to normal CSF  $\pm$  Cu, Zn (2  $\mu\text{M}$ ) or the Cu/Zn-selective chelator TPEN (150  $\mu\text{M}$ ), and incubated for 4 h. The amount of A $\beta$  remaining in the CSF was then quantified by western blot and densitometry. All data (means  $\pm$  S.E.M.) have been normalized to the density of the 4 kD immunoreactive A $\beta$  density of the CSF + A $\beta$  sample. Samples were assayed in triplicate, and the results are typical of  $N = 6$  experiments. \*\* $p < 0.01$  vs. CSF + A $\beta$  sample.

in their inactive apo-forms in CSF, and that adding Cu and Zn activates them (Fig. 3). Therefore, the relationship between CSF metals and A $\beta$  that we identified (Figs. 1 and 2, Table 1) probably reflects the interaction of metals with A $\beta$  or with enzymes that cleave A $\beta$  or process APP in the brain interstitium. Cu and Zn could promote non-amyloidogenic APP processing by metalloproteinases such as TACE and ADAM-10 (Buxbaum et al., 1998), or promote the degradation of A $\beta$  by interstitial metalloproteinases neprilysin and IDE. Alternatively, elevated metals could promote the sequestration of A $\beta$  by  $\alpha$ -2 macroglobulin (Du et al., 1997). In each of these instances, A $\beta$  would be degraded as metal levels increased, consistent with our current findings. These findings support endogenous brain metal metabolism as being a critical factor in determining steady state levels of A $\beta$ . Aluminum is not known to participate in normal neurochemistry, hence it is not surprising that we found no association of Al levels with A $\beta$ . This finding is also consistent with multiple epidemiological studies that have failed to sustain the once-held suspicion that Al intoxication could increase the risk for AD (Flaten, 2001). Similarly, selenium is not normally found in the metal ionic state in tissues, but rather as selenomethionine or selenocysteine and would not be expected to influence A $\beta$  metabolism by the metalloproteinase mechanism envisaged here.

This newly defined close association of CSF A $\beta$  with biological metal levels in health may explain the vulnerability of A $\beta$  to abnormal interaction with these metals, which could trigger AD (Bush, 2003). Therefore, we also explored whether the abnormal interaction of metals with A $\beta$  in the brain interstitium in AD is reflected in the CSF. There are very few reports on CSF metal levels in AD. One study reported a significant elevation of copper (Basun et al., 1991), a second study described decreased zinc levels in living AD subjects (Molina et al., 1998). We did not detect such changes, but there were several limitations that may have diminished the power of the study to discriminate CSF metal levels associated with clinical or neuropathological AD. The clinical dementia assessment was performed up to 8.7 years prior to the measurement of A $\beta$ 42 and metals in post-mortem CSF. Therefore, it is likely that some non-demented individuals developed clinical dementia before death, and that demented participants would have further progressed. Also, the number of AD cases was relatively small. We did not observe an association of CSF metal levels to neuritic (amyloid) plaques, but it has been previously suggested the amyloid plaques do not correlate with total A $\beta$ 42 burden in brain tissue (McLean et al., 1999). Also, there were no brain A $\beta$ 42 values to compare with the CSF values. Future studies that analyze the association of CSF metals and CSF A $\beta$ 42 in living subjects are needed to clarify these associations.

Since an intimate biochemical relationship between A $\beta$  and biological metals is revealed by our results, the findings also underscore the potential potency of pharmacological approaches targeting A $\beta$ –metal interactions. One would expect, for example, high-affinity chelators to inhibit A $\beta$

degradation if they could enter the CSF compartment. This could be viewed favorable since low CSF A $\beta$  levels have predictive value for developing AD (Hansson et al., 2006; Strozzyk et al., 2003). However treatment with clioquinol (CQ), a zinc/copper chelator, lowered plasma A $\beta$  in a double-blind phase 2 pilot study in AD subjects (Ritchie et al., 2003). Since plasma A $\beta$  is in a dynamic equilibrium with CSF A $\beta$  (Bateman et al., 2006) it is tempting to assume that the CQ-induced drop in plasma A $\beta$  would have been associated with a drop in CSF A $\beta$  and therefore might be sinister. However, the levels of A $\beta$  in the CSF/plasma compartments have only been established to be in a dynamic equilibrium over short time scales in healthy humans (e.g. hours). The nature of the steady-state equilibrium may be quite different on a time scale measured in weeks to months (as in clinical trials or prospective biomarker surveys), or in the presence of a brain amyloid reservoir seen in AD. In attempting to interpret the meaning of the changes in plasma A $\beta$  in the CQ trial, it is important to note that the CQ-treated AD subjects exhibited a significant arrest in cognitive deterioration compared to placebo controls during the epoch after 24 weeks of treatment where the plasma A $\beta$  levels rose. Also, CQ treatment has been reported to stabilize clinical deterioration over 9–14 months in an open-label study of familial AD (Ibach et al., 2005). Therefore, the rise in plasma A $\beta$  associated with CQ treatment is unlikely to be sinister.

Another important point to consider about the mechanism of action of CQ in this context, is that it is a low-affinity chelator and does not remove metals from tissue. Unlike high-affinity chelators, CQ actually raises brain copper and zinc levels (Cherny et al., 2001). Animal studies have shown that despite limited blood–brain barrier penetration, CQ concentrates in brain amyloid pathology and is cleared within hours from the brain compartment and plasma (Opazo et al., 2006), so its site of action in amyloid clearance (Cherny et al., 2001) is unlikely to be the CSF. We hypothesize that CQ binds copper and zinc in amyloid aggregates and so promotes A $\beta$  dissociation and clearance, with the metals returning to relatively metal-deficient tissue. It is therefore difficult to predict the consequent change in CSF A $\beta$  levels in the short and long term, as illustrated by a phase 1 study with low doses of CQ showing CSF A $\beta$  levels to rise at day 7 of treatment and then fall at day 21 (Regland et al., 2001). This may be due to dissociated A $\beta$  from plaques transiently exiting the brain via the CSF. A second-generation derivative of CQ with much better blood–brain barrier permeability, named PBT2 (<http://www.alzforum.org/drg/drc/default.asp?type=drug&Name=PBT2>), has nearly completed a double-blind phase 2 clinical trial where plasma and CSF A $\beta$  are assayed over a period of 12 weeks of treatment. Data from this study could illuminate the complexities involved in perturbing the equilibria between the brain amyloid compartment, CSF A $\beta$  and plasma A $\beta$  using this pharmacological approach.

The close inverse association that we have identified between CSF A $\beta$  levels and biological metals underscore

the importance of brain metal homeostasis as a potential factor in the evolution of AD pathology. These data would also be consistent with a role for APP and its derivatives in participating in metal regulation in brain tissue, as previously considered (Maynard et al., 2002, 2006).

## Disclosure statement

Drs. Bush, Adlard and Cherny are shareholders in and consultants for Prana Biotechnology Ltd. The company had no involvement in the funding, design or interpretation of the studies, and no input in the drafting of the manuscript.

## Acknowledgements

This study was supported by NIH (National Institute on Aging); Grant Numbers 1R01 AG12686 (to AIB), 1 U01 AG19349-01, 5 R01 AG017155-04, and in part by the Intramural Research Program of the NIH, National Institute on Aging, as well as (to AIB) the Australian Research Council Federation Fellowship, the National Health and Medical Research Council of Australia, the Alzheimer Association Zenith Award and the American Health Assistance Foundation. The Victorian Brain Bank Network is supported by The University of Melbourne, The Mental Health Research Institute of Victoria, Victorian Forensic Institute of Medicine and funded by Neurosciences Australia and the National Health & Medical Research Council.

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