Supplementary Information for:

Decreased serum zinc is an effect of ageing and not Alzheimer's disease

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Methods and materials

Subjects

Participants were recruited from the AIBL study, which is collected from two geographical sites: Melbourne, Victoria; and Perth, Western Australia. Subjects over the age of 65 years and fluent in English were divided into three groups; cognitively healthy individuals (HC), participants with mild cognitive impairment (MCI) based on the established criteria,[1,2] and participants diagnosed with *possible* or *probable* AD as defined by NINCDS-ADRDA criteria.[3] AIBL was approved by the institutional ethics committees of Austin Health, St. Vincent's Health, Hollywood Private Hospital, and Edith Cowan University. Written informed consent was obtained from all study participants.

Serum and erythrocyte collection and preparation

Serum was prepared from whole blood drawn by venepuncture from overnight fasted participants. Filled Sarstedt s-monovette serum-gel 7.5ml tubes (Sarstedt, Nümbrecht, Germany) were left standing upright for a minimum of 20 minutes at room temperature. Samples were centrifuged at 1,800 *g* for 15 minutes at 20°C with braking on. The serum was then split into 250 µl aliquots, and stored in Nunc Cryobank polypropylene tubes (Nunc, Rochester, NY, USA) in liquid nitrogen vapour tanks until required for metal analysis.

Whole blood was collected from overnight fasted participants with a 27 gauge needle, into Sarstedt S-Monovette® Lithium-Heparin 7.5 mL tubes. The blood was

spun at 3,200 *g* for 30 minutes at room temperature and the plasma was carefully removed. Erythrocytes were then washed 3 times in 0.9% (w/v) normal saline. Erythrocytes were distributed by tube inversion, and then centrifuged at 650 *g* for 10 minutes at 20 °C. The supernatant was removed and the tubes centrifuged at 1,500 *g* for a further 10 minutes at 20 °C. Erythrocytes were then resuspended to a volume of 6 mL in phosphate buffered saline (PBS) (pH 7.4) and aliquoted into polypropylene (Nunc) tubes for storage in liquid nitrogen.

Inductively coupled plasma-mass spectrometry (ICP-MS)

Samples were all thawed at room temperature on the day of analysis. Serum was briefly spun at 1,800 g before being diluted 1:10 with 1% HNO_3. 50 μL of washed erythrocytes were digested in equivalent volumes of concentrated (65%) HNO_3 and H_2O_2 (Merck Milipore) at 80°C for 5 minutes, then diluted 1:20 with 1% HNO_3. Zinc concentration was determined using an Agilent Technologies 7700x ICP-MS system. The sample introduction system used a Teflon MiraMist parallel path nebulizer (Burgener Research Inc.) and standard Scott-type double-pass spray chamber (Glass Expansion). Helium was used as a collision gas. ICP-MS conditions were replicated from previously reported studies from our laboratory.[4] The instrument was calibrated using multi-element standards (Accustandard, ICP-MS-2-1, ICP-MS-3-1, ICP-MS-4-1; total of 44 elements) containing zinc at 0, 5, 10, 50, 100 and 500 ppb with ^{89}Y as the internal standard. ^{64}Zn and ^{66}Zn were the monitored isotopes of zinc. Seronorm™ L1 and L2 (Sero) were reconstituted in 1% HNO_3 (1:20 dilution factor) prior to analysis.

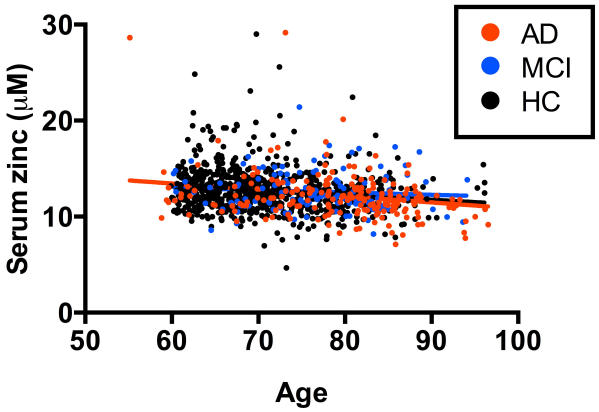
Size exclusion chromatography-ICP-MS

Size exclusion chromatography was performed following the methods outlined previously.[5] Briefly, 25 μL of serum was injected onto an Agilent BioSEC300 (4.6 x 300 mm, 300 Å pore size, 3 μm particle size; Agilent) column in an Agilent 1200 Series LC system. Separation was carried out at 30 °C using a 200 mM ammonium

acetate buffer (pH 7.7) at 0.4 mL min^{-1}. Outflow from the column was directed to an Agilent 7700x ICP-MS via a 60 cm length of PEEK tubing.

Statistical analysis

Subject demographic data were compared using ANOVA, Chi Square and Kruskal-Wallis tests. A Kolmogorov-Smirnov test demonstrated zinc concentrations at baseline and 18-month follow-up for overall and sex-partitioned populations were normally distributed and a Bartlett's test for equality of variances did not suggest that the population of males had a different variance to the population of females. A generalised linear model approach was used to define differences in serum zinc for both marginalized means and covariate-adjusted (age, sex and *ApoEε4* allele status) comparisons of HC vs MCI and HC vs AD groups. Proportional odds logistic regression was used to define differences for the three group comparisons (HC, MCI, AD) for both marginalised means and covariate-adjusted analyses. Statistical analyses were performed with Prism 6.0 (Graphpad Inc) and the R statistical environment (Version 3.02). Significant *p*-values were < 0.05, while a Bonferroni adjusted alpha (0.05/3) was used to assess *p*-values for group-wise analyses.



Supplementary Figure 1: Serum zinc concentration versus age in AD, MCI and HC cases.

Table S1: Analytical figures of merit across all experimental batches. BEC = background equivalent concentration and DL = detection limit (*n* = 25). * based on measured concentration of 74.1 ± 3.9 μg L^{-1} (*n* = 6).

	Sample replicates	True concentration (μg L^{-1})	Measured concentration (μg L^{-1})	% recovery
SeroNorm L1	12	86.9 ± 3.6	89.2 ± 5.0	102.6 ± 5.8
SeroNorm L2	12	126.0 ± 10.3	126.2 ± 12.8	100.2 ± 10.1
Serum IV + 5 ppb spike	6	79.1*	78.6 ± 3.1	99.4 ± 3.9
Serum IV + 50 ppb spike	6	124.1*	123.7 ± 6.3	99.7 ± 5.0%
BEC			0.892 ± 0.65 μg L^{-1}	
DL			0.511 ± 0.871 μg L^{-1}	
R^2			0.9998 ± 0.0002	

Table S2: Zinc concentration by clinical classification and *ApoE* allele status.

		n	Serum Zn ± 1 SD (μM)
HC	*E2/E2*	5	11.45 ± 2.25
	E3/E2	107	12.73 ± 3.38
	E3/E3	421	12.65 ± 2.20
	E4/E2	20	12.79 ± 4.10
	E4/E3	162	12.97 ± 2.36
	E4/E4	19	12.64 ± 1.67
MCI	*E3/E2*	10	12.66 ± 1.23
	E3/E3	50	12.78 ± 2.40
	E4/E2	4	12.53 ± 1.71
	E4/E3	47	12.49 ± 2.07
	E4/E4	13	12.32 ± 1.49
AD	*E3/E2*	6	11.22 ± 0.99
	E3/E3	70	12.64 ± 3.56
	E4/E2	2	11.60 ± 1.48
	E4/E3	95	12.01 ± 2.57
	E4/E4	29	12.04 ± 1.46

Supplementary references:

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