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Decreased Plasma Iron in Alzheimer's Disease Is Due to Transferrin Desaturation

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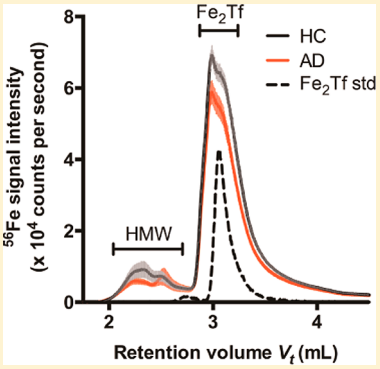
Supporting Information

ABSTRACT: Plasma iron levels are decreased in Alzheimer's disease (AD) and associated with an idiopathic anemia. We examined iron-binding plasma proteins from AD patients and healthy controls from the Australian Imaging, Biomarkers and Lifestyle (AIBL) Flagship Study of Ageing using size exclusion chromatography-inductively coupled plasma-mass spectrometry. Peak area corresponding to transferrin (Tf) saturation was directly compared to routine pathological testing. We found a significant decrease in transferrin-associated iron in AD that was missed by routine pathological tests of transferrin saturation, and that was able to discriminate between AD and controls. The AD cases showed no significant difference in transferrin concentration, only a decrease in total transferrin-bound iron. These findings support that a previously identified decrease in plasma iron levels in AD patients within the AIBL study is attributable to decreased loading of iron into transferrin, and that this subtle but discriminatory change is not observed through routine pathological testing.



KEYWORDS: *Alzheimer's disease, iron, size exclusion chromatography-inductively coupled plasma-mass spectrometry, transferrin, transferrin saturation*

There is mounting evidence that brain iron homeostasis is perturbed in Alzheimer's disease (AD).[1−3] Accumulation of iron in the brain is a feature of normal ageing that, coupled with a loss of metal homeostasis, can impart neurotoxicity through unchecked redox activity.[4] Recent post-mortem[5,6] and in vivo studies[7] have identified a measurable change in brain iron levels, though available data is very heterogeneous.[8] In the periphery, reports of circulating iron levels in AD are equally inconsistent; a recent meta-analysis identified 5 separate studies totaling 153 AD patients and 545 controls, of which 2 reported a decrease in plasma iron levels while the remaining 3 failed to identify a significant change.[9] This suggests that if a change in circulating iron is present in AD, it is likely a subtle effect that may be confined to a specific iron-binding protein, and that a large cohort of AD and control subjects is necessary to obtain statistically adequate numbers to further investigate the potential iron may have as a plasma-based biomarker of AD.

The Australian Imaging, Biomarkers and Lifestyle Flagship Study of Ageing (AIBL) provides a unique resource for this endeavor as a large, well-characterized cohort of over 1000 individuals,[10] including over 200 diagnosed cases of AD. At the baseline time point of this longitudinal study, subjects gave 80 mL of blood, which was then used for clinical pathology, apolipoprotein E (*ApoE*) genotyping, and analytical biochemistry, with a portion fractionated and cryogenically stored. We have previously used this resource to demonstrate that a perceived decrease in plasma zinc concentration in AD is in fact an effect of age,[11] which was possible only due to the high statistical power of AIBL.

Recent data from AIBL has also shown that disturbed brain iron metabolism is reflected in the periphery by a decrease in plasma iron and hemoglobin (Hb).[12] This finding is important because a systemic deficit in iron trafficking could contribute to morbidity associated with AD, since the catalytic properties of iron are essential for a number of metabolic processes including heme formation, neurotransmitter synthesis, and myelination of axons. A possible mechanism driving iron dyshomeostasis in the brain has been identified as involving the iron transporter

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transferrin (Tf) via immunohistochemistry,[13,14] though this post-mortem assay provides information only on protein distribution, as opposed to metal status, and has little value as a potential biomarker. However, in situ assay of brain iron levels via T2* MRI has shown possible use as a biomarker of AD,[6] and data from AIBL suggestive of iron-binding protein changes in AD[12,15] indicative of a systemic deficit in iron homeostasis[16,17] demands further examination with respect to systemwide iron metabolism.

For this prospective study, we examined a randomly selected cohort of nonanemic AD (n_{AD} = 34) and healthy controls (HC; n_{HC} = 36) plasma samples from AIBL to determine whether Tf or Tf–iron binding is responsible for the decreased plasma iron levels we have previously observed in AD patients. To do this, we employed native separation of iron-binding proteins by size exclusion chromatography (SEC), which was hyphenated to inductively coupled plasma-mass spectrometry (ICP-MS) for metal-specific detection.

The average iron-binding SEC-ICP-MS protein profile (Figure 1a; see Supporting Information Figure S1 for individual chromatograms) resolved iron-containing species into two distinct peaks: the first attributable to high molecular weight species (HMW; including the major iron-storage protein ferritin (FTN) at V_R = 2.07–2.76 mL) and the second corresponding to the retention volume of a purified human Tf standard (V_R = 3.06 ± 0.01 mL; n = 3). The results of a Student's t test on each chromatographic data point (Figure 1b) found that only the peak corresponding to iron-bound Tf was significantly decreased (P < 0.017; P_{min} < 0.001 at the maximum Tf peak height) between the AD and HC groups. Comparing the total chromatographic area, we again found the difference in our normally distributed data (Shapiro-Wilks test; P_{HC} = 0.986; P_{AD} = 0.278) was confined to the peak corresponding to iron-bound Tf (P = 0.01; Figure 1c). The low peak capacity of SEC results in coelution of Tf with human serum albumin (HSA), though any contribution from iron bound to HSA is unlikely, as iron-HSA binding has only been observed in pathological iron *overload* and affinity of iron to HSA is comparatively minor relative to Tf.[18] We constructed a receiver operating characteristic (ROC) curve to examine the potential of iron–Tf assay by SEC-ICP-MS as a biomarker for AD (Figure 1d). The curve showed that Tf-associated iron could discriminate between HC and AD cases (area under curve = 0.70; P = 0.003).

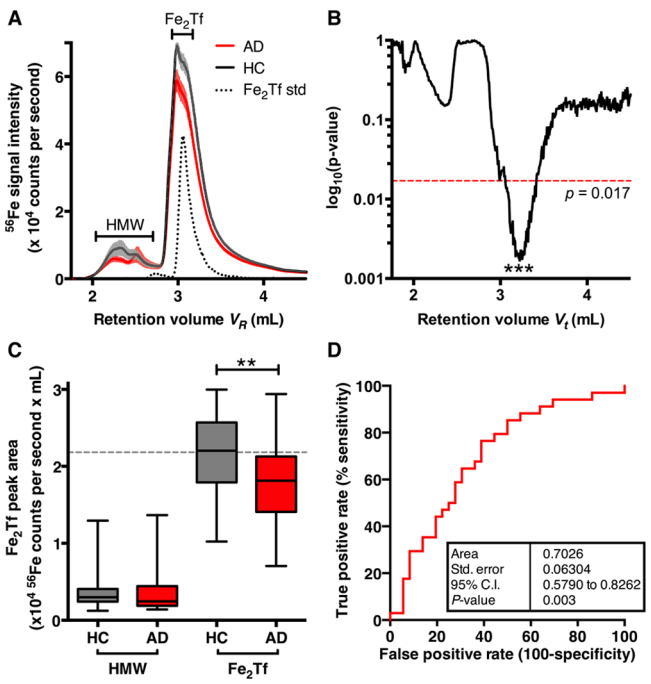

Figure 1. Transferrin is significantly desaturated in Alzheimer's disease. (A) Averaged iron-56 (^{56}Fe) SEC-ICP-MS chromatograms of plasma from AD (red line ± SEM, n = 34) and control subjects (black line ± SEM, n = 36). Dashed black line represents single injection of purified human iron-laden transferrin (Fe_2Tf) standard. Fe-binding species were categorized as either high molecular weight (HMW) species or Tf-bound. (B) Student's t test of each data point collected by the ICP-MS reveals a significant difference in iron-bound Tf (V_R = 3.06 mL; P < 0.017 following false discovery rate correction; ***P < 0.001). (C) The area of each iron-containing peak confirmed that this change was restricted to iron-bound Tf (unpaired, two-tailed Student's t test; **p = 0.01). The dashed gray line shows that, using an ROC curve (D), at a cutoff iron-bound Tf peak area of 2.2 × 10^{-4} iron-56 counts per second, sensitivity = 80% and selectivity = 50% (positive predictive value = 63%; negative predictive value = 84%).

This decrease in iron-bound Tf was not due to a decrease in total plasma Tf levels (Figure 2a). However, as reported by the clinical pathology laboratory, Tf saturation was not significantly decreased in the AD cohort (Figure 2b). From that clinical reporting service, Tf saturation is calculated as the total plasma iron/Tf ratio, based on the assumption that Tf is the primary iron-transport protein.[19] We found that iron-bound Tf accounts for 86.7 ± 8.5% (±1 standard deviation; calculated from the percentage area of total iron chromatographic peaks attributable to iron-bound Tf peak) of total plasma iron in the combined cohorts, with the remaining amount attributable to HMW species (Figure 2c), principally expected to be from FTN and high mass protein aggregates. Indeed, linear regression of the iron-bound Tf peak area and total plasma iron from the samples analyzed showed only a moderate correlation (r^2 = 0.7278; P < 0.001; Figure 2d) that did not differ between clinical classification (P = 0.3). Considering that clinical pathology assays use the assumption that Tf saturation is independent of all other iron-binding species, a stronger correlation (r^2 approaching 1[20]) would be required to demonstrate that plasma iron concentration alone is indeed an accurate proxy for Tf saturation. Instead, our data suggests that the contribution from non-Tf bound iron in fact obscures true Tf saturation, particularly in the case of AD where decreased iron-Tf binding is a subtle but significant effect and the relationship between true Tf saturation and total plasma iron does not account for all sources of variance. There was no correlation between the peak area of the HMW species and Tf saturation measured by SEC-ICP-MS (Supporting Information Figure S2). By substituting the peak area of iron-bound Tf as a more direct measure of Tf saturation, we found a significant decrease in the AD cohort (Figure 2e), consistent with a significant decrease in total plasma iron level, measured by both SEC-ICP-MS and traditional solution nebulization (SN)-ICP-MS (Figure 2f).

Considering the established risk associated with *ApoE* ε4 polymorphism and AD, and the availability of *ApoE* genotyping in AIBL, we also examined Tf saturation levels according to *ApoE* genotype, independent of clinical classification and found no significant relationship between iron-bound Tf levels and *ApoE* (Supporting Information Figure S3).

Our findings indicate that there is a deficiency of iron-loading into Tf in AD, which explains the decrease in plasma iron levels we recently reported.[12] These data are also consistent with previous reports of Tf and Tf receptor alleles affecting the risk for

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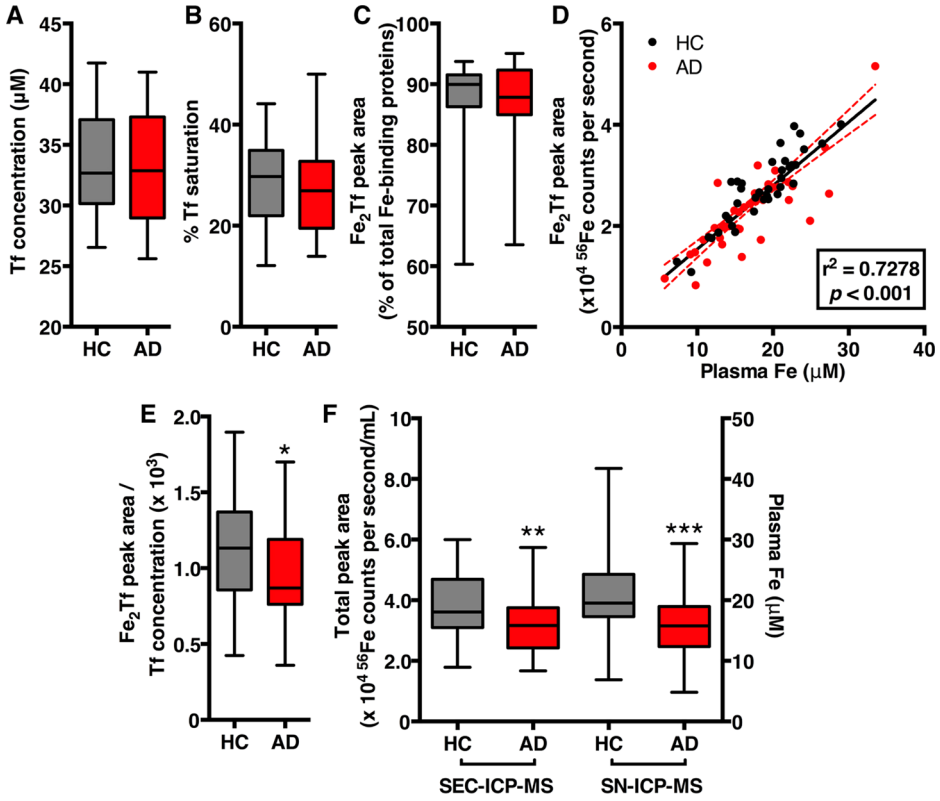

Figure 2. Transferrin saturation reported by clinical pathology does not detect the deficit observed. (A) Tf protein concentration did not significantly differ between AD and HC groups, suggesting the observed decrease in iron-bound Tf is iron-dependent. (B) Pathological Tf saturation assay did not replicate the observed decrease in iron-bound Tf. (C) Tf saturation assay assumes total plasma iron is a proxy for iron-Tf binding, though in both groups most, though not all iron is attributed to Tf. (D) Accordingly, correlation between iron-bound Tf peak area and total plasma iron levels indicates a negative impact of Tf saturation accuracy. (E) Using the ratio of the iron-bound Tf peak to Tf concentration (in place of clinical Tf saturation) reveals a significant decrease in Tf-bound iron in AD plasma ($P < 0.05$), (F) in line with a decrease in total plasma iron measured by both integration of SEC-ICP-MS chromatograms ($P < 0.01$; left axis) and independent solution nebulization (SN)-ICP-MS assay ($P < 0.001$; right axis).

AD.[21,22] Zinc has been shown to reduce incorporation of iron into Tf though competitive binding in vitro,[23] though paradoxically serum zinc is also decreased in AIBL.[15]

Perhaps the most compelling explanation for decreased Tf saturation is impaired ceruloplasmin (Cp) activity in AD. Cp is a multicopper oxidase that facilitates loading of ferric iron onto Tf.[24] In AIBL, total Cp levels are not decreased in AD,[12] though decreased non-Cp copper has been observed in this cohort,[25] as has an increase in others.[26] It should also be noted that clinicopathology assay of Cp concentration is not a measure of activity, which is dictated by the copper status of the protein and has been shown to be decreased in Parkinson's disease independent of protein levels.[27] Several other studies have identified a decrease in Cp activity in AD,[28,29] including evidence of fragmentation of Cp in serum.[30] Torsdottir et al.[31] reported that decreased Cp activity was not associated with Tf saturation, though again this used the immunoturbidimetric Tf saturation assay that we believe is inferior to our more specific SEC-ICP-MS method. Further, assessment of non-Cp copper similarly uses a mathematical assumption regarding the complete occupation of Cp copper binding sites and neglects possible influence of other copper-binding proteins including HSA (which, unlike iron, has a high affinity to copper[18]), transcuprein, and low molecular weight ligands; thus, clinicians have recommended this method be used with extreme caution.[32] Even a minor loss of copper from Cp, which is necessary for its Tf-loading ferroxidase activity,[33] may be responsible for our observed subtle decrease in Tf saturation, and redistribution of this copper to other circulating cuproproteins might explain discrepancies in reported non-Cp copper concentrations in AD.

Regardless, the reasons as to why plasma Tf saturation is decreased in AD remains unclear, though it appears indicative of a more widespread metabolic deficit with regard to metal homeostasis. With further investigation, assay of Tf saturation by SEC-ICP-MS provides a potential AD biomarker. This approach provides a more specific report of iron-Tf binding than the indirect clinical assay, as other competing biological iron ligands (such as FTN) are isolated from the iron−Tf complex according to retention time. In the case of AD, the changes in iron−Tf binding were masked by the poor specificity of the clinical Tf saturation assay.

METHODS

Study Approval. AIBL was approved by the institutional ethics committees of Austin Health, St. Vincent's Health, Hollywood Private Hospital, Edith Cowan University, and the University of Melbourne Human Research Ethics Committee. Written informed consent was obtained from all study participants.

Subject Information. A subgroup of sex- and age-balanced, nonanemic (Hb >120 g L^{-1} for males, >130 g L^{-1} for females) AD (n = 34) and HC (n = 36; see Table 1) were selected from the wider AIBL study (details of recruitment and subject characterization can be found in Ellis et al.[10]). All data reported are from the baseline (t = 0) inception cohort.

Reagents. Ammonium hydroxide (NH_4OH) and ammonium acetate (NH_4CH_3COO) were trace element grade and obtained from Sigma (Castle Hill, Australia). Purified human Tf and FTN standards

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Table 1. AIBL Cohort Demographics and Relevant Iron Clinical Pathology for Individuals Studied

	healthy controls (n = 36)	Alzheimer's disease (n = 34)
age (SD), years	66.8 (4.8)	74.0 (7.1)
sex (females)	50.0%	50.0%
hemoglobin (SD), g L^{-1}	145.1 (8.5)	141.3 (8.5)
males	150.0 (7.9)	145.9 (7.6)
females	140.2 (6.0)	135.9 (6.7)
ferritin (SD), g L^{-1}	153.9 (18.0)	187.4 (34.8)
ApoE ε4 carriers	31.6%	55.3%
ε4/ε3	31.6%	39.5%
ε4/ε4	0%	15.9%

were also obtained from Sigma. Buffers were made using Milli-Q water (18.2 MΩ)

Size Exclusion Chromatography-Inductively Coupled Plasma-Mass Spectrometry (SEC-ICP-MS). SEC was performed on an Agilent 1200 HPLC equipped with a BioSEC300 (4.6 × 300 mm, 300 Å pore size, 3 μm particle size; Agilent) size exclusion column. The column was developed with 200 mM NH_4CH_3COO (pH 7.7) at a flow rate of 0.4 mL min^{-1} at a temperature of 30 °C. Elution of analytes was monitored using absorbance at 280 nm and the outflow from the variable wavelength detector was connected directly to a nebulizer (MiraMist design; Burgener) fitted to an Agilent 7700x ICP-MS via a 60 cm length of polyetheretherketone (PEEK) tubing (0.005 in. I.D.). The ICP-MS was run in standard multielemental detection mode with helium (3.1 mL min^{-1}) to reduce polyatomic interferences.[34] Standard Tf was injected in triplicate over the course of the experiment to ensure V_R remained stable.

Plasma Samples. Li-heparin samples were stored in liquid nitrogen (−178 °C) prior to analysis. Samples were thawed on ice for 1 h then centrifuged for 5 min at 16 100*g*. The supernatant was recovered, and 25 μL was injected.

Clinical Pathology. Clinical pathology was performed by Melbourne Health in Melbourne, and PathWest Laboratory Medicine Western Australia in Perth. Plasma iron was quantitatively determined using a photometric color test as follows. Plasma was acidified to release protein-bound iron, which was then reduced to Fe^{3+} in the presence of hydrochloric acid and sodium ascorbate. The chromogen 2,4,6-tri-(2-pyridyl)-5-triazine was added, and bichromatic absorbance measured at 600/800 nm. Tf protein concentration was determined using an immunoturbidimetric assay. Plasma was incubated with goat antihuman Tf antibodies that formed insoluble aggregates, the absorbance of which was directly proportional to Tf concentration in plasma. Both assays were manufactured by Olympus. Routine clinical Tf saturation is calculated according to the following:

$$\%\mathrm{Tf}_{\mathrm{sat}} = \frac{[\mathrm{Fe}]}{[\mathrm{Tf}]} \times 25.06$$

Statistics. Statistical analysis was performed using Prism 6 (GraphPad). For comparisons of multiple SEC-ICP-MS chromatograms (n_{AD} = 34; n_{HC} = 36 per group per time point), we performed an unpaired, two-tailed Student's t test on each individual time-resolved chromatographic data point with a false discovery rate correction.[35] Unless stated otherwise, direct comparisons of two measured conditions was performed using unpaired, two-tailed Student's t tests in Prism. Interpretation of the ROC curve was performed as directed by Florkowski.[36]

■ ASSOCIATED CONTENT

Supporting Information

Individual chromatograms from HC and AD samples (Figure S1); test for correlation between HMW and Tf-associated iron (Figure S2); and Tf saturation by *ApoE* genotype (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

D.J.H. and B.R.R. designed the research. D.J.H., A.R., I.V., and B.R.R. performed the experiments. D.J.H., J.D.D., N.G.F., C.J.F., and B.R.R. analyzed the data. R.G., P.A.D., R.A.C., C.L.M., and A.I.B. provided material support. D.J.H., A.I.B., and B.R.R. wrote the manuscript. All authors edited the manuscript.

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Notes

The authors declare the following competing financial interest(s): Rudolf Grimm is an employee of Agilent Technologies. Robert A. Cherny is a shareholder of and consultant to Prana Biotechnology, Ltd. Colin L. Masters is a consultant to Prana Biotechnology, Ltd. Ashley I. Bush is a shareholder in Prana Biotechnology, Ltd; Mesoblast, Ltd; Cogstate Ltd; Brighton, LLC; Eucalyptus, LLC; and is a paid consultant for Collaborative Medicinal Discovery, LLC and Brighton, LLC. All other authors declare that no conflict of interest exists.

■ DEDICATION

This work is dedicated to our friend and colleague, Dr. Alan Rembach, who passed away during the preparation of this manuscript.

■ ABBREVIATIONS

AD, Alzheimer's disease; AIBL, Australian Imaging, Biomarkers and Lifestyle Flagship Study of Aging; *ApoE*, apolipoprotein E; Cp, ceruloplasmin; FTN, ferritin; Hb, hemoglobin; HC, healthy controls; HSA, human serum albumin; HMW, high molecular weight; ICP-MS, inductively coupled plasma-mass spectrometry; ROC, receiver operating characteristic; SEC, size exclusion chromatography; Tf, transferrin; V_R, retention volume

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