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Oxidation of iron under physiologically relevant conditions in biological fluids from healthy and Alzheimer's disease subjects

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ABSTRACT

Ferroxidase activity has been reported to be altered in various biological fluids in neurodegenerative disease, but the sources contributing to the altered activity are uncertain. Here we assay fractions of serum and cerebrospinal fluid with a newly-validated triplex ferroxidase assay. Our data indicate that while ceruloplasmin, a multicopper ferroxidase, is the predominant source of serum activity, activity in CSF predominantly derives from a <10kDa component, specifically from polyanions such as citrate and phosphate. We confirm that in human biological samples, ceruloplasmin activity in serum is decreased in Alzheimer's disease, but in CSF a reduction of activity in Alzheimer's disease originates from the polyanion component.

Keywords: Ferroxidase, ceruloplasmin, iron, neurodegenerative disease, polyanion, oxidation

Iron is required as a cofactor in many metabolic processes. In aerobic conditions, the redox cycling between ferric (Fe^{3+}) and ferrous (Fe^{2+}) iron is controlled by protein chaperoning[1] to prevent the generation of reactive hydroxyl radicals[2]. Ferroxidase activity aerobically catalyses ferrous iron oxidation producing water, so mitigating hazardous reactive oxygen species (ROS) production[3]. Iron oxidation[2], with subsequent Fe^{3+} loading into extracellular transferrin (TF)[3, 4], is important in cellular iron homeostasis by facilitating iron efflux through ferroportin (Fpn), the only known iron export pore protein.

In the brain, multicopper ceruloplasmin (CP) is considered the predominant ferroxidase for iron export. It is primarily secreted by choroid plexus epithelial cells, although there is also ceruloplasmin GPI-anchored to the astrocyte membranes[5]. Some less-abundant multicopper proteins within the brain also have ferroxidase capability, such as hephaestin (HEPH), which is primarily expressed in oligodendrocytes[6]. The importance of ferroxidase activity in the central nervous system (CNS) has been demonstrated through ablation of CP in knockout mice and in aceruloplasminemia patients, where loss of function mutations of CP lead to brain regional iron retention and neurological deficits[7, 8]. Plasma CP can cross the blood brain barrier, and rescues neurological features in a mouse model of Parkinson's disease (PD)[7]. Decreased CP oxidase activity has been reported in serum from patients suffering from Alzheimer's disease (AD)[9] and PD[10].

The incorporation of iron into TF has been used to measure ferroxidase activity indirectly[4, 11], but two main controversies on its accuracy arise from a reliance on non-physiological (low) pH in order to suppress auto-oxidation, as well as the requirement for indirect colorimetric detection of holo-TF formation, which is a rate-limiting

extraneous step in appraising the enzyme kinetics[1]. A more direct measurement of ferroxidase activity is to assay Fe^{2+} loss[12, 13] and Fe^{3+} gain[12, 14] but buffer conditions are not physiological in these systems, and TF is absent. While each assay was originally used to determine activity in serum, they have been adapted increasingly to assay activity in other biological samples including human cerebrospinal fluid (CSF)[13-15]. To achieve measurement of ferroxidase activities under physiological conditions we recently developed a triplex ferroxidase assay that assesses 'Ferrous Loss', 'Ferric Gain' and 'TF Loading' in tandem under biologically relevant pH and salinity. Here, we report the first assays of CSF and serum using this more physiologically appropriate system.

Human serum (0.25 mg/ml) was separated into filtrate (containing serum fluid, electrolytes, molecules and polypeptides <10kDa) and retentate (containing molecules and proteins >10kDa) fractions (Fig. S1). Simultaneous measurement of 'Ferric Gain', 'TF Loading' and 'Ferrous Loss' in human serum was determined in the presence or absence of apo-TF for all fractions (Fig. 1). Serum activity was achieved with all readouts from the triplex assay to provide the rate of total serum activity without TF as measured by 'Ferric Gain' at 5.21 ± 0.28 μM Fe^{3+}/min (Fig. 1Ai). As predicted[1], the rate of oxidation was increased by the presence of TF in the TF loading assay (7.48 ± 0.31 μM Fe^{3+}/min) (Fig. 1Aii). Most of the activity in total serum fractionated into the retentate (Ferric gain: 4.01 ± 0.16 μM Fe^{3+}/min [77%], TF loading: 6.67 ± 0.20 μM Fe^{3+}/min [89%]).

Multicopper ferroxidase inhibition with sodium azide[12] verified that CP was the major component of retentate activity (Fig. 1B). However, despite all activity being ablated when using the Ferric Gain assay (Fig. 1Bi), a small proportion of retentate activity

was not inhibited by azide when measured by TF loading (1.44 ± 0.13 μM Fe^{3+}/min) (Fig. 1Bii). This is consistent with previous TF loading reports identifying the presence of a non-CP ferroxidase activity in serum[11, 17]. End point quantitation of Fe^{2+} loss corroborated the production of Fe^{3+} during the assays in both the presence and absence of TF (Fig. 1C).

Triplex assay parameters for CSF were found to be as previously determined for purified CP[1] and human serum (Fig. 1), at an optimal CSF volume of 20 μl (Fig. S2B). In contrast to serum fractions (Fig. 1), the predominant ferroxidase activity in CSF was found in the filtrate fraction (<10kDa) by both 'Ferric Gain' (Total = 3.47 ± 0.19 μM Fe^{3+}/min, Filtrate = 3.06 ± 0.17 μM Fe^{3+}/min [88%]) (Fig. 2Ai) and 'TF loading' (Total = 2.60 ± 0.35 μM Fe^{3+}/min, Filtrate = 2.10 ± 0.18 μM Fe^{3+}/min [81%]) (Fig. 2Aii) assays.

In assaying the same volume (5μl) of serum and CSF in both the 'Ferric Gain' and 'TF loading' components of the triplex assay, activity was markedly greater in serum (Fig. S3A). Upon fractionation of either biological fluid, retentate had markedly greater activity from serum (Fig. S3B) and negligible activity from CSF (Fig. 2A). Despite this greater percentage of filtrate activity in total CSF, when compared to the same serum volume this fraction's activity only had a minor elevation in Ferric Gain velocity (CSF: 1.12 ± 0.06 μM Fe^{3+}/min, Serum: 0.54 ± 0.04 μM Fe^{3+}/min)(Fig. S3Ci) and was comparable by TF loading (CSF: 1.79 ± 0.10 μM Fe^{3+}/min, Serum: 1.81 ± 0.12 μM Fe^{3+}/min) (Fig. S3Cii).

Azide (2.5mM) did not inhibit CSF activity (Fig. 2B), excluding the source of the activity being a multicopper ferroxidase e.g. CP. The absence of CP activity in human CSF is in alignment with normal levels of CP (~1.5 μg/ml)[18] being <1% of those

found in serum (200-500μg/ml)[19]. Consistent with the previously reported level of CP in CSF, recombinant CP at this concentration (~2.5 μg/ml) is outside the active range of our assay (<20nM; Fig. S4). Our findings are similar to the activity that has been reported to arise from <10kDa species in CSF from healthy and sporadic Creutzfeldt-Jakob disease (sCJD) patients[14], but at variance with a report that ferroxidase activity in CSF originates from CP[13].

To identify whether the ferroxidase activity from the filtrate was of protein enzyme origin, fractionated serum and CSF were treated with proteinase K. In serum, the protease abolished the retentate activity, but had no effect on the filtrate activity (Fig. 3A & S5A). Protease incubation with CSF had no effect on the total activity or the filtrate fraction (Fig. 3B & S5B).

The capacity for polyanions to oxidize iron and facilitate the incorporation of Fe^{3+} into TF is a confounding factor in measuring enzymatic ferroxidase activity[1]. We quantified the polyanion content in serum and CSF to identify whether these could be a source of the apparent ferroxidation activity in the filtrates. Phosphate and citrate are prevalent polyanions to oxidize iron in biological samples. We found that phosphate levels were greater in serum (Fig. 4Ai) whereas citrate levels were observed to be greater in CSF (Fig. 4Aii). To highlight the polyanion influence on ferroxidase activity in CSF, variability in the combined phosphate and citrate levels within CSF filtrates was observed to correlate with activity as measured by Ferric gain ($P=0.010$, $r^2=0.465$) and TF loading ($P=0.006$, $r^2=0.509$) (Fig. 4B). Ferric gain assay identified that both phosphate ($P=0.040$, $r^2=0.391$) and citrate ($P=0.029$, $r^2=0.363$) contributed to this correlation (Fig. S6A) and a similar trend was observed with TF loading (Fig. S6B).

We applied our assays under physiological conditions to assay serum and serum fractions from AD patients (n=10) and age-matched healthy controls (n=10) (Table S1). Although activities in whole serum samples were not different in AD patients by either Ferric gain or TF loading assays (Fig. 5A), there was a significant decrease in ferroxidase activity in the serum retentate fractions by these assays (Ferric gain -13%, TF loading -10%, Fig. 5A). This is analogous with the ~10% decrease in ceruloplasmin specific activity reported in a larger sampling of AD compared to control sera[9]. There was no difference between the AD and control groups in the serum filtrate activity measured by either assay, consistent with the finding of no difference in either serum phosphate, citrate levels (Fig. 5C) or when these were combined (data not shown).

We also applied our assays under physiological conditions to evaluate CSF and CSF fractions from AD patients (n=10) and age-matched healthy controls (n=10) (Table S1). Total CSF and retentate samples were not altered in AD, but AD CSF filtrates had significantly decreased activity by both Ferric gain and TF loading assays (Fig. 5B). With activity mostly originating from polyanions within the filtrate, citrate and phosphate levels were analysed but neither were altered in AD compared to healthy controls when measured individually (Fig. 5C) or in combination (data not shown).

Our data provide simultaneous analysis on all steps of iron oxidation in biological fluids under physiological conditions, relevant for neurological disorder screening. Utilizing this assay confirms that serum activity largely resides in a >10kDa fraction that can be inhibited by azide, consistent with CP. In contrast, the majority of the activity in human CSF originates from the <10kDa fraction. Lack of a high molecular weight enzymatic activity is consistent with the low CP concentration reported in

CSF. We[1, 2] and others[14] have previously proposed that abundant polyanions such as phosphate and citrate could have a substantial affect on iron oxidation within a biological environment and play a role in cellular export when multicopper ferroxidases are absent. Indeed, we confirm that the iron oxidation activity derived from CSF is protease resistant and correlates with the polyanion content. The abundance of these polyanions can fuel iron oxidation to the extent that assays can misinterpret the reaction as enzymatic, and hence report a "pseudo-ferroxidase" activity.

Previous measurements of ferroxidase activity using older assays under non-physiological conditions (e.g. pH, temperature and buffer) have identified moderate changes in neurodegenerative diseases associated with iron disruption. We have been concerned about the reliability of only measuring one component of the ferroxidase reaction, especially under non-physiological conditions. Instead, a multi-component analysis of physiological activity and the elimination of the residual pseudo-ferroxidase activity derived from a non-protein component in the sample is more accurate.

The discovery that these disease associated changes in serum predominately originate from an azide inhibitable component suggests that while there is no detectable difference in serum copper or CP levels[19], there may be less copper bound to CP in AD patients. This would be different to PD where a lower CP expression correlates with age of Parkinsonian onset[20]. Either a decreased copper-dependent activity of CP expression would likely cause the decreased iron saturation of TF recently reported for AD[21]. While the negligible ferroxidase activity in CSF is unaltered in AD, we found a decrease in iron oxidation mediated by small molecular weight species,

similar to other neurodegenerative diseases such as sCJD and PD[13, 14]. We could not confirm that this change in CSF was attributable to decreased citrate and phosphate levels, and other small molecular weight molecules may contribute. Monitoring the activity in neurological conditions such as multiple sclerosis that have a reported CSF polyanion imbalance[22] may assist in identifying these other contributory small molecular weight molecules.

METHODS

Reagents. Reagents were purchased from Sigma Australia at an analytical grade, unless stated otherwise. When required as a positive control, purity of human CP (Vital Products) was enhanced by repeated washes with Milli-Q® H_20 through a 30kDa cutoff Amicon Ultra-15 (Millipore).

Biological sample preparation. Human serum samples from healthy controls and from subjects with Alzheimer's disease (AD) were obtained from The Australian Imaging, Biomarker and Lifestyle Flagship Study of Ageing (AIBL) study[16], and CSF samples from the same diagnostic categories were obtained from the National Dementia Diagnostic Laboratory, University of Melbourne. All procedures were carried out in accordance with the Australian National Health & Medical Research Council's National Statement on Ethical Conduct in Human Research (2007) and the Victorian Human Tissue Act 1982.

The CSF and serum samples were also kept at -80^{o}C in aliquots (50µl) until required, to reduce freeze thawing and maximise ferroxidase activity. To obtain retentate and filtrate fractions for each biological material, samples were centrifugally fractionated (14,000g, 30 min, 4^{o}C) through a 10kDa filter (Amicon Ultra 0.5ml MMCO 10kDa). The retentate fraction (>10kDa) was resuspended in Milli-Q® H_20 to a comparable

volume as the filtrate (<10kda) (Fig.S1). The retentate was then washed once with the same volume of Milli-Q® H_20 to remove retained filtrate. In experimental conditions that required the elimination of protein enzymatic activity, samples were treated with 50μg/ml of proteinase-K overnight at 37^{o}C.

Ferroxidase assays. Assay conditions originally reported for *in vitro* analysis[1] were found to be optimal also for measuring activity from CSF and serum. Only the duration of the assay and the volume of CSF or concentration of serum required optimization (Fig. S2). As previously described[1], preparation of apo-transferrin (apo-TF; >98% purity) and ferrous sulfate ($FeSO_4$) in Milli-Q® H_20 within an hour prior to use minimizes substrate deterioration through auto-oxidation. For kinetic analysis of 'Ferric gain' (measured at Abs_{310}/min) and 'TF loading' (measured at Abs_{460}/min) velocities, the reagents (final concentrations) were added in the following order to a 200µl reaction volume: HBS (HEPES 50 mM, NaCl 150 mM, pH 7.2), biological sample (optimally determined as 0.25mg/ml of serum or 20µl CSF), +/- apo-Tf (50µM), $FeSO_4$ (100uM) and ddH_20 made up to the total reaction volume. Confirmation of 'Ferrous loss' at the end of the log phase of the reaction was measured by the addition of Ferene S (500µM) (Abs_{590} at times dependent on sample type). All experiments were performed on the Power Wave HT (BioTek) microplate spectrophotometer using the iron extinction coefficient published previously[1] and confirmed to follow the Beer-Lambert Law. When required, sodium azide (2.5 mM) was added to block multicopper ferroxidase activity.

Statistical Analysis and data representation. Results were analysed using a combination of Excel (Microsoft 2011) and Prism (v5.0 Graphpad, Software Inc.). Difference between the means were calculated by one-way ANOVA followed by

Dunnett's test to confirm the existence of correlations between activity and polyanion concentration or between AD and healthy control. $P<0.05$ was taken to be statistically significant and results are expressed as the means ± SEM unless presented otherwise. 'Ferric gain' plots represent the differential amount of Fe(III) that results from biological sample oxidation, 'Transferrin loading' identifies the amount of Fe^{3+} that was loaded onto apo-transferrin and lastly 'Ferrous loss' illustrates the amount of Fe^{2+} that remains from the conversion of Fe^{2+} to Fe^{3+} during the oxidation process by the sample.

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Author contributions

LQL and BXW equally contributed to the research by acquisition and interpretation of data while PJC and JAD designed and supervised the research. LQL, JAD and AIB drafted the manuscript with critical review from the rest of the authors.

Conflict of Interest

All authors do not have any conflict of interest.

Supplementary information

Table S1: Demographic and clinical features of patients.

Figure S1: Schematic illustrating the centrifugal filtration collection procedure in biological samples

Figure S2: Optimization of ferroxidase activity for serum and CSF in HBS pH 7.2.

Figure S3. Ferroxidase activity in comparable volumes of serum and CSF.

Figure S4: Determining the sensitivity of the multiplex assay to CP.

Figure S5: Determining enzymatic activity in serum and CSF.

Figure S6: Separate Phosphate and Citrate analysis in CSF filtrate.

Figure S7. Determining ferrous loss by ferroxidase activity in human control and Alzheimer's disease biological fluid.

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FIGURE LEGENDS

Figure 1: Characterizing ferroxidase activity in human serum. A. Total, retentate and filtrate fractions of human serum ferroxidase activity were kinetically quantified for Fe^{3+} production (Ferric Gain) (**i**) and apo-TF loading (**ii**) over 5min. **B**. Identical parameters as **A** were measured for retentate and filtrate fractions in the presence of sodium azide (2.5mM) for Fe^{3+} production (**i**) and apo-TF loading (**ii**). **C.** After 10min, remaining Fe^{2+} (Ferrous Loss) post ferroxidase conversion from all serum fractions +/- sodium azide (2.5mM) was measured at endpoint with the addition of ferene *S* in the absence **(i)** or presence **(ii)** of TF. Final optimal conditions for carrying out the triplex assay in human serum were: HBS buffer (50mM HEPES, 150mM NaCl, pH 7.2); Serum (0.25mg/ml); +/- sodium azide (2.5mM); $FeSO_4$ (100μM); +/- apo-TF (50μM). Temperature was constant at 24^{o}C throughout the assay and results blanked against the reading at the first time point. The individual data points shown are means ± S.E., n= 8 read in duplicates.

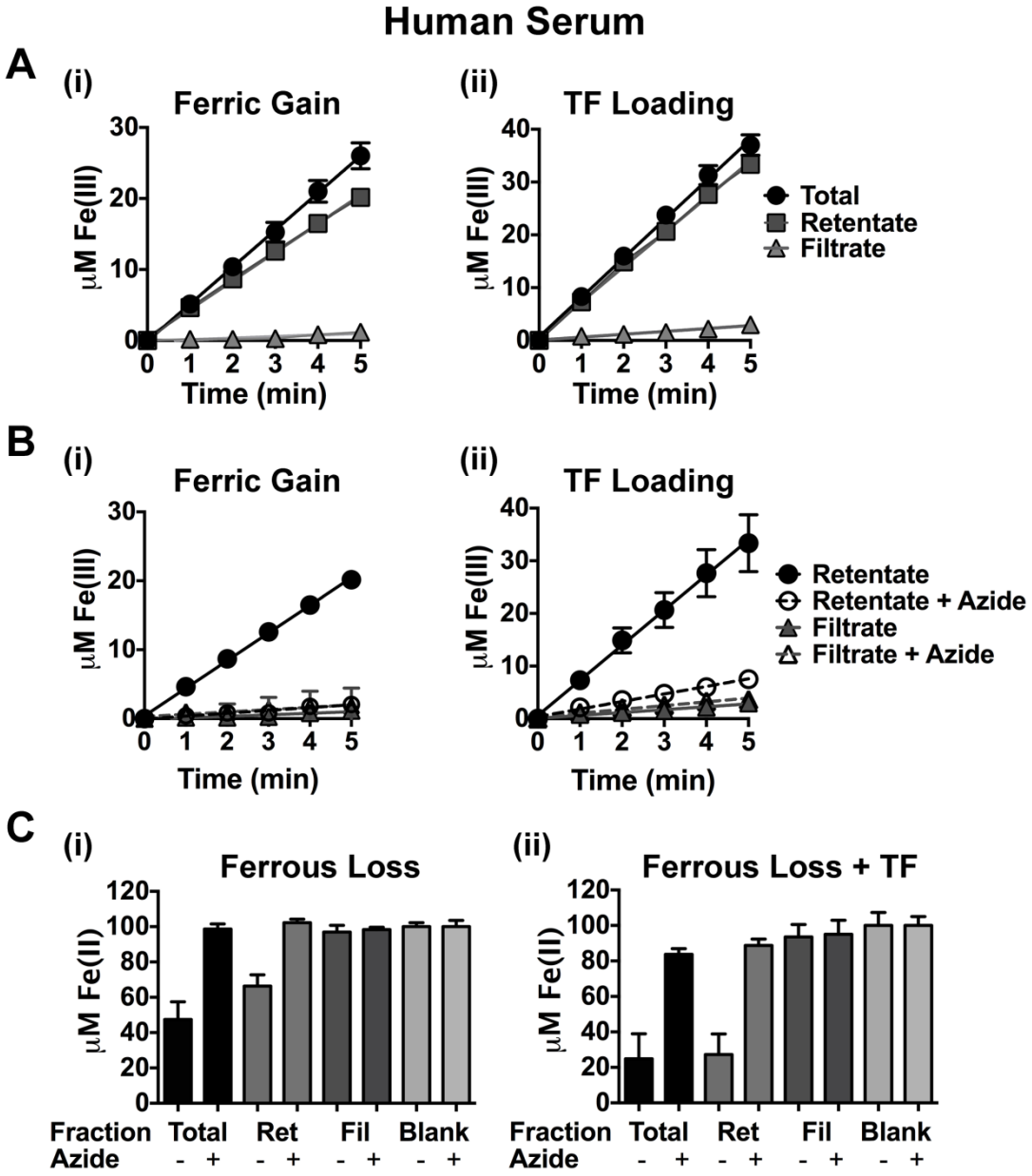
Figure 2: Characterizing ferroxidase activity in human CSF. A. Velocity rate of iron oxidation was kinetically measured for total, retentate and filtrate fractions of human CSF by Ferric Gain (**i**) and Fe^{3+} loaded into apo-Tf (**ii**) within 5 min. **B.** Conditions replicated from **A** were used to measure retentate and filtrate fractions of human CSF ferroxidase activity in the presence of sodium azide (2.5mM). **C.** 10 min after ferroxidase conversion remaining Fe^{2+} was measured by ferene *S* in the absence (**i**) or presence (**ii**) of TF to confirm ferroxidase activity from CSF total, retentate and filtrate fractions. The same conditions as for serum were found to be optimal for CSF (20 μl). The individual data points shown are means ± S.E., n= 8 read in duplicates.

Figure 3: Determining enzymatic activity in serum and CSF. A. Total, retentate and filtrate fractions of human serum was incubated with proteinase K (50µg/ml) overnight at 37^{o}C before ferroxidase activity was quantified for Fe^{3+} production (**i**) and apo-TF loading (**ii**) after 5min. **B**. Identical parameters as **A** were measured for human CSF to measure Fe^{3+} production (**i**) and apo-TF loading (**ii**). The triplex assay conditions were as optimally determined for human serum and CSF. Temperature was constant at 24^{o}C throughout the assay and results blanked against the reading at the first time point. The individual data points shown are means ± S.E., n= 3 read in duplicates. ***=P<0.001 & NS=not significant by 2-tailed T-test analysis.

Figure 4: Phosphate and Citrate analysis in serum and CSF. A. To represent polyanion levels in biological fluids, total serum and CSF samples were assayed for phosphate (i) and citrate (ii). **B**. Activity obtained by Ferric Gain (**i**) and TF Loading (**ii**) was correlated with the combined phosphate and citrate concentrations for the filtrate fraction. In **A,** each biological fluid was grouped for means ± S.E., n= 10 read in triplicate. ***=P<0.001 by 2-tailed T-test analysis. For **B,** the means of each individual data point was correlated before analysis by 2-tailed T-test.

Figure 5. Determining the ferroxidase activity in human control and Alzheimer's disease biological fluid. A. Alzheimer's disease (AD) ferroxidase activity in total serum as well as the retentate and filtrate fractions were compared to age-matched healthy controls (HC) by Ferric Gain in the absence of TF **(i)** or apo-TF loading **(ii)**. Conditions for carrying out the triplex assay in human serum were as optimized in Fig. 1. **B**. As with **A**, ferroxidase activity in AD CSF was compared to HC by Ferric Gain in the absence of TF **(i)** or apo-TF loading **(ii)** using the optimal conditions identified in Fig. 2. **C**. Within the filtrate fraction found to contain non-enzymatic activity, phosphate (**i**) and citrate (**ii**) levels in AD were compared to HC in serum and

CSF. Individual data points are means read in triplicates, each disease group are means ± S.E, with each group containing n= 10. *=P<0.05 as analysed by 2-tailed T-test.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Ferrous Loss + TF

Figure 1

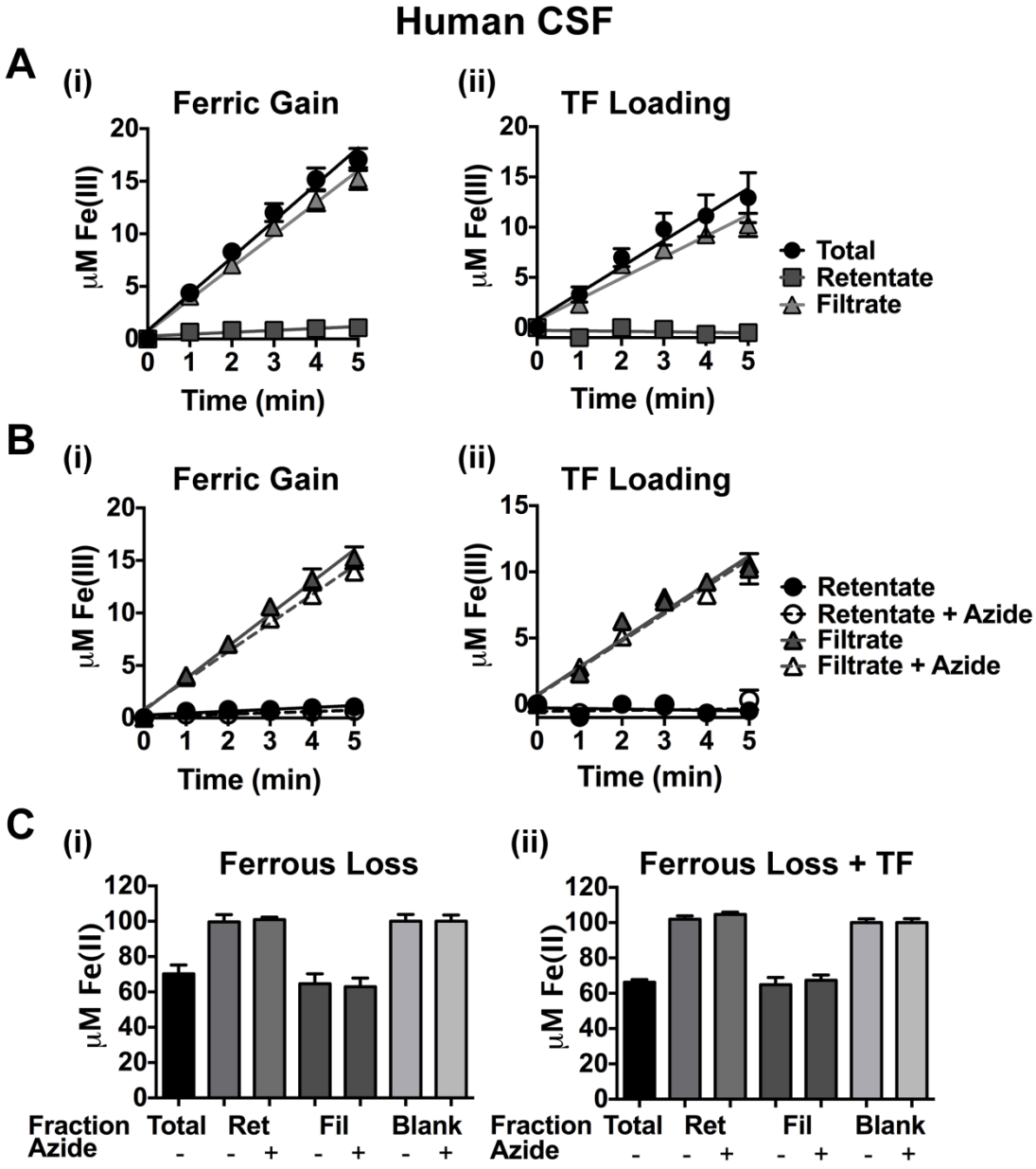
Human CSF
A
(i)
Ferric Gain
μM Fe(III)
Time (min)
(ii)
TF Loading
Total
Retentate
Filtrate
B
(i)
Ferric Gain
(ii)
TF Loading
Retentate
Retentate + Azide
Filtrate
Filtrate + Azide
C
(i)
Ferrous Loss
μM Fe(II)
(ii)
Ferrous Loss + TF
Fraction Total Ret Fil Blank
Azide - - + - + - +

Figure 2

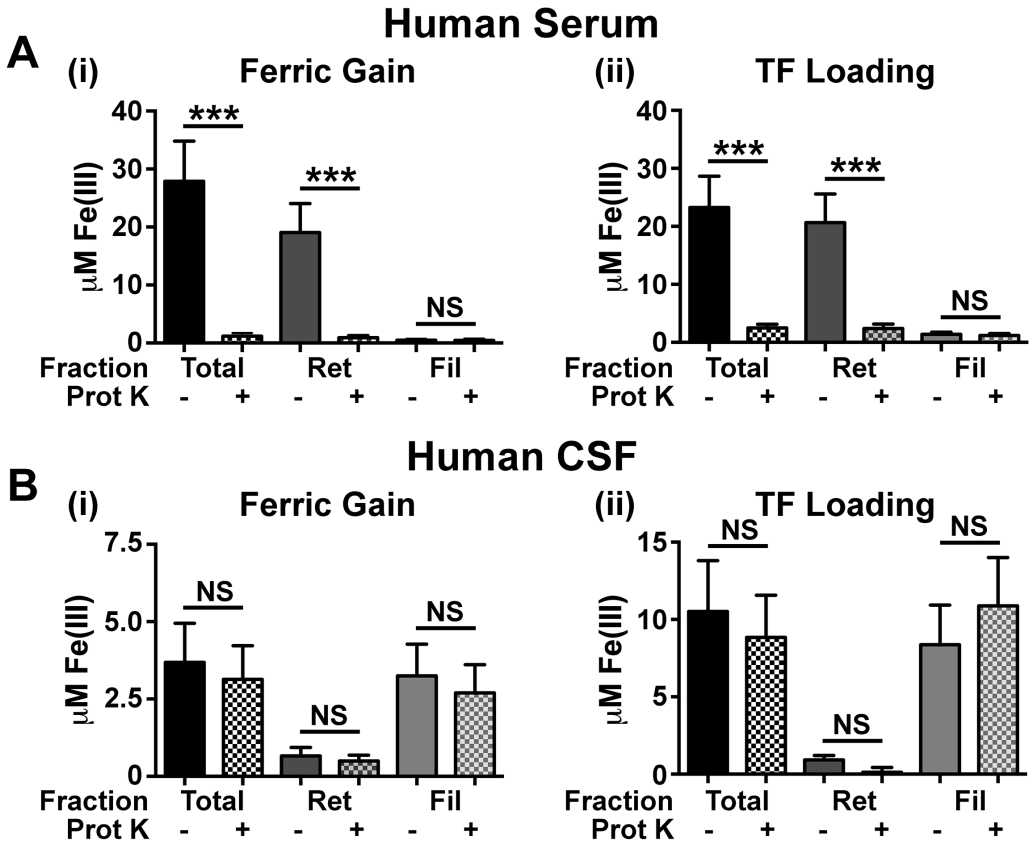
Human Serum
A
(i)
Ferric Gain
(ii)
TF Loading
μM Fe(III)
40
30
20
10
0
***
***
NS
Fraction
Total
Ret
Fil
Prot K
- + - + - +
Human CSF
B
(i)
Ferric Gain
(ii)
TF Loading
μM Fe(III)
7.5
5.0
2.5
0
15
10
5
NS
NS
NS
NS
Fraction
Total
Ret
Fil
Prot K
- + - + - +

Figure 3

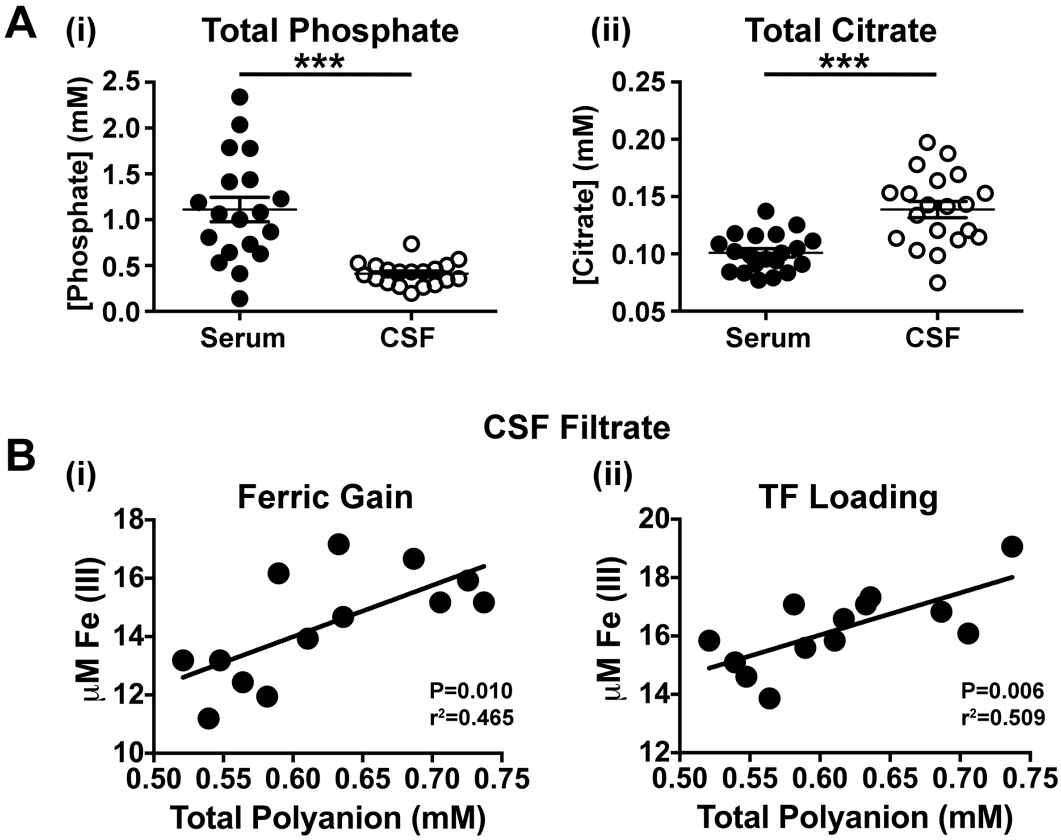
A
(i)
Total Phosphate
***
[Phosphate] (mM)
2.5
2.0
1.5
1.0
0.5
0.0
Serum
CSF
(ii)
Total Citrate
***
[Citrate] (mM)
0.25
0.20
0.15
0.10
0.05
Serum
CSF
B
CSF Filtrate
(i)
Ferric Gain
μM Fe (III)
18
16
14
12
10
P=0.010
r²=0.465
0.50 0.55 0.60 0.65 0.70 0.75
Total Polyanion (mM)
(ii)
TF Loading
μM Fe (III)
20
18
16
14
12
P=0.006
r²=0.509
0.50 0.55 0.60 0.65 0.70 0.75
Total Polyanion (mM)

Figure 4

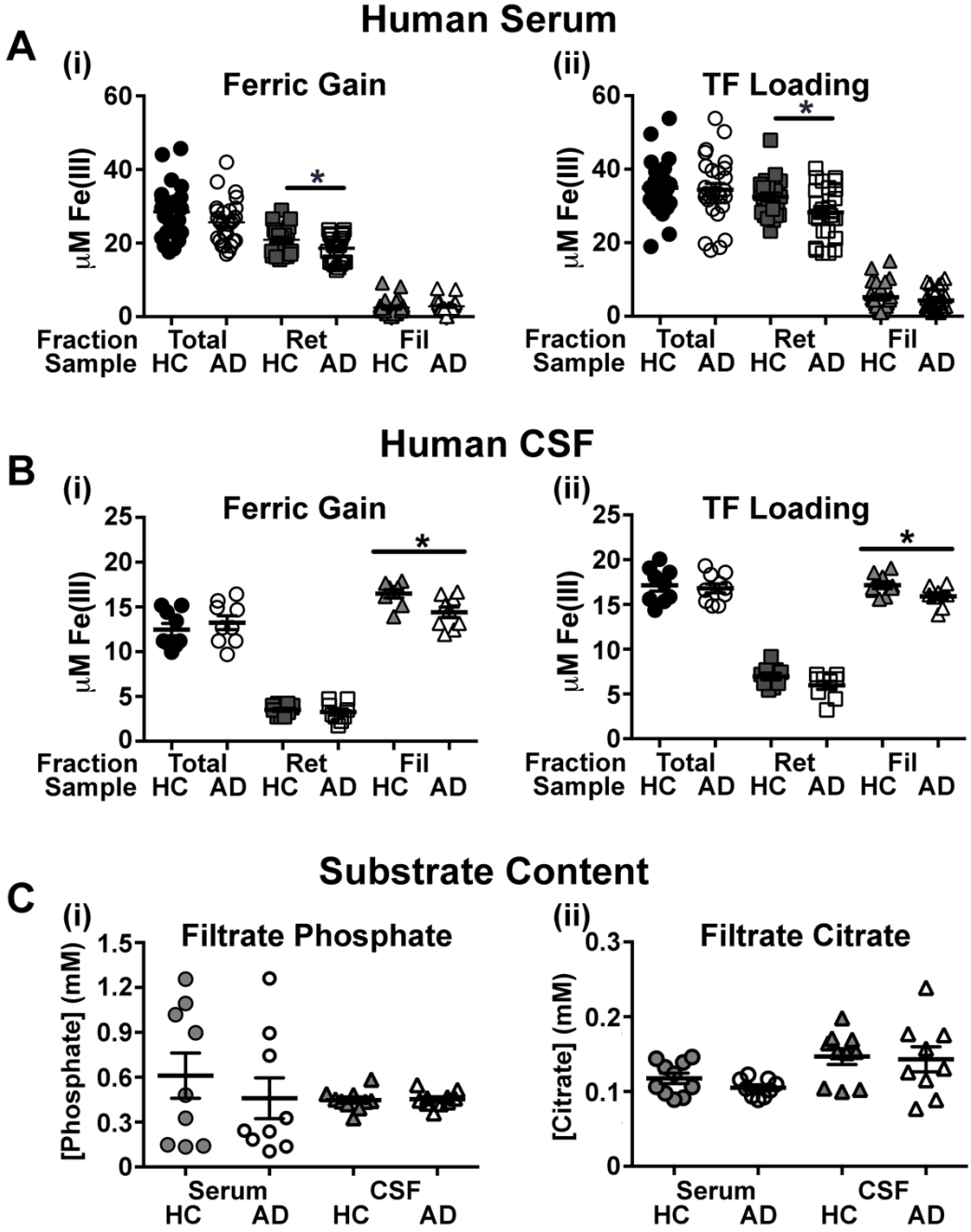
Human Serum
A
(i)
Ferric Gain
(ii)
TF Loading
μM Fe(III)
60
40
20
0
*
Fraction Total Ret Fil
Sample HC AD HC AD HC AD
Human CSF
B
(i)
Ferric Gain
(ii)
TF Loading
μM Fe(III)
25
20
15
10
5
0
*
Fraction Total Ret Fil
Sample HC AD HC AD HC AD
Substrate Content
C
(i)
Filtrate Phosphate
[Phosphate] (mM)
1.5
1.2
0.9
0.6
0.3
0
Serum CSF
HC AD HC AD
(ii)
Filtrate Citrate
[Citrate] (mM)
0.3
0.2
0.1
0
Serum CSF
HC AD HC AD

Figure 5