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## Enzymatic oxidation of Fe<sup>2+</sup> under physiologically relevant conditions in biological fluids from healthy and Alzheimer's disease subjects.

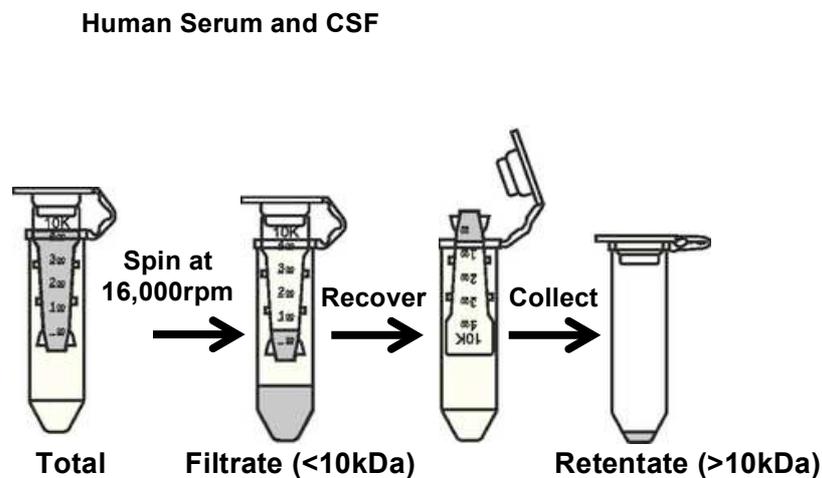
Linh Q. Lam<sup>1,2,\*</sup>, Bruce X. Wong<sup>1,3,\*</sup>, Tony Frugier<sup>2</sup>, Qiao-Xin Li<sup>1</sup>, Steven J. Collins<sup>4</sup>, Ashley I. Bush<sup>1</sup>, Peter J. Crack<sup>2,b,#</sup> & James A. Duce<sup>1,3,a,#</sup>.

### SUPPLEMENTAL DATA

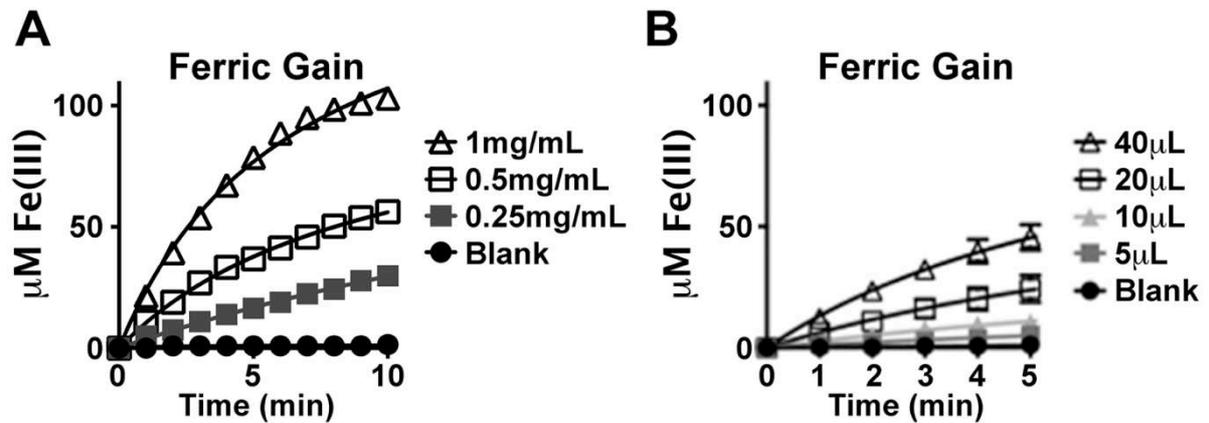
| Case     | Gender | Age  | diagnosis |
|----------|--------|------|-----------|
| 15/0063C | Female | 61   | HC*       |
| 15/0066C | Male   | 49   | HC*       |
| 16/0002C | Male   | 70   | HC*       |
| 16/0005C | Female | 44   | HC*       |
| 16/0018C | Male   | 57   | HC*       |
| 16/0021C | Male   | 59   | HC*       |
| 16/0026C | Male   | 55   | HC*       |
| 16/0030C | Female | 57   | HC*       |
| 16/0034C | Female | 43   | HC*       |
| 16/0037C | Female | 51   | HC*       |
| 43       | Female | 84   | HC#       |
| 24       | Female | 81   | HC#       |
| 462      | Female | 92   | HC#       |
| 792      | Male   | 82   | HC#       |
| 176      | Male   | 71   | HC#       |
| 704      | Female | 70   | HC#       |
| 750      | Male   | 79   | HC#       |
| 356      | Female | 72   | HC#       |
| 422      | Male   | 82   | HC#       |
| 58       | Female | 80   | HC#       |
| 862      | Female | 69   | HC#       |
| 13       | Female | 81.2 | HC#       |
| 88       | Female | 77.5 | HC#       |
| 105      | Male   | 72.6 | HC#       |
| 573      | Male   | 77.3 | HC#       |
| 1104     | Male   | 80.1 | HC#       |
| 1446     | Male   | 80.8 | HC#       |
| 1459     | Female | 70.3 | HC#       |
| 15/0062C | Female | 71   | AD*       |
| 15/0076C | Female | 69   | AD*       |
| 15/0081C | Male   | 75   | AD*       |
| 16/0017C | Male   | 53   | AD*       |
| 16/0019C | Female | 74   | AD*       |
| 15/0021C | Female | 63   | AD*       |
| 15/0041C | Male   | 78   | AD*       |
| 16/0023C | Male   | 61   | AD*       |
| 16/0024C | Female | 65   | AD*       |
| 16/0048C | Female | 71   | AD*       |
| 575      | Male   | 71   | AD#       |
| 7        | Female | 87   | AD#       |
| 521      | Female | 74   | AD#       |
| 345      | Male   | 82   | AD#       |
| 726      | Male   | 72   | AD#       |
| 789      | Male   | 79   | AD#       |

|      |        |      |     |
|------|--------|------|-----|
| 744  | Male   | 68   | AD# |
| 564  | Male   | 85   | AD# |
| 913  | Female | 82   | AD# |
| 1144 | Female | 78   | AD# |
| 100  | Female | 85.2 | AD# |
| 102  | Female | 74.2 | AD# |
| 361  | Male   | 77.7 | AD# |
| 372  | Female | 74.4 | AD# |
| 609  | Female | 78.8 | AD# |
| 851  | Male   | 92.6 | AD# |
| 890  | Female | 84.6 | AD# |
| 1092 | Female | 72.7 | AD# |

**Table S1: Demographic and clinical features of patients.** Details of patients used for this study n=56. Anti-mortem CSF used are depicted by \* and anti-mortem serum depicted by #. Samples were categorised by the following diagnosis; HC= healthy control and AD= Alzheimer's disease.

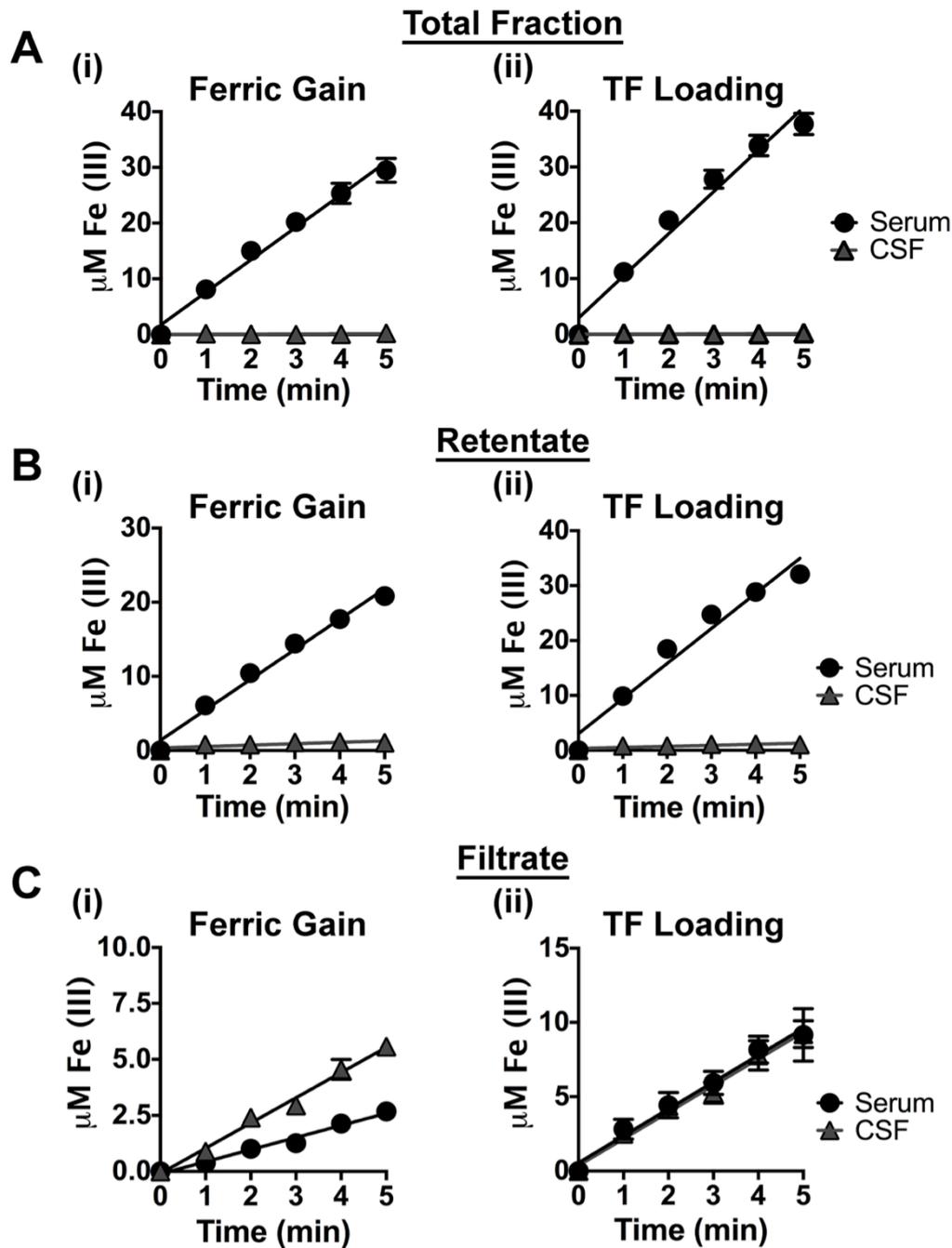


**Figure S1: Schematic illustrating the centrifugal filtration collection procedure in biological samples.** Centrifugation of total human serum and CSF to obtain filtrate (containing serum fluid, electrolytes, molecules and proteins < 10kDa) and retentate (containing molecules and proteins > 10kDa). The retentate fraction was diluted in Milli-Q® H<sub>2</sub>O to the same volume as filtrate to ensure comparable concentrations to the total fraction.



**Figure S2: Optimization of ferroxidase activity for serum and CSF in HBS pH 7.2. A.** Measuring the rate of velocity of  $\text{Fe}^{3+}$  production over 10 min for a serum concentration range (0-1mg/ml) identified 0.25mg/ml as the optimal concentration to produce a linear rate of increase. **B.** In similar conditions as used for serum, kinetic measurements of CSF volumes from 5-40µl indicated that 20µl was adequate to quantify  $\text{Fe}^{3+}$  produced over 5min. With both biological fluids, temperature was constant at 24°C throughout the assay and results blanked against the first time point reading. The individual data points shown are means  $\pm$  S.E., n=2 read in duplicates.

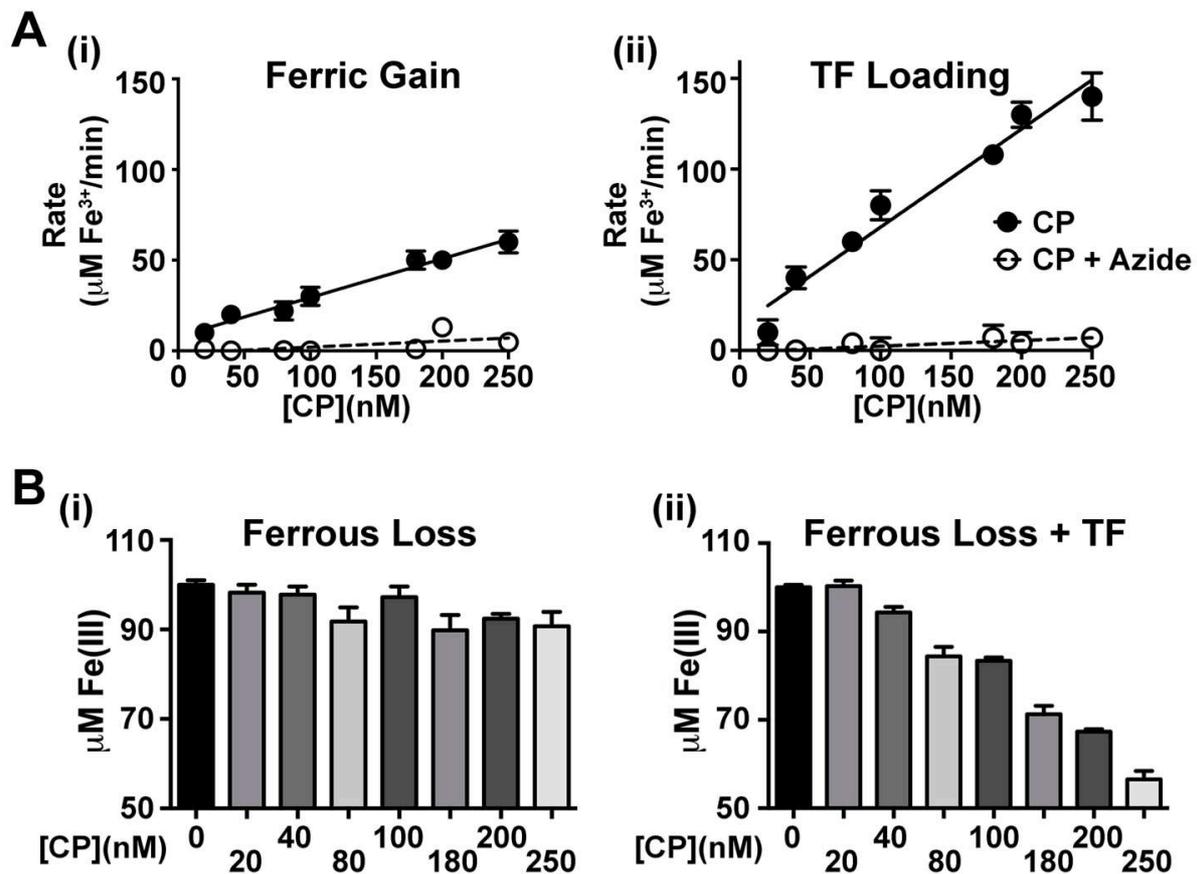
## Human Serum and CSF



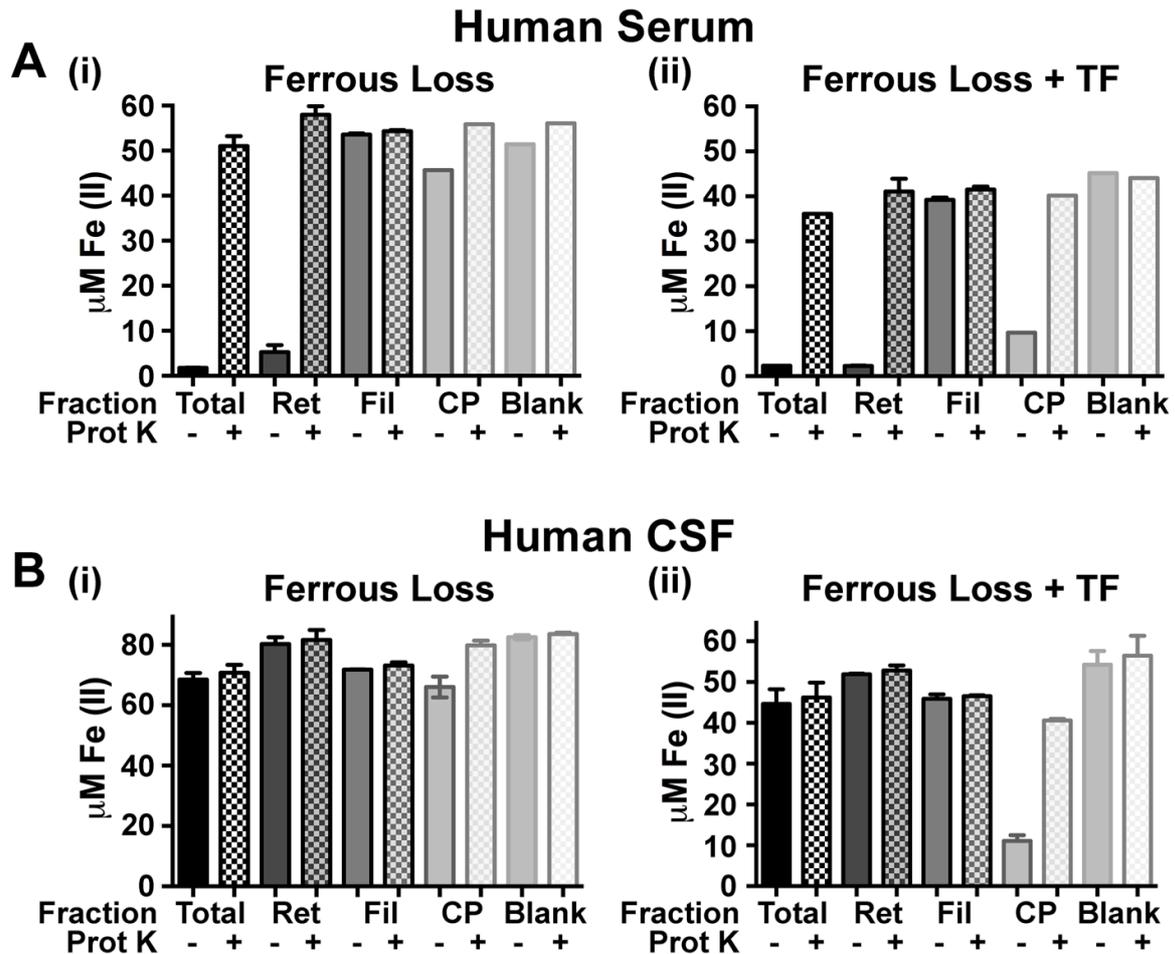
**Figure S3. Ferroxidase activity in comparable volumes of serum and CSF.** A. 5 $\mu\text{l}$  of either total human serum or CSF were kinetically quantified for  $\text{Fe}^{3+}$  production (Ferric Gain) (i) and apo-TF loading (ii) over 5min. B. As in A, 5 $\mu\text{l}$  of retentate fraction from serum or CSF were kinetically quantified for  $\text{Fe}^{3+}$  production (Ferric Gain) (i) and apo-TF loading (ii) over 5min. C. Due to the reduced filtrate activity obtained from serum, 20 $\mu\text{l}$  was required to measure retentate fraction from serum or CSF kinetically by  $\text{Fe}^{3+}$  production (Ferric Gain) (i) and apo-TF loading (ii) over 5min.

## SUPPLEMENTAL INFORMATION

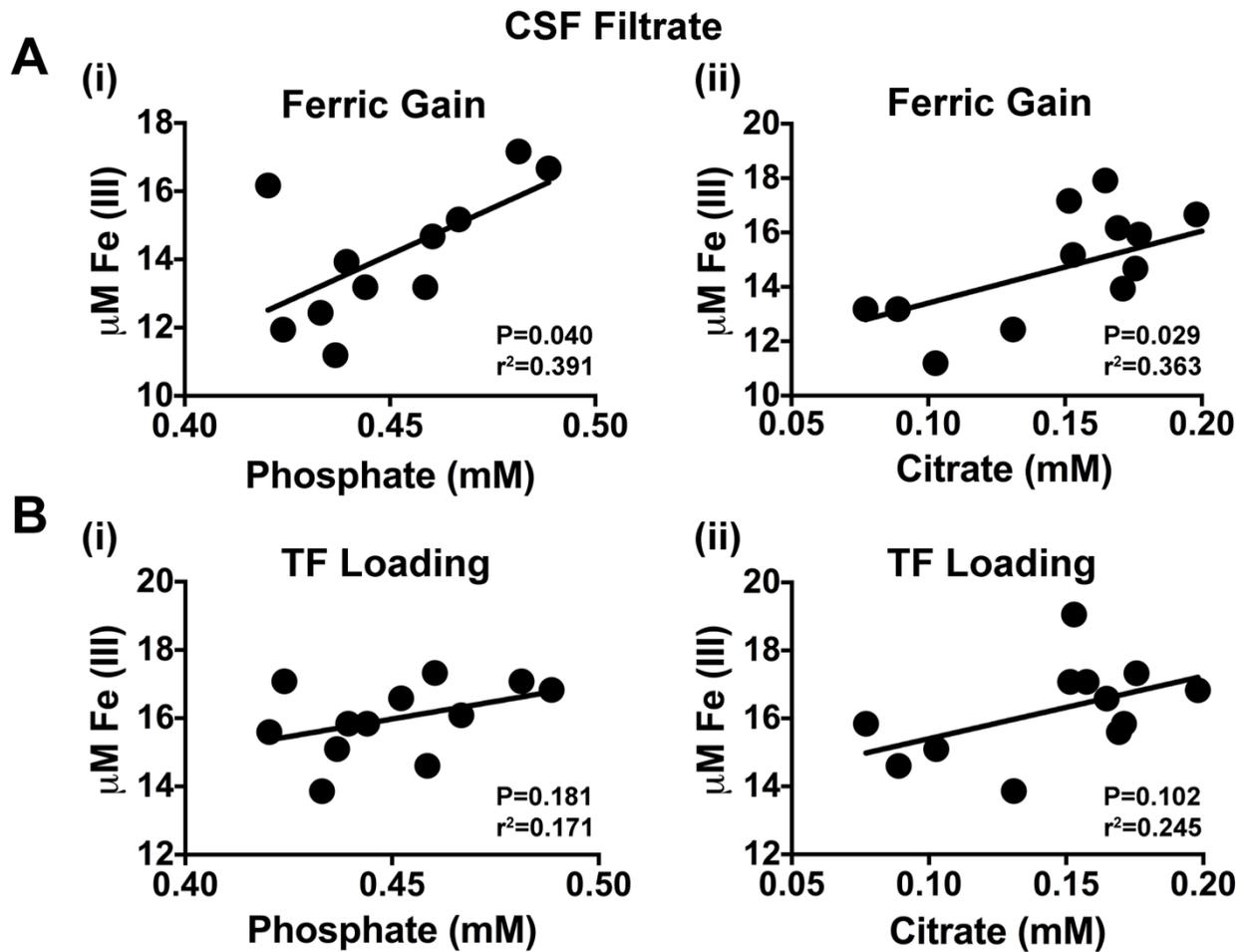
All other conditions used for carrying out the triplex assay in human serum and CSF were as described in Figs. 1 & 2; HBS buffer (50mM HEPES, 150mM NaCl, pH 7.2); FeSO<sub>4</sub> (100μM); +/- apo-TF (50μM). Temperature was constant at 24°C throughout the assay and results blanked against the reading at the first time point. The individual data points shown are means ± S.E., n= 3 read in duplicates.



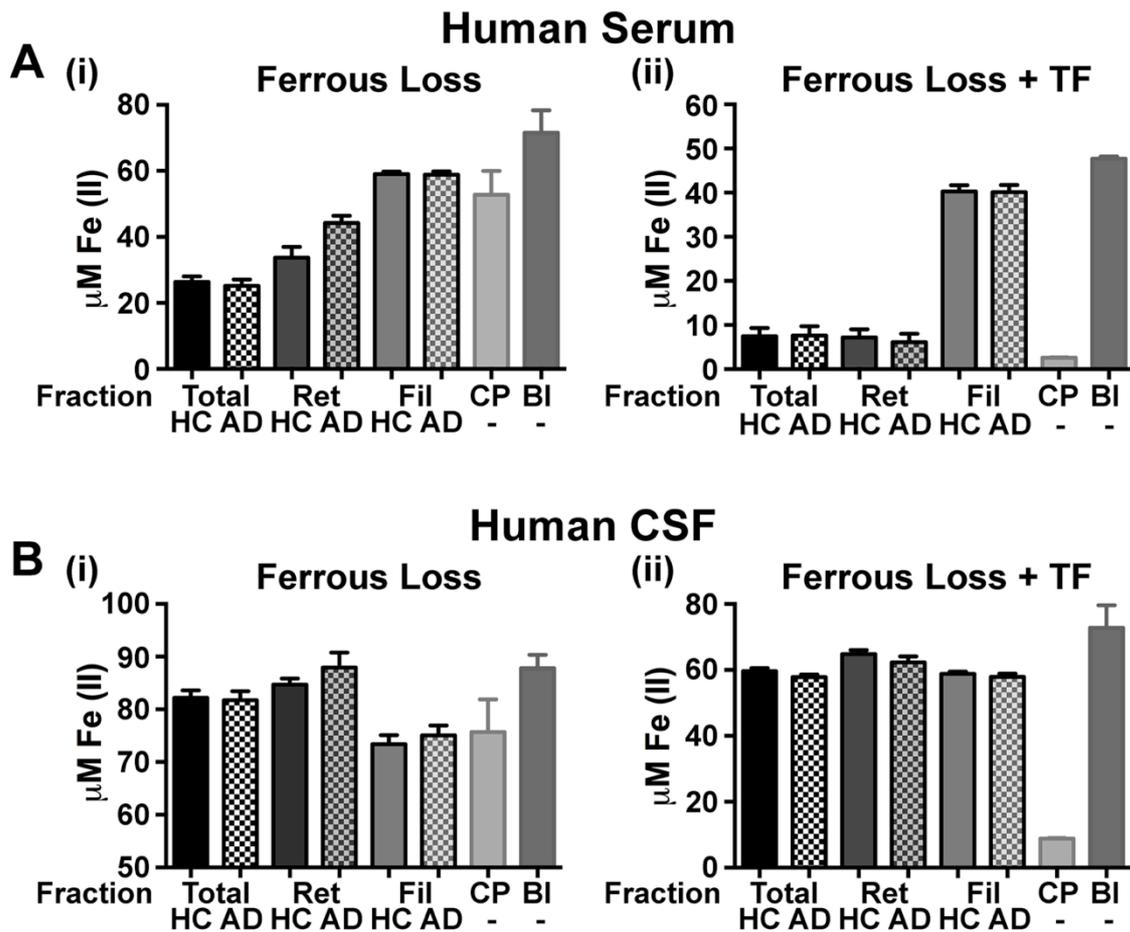
**Figure S4: Determining the sensitivity of the multiplex assay to CP.** **A.** Ferroxidase activity of purified CP at varying concentrations (0 – 250nM) was calculated kinetically using Ferric gain (i) and apo-TF loading (ii) over 10min. **B.** After 10min, remaining  $\text{Fe}^{2+}$  post CP conversion was measured at endpoint with the addition of a  $\text{Fe}^{2+}$  selective chromogen ferene S in the absence (i) or presence (ii) of TF. 250nM of purified CP is an optimal concentration to observe CP ferroxidase activity {Wong, 2014 #2}, but this data now indicates the assay to be sensitive enough to detect an azide inhibitable CP at a concentration as low as 20nM. Final optimal conditions for carrying out the triplex assay in human serum were as previously reported {Wong, 2014 #2}: HBS buffer (50mM HEPES, 150mM NaCl, pH 7.2); CP (20 – 250nM); +/- sodium azide (2.5mM);  $\text{FeSO}_4$  (100 $\mu\text{M}$ ); +/- apo-TF (50 $\mu\text{M}$ ). Temperature was constant at 24 $^\circ\text{C}$  throughout the assay and results blanked against the reading at the first time point. The individual data points shown are means  $\pm$  S.E.M, n= 2 read in duplicates.



**Figure S5: Determining enzymatic activity in serum and CSF.** In support of Figure 3 that demonstrates Ferric Gain and apo-TF loading components to the triplex assay, ferrous loss was also measured. **A.** Total, retentate and filtrate fractions of human serum was incubated with proteinase K (50 $\mu$ g/ml) overnight at 37 $^{\circ}$ C before ferroxidase activity was measured by ferrous loss +/- apo-TF after 10min. **B.** Identical parameters as **A** were measured for human CSF to measure Fe<sup>2+</sup> loss in the absence (i) or presence (ii) of apo-TF. The triplex assay conditions were as optimally determined for human serum and CSF. Temperature was constant at 24 $^{\circ}$ C throughout the assay and results blanked against the reading at the first time point. CP (250nM) was used as a positive control whereas the blank (Bl) indicated Fe<sup>2+</sup> loss caused by auto-oxidation within the assay conditions. The individual data points shown are means  $\pm$  S.E., n= 3 read in duplicates.



**Figure S6: Separate Phosphate and Citrate analysis in CSF filtrate.** A. In support of Figure 4B, activity obtained by Ferric Gain (A) and TF loading (B) was correlated with phosphate (i) or citrate (ii) concentration for CSF filtrate. Samples were the same as used for the combined polyanion correlation in Figure 4. Despite the lack of significance in TF loading when analysed for the separate polyanions, we show in Figure 4Bii a strong correlation when phosphate and citrate concentrations were added, indicating that both components contributed to the iron oxidation and subsequent loading into TF. The means of each individual data point were calculated before correlation and statistical analysis by 2-tailed T-test.



**Figure S7. Determining ferrous loss by ferroxidase activity in human control and Alzheimer's disease biological fluid.** In support of Figure 5 that demonstrates Ferric Gain and apo-TF loading components to the triplex assay, ferrous loss was also measured. **A.** Total, retentate and filtrate fractions from healthy control and AD serum were measured by ferrous loss in the absence (i) or presence (ii) of apo-TF. This end-stage measurement was carried out after 10min kinetic analysis of Ferric gain and apo-TF loading. **B.** Identical parameters as **A** were measured for human CSF from healthy control and AD to measure  $\text{Fe}^{2+}$  loss +/- apo-TF. The triplex assay conditions were as optimally determined for human serum and CSF. Temperature was constant at  $24^{\circ}\text{C}$  throughout the assay and results blanked against the reading at the first time point. CP (250nM) was used as a positive control whereas the blank (BI) indicated  $\text{Fe}^{2+}$  loss caused by auto-oxidation within the assay conditions. The individual data points shown are means  $\pm$  S.E., with each group containing n=10 read in duplicates.