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Enzymatic oxidation of Fe^{2+} under physiologically relevant conditions in biological fluids from healthy and Alzheimer's disease subjects.

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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.

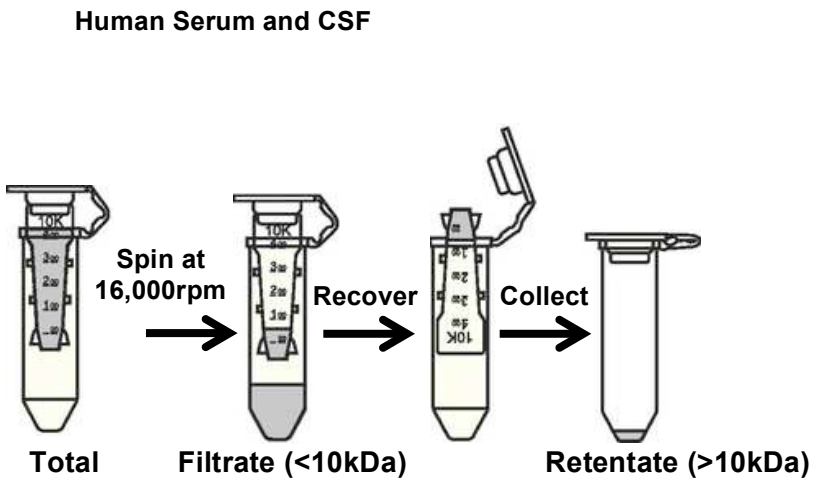

Figure S1: Schematic illustrating the centrifugal filtration collection procedure in biological samples. Centrifugation of total human serum and CSF to obtain filtrate (containing serum fluid, electrolytes, molecules and proteins < 10kDa) and retentate (containing molecules and proteins > 10kDa). The retentate fraction was diluted in Milli-Q® H_20 to the same volume as filtrate to ensure comparable concentrations to the total fraction.

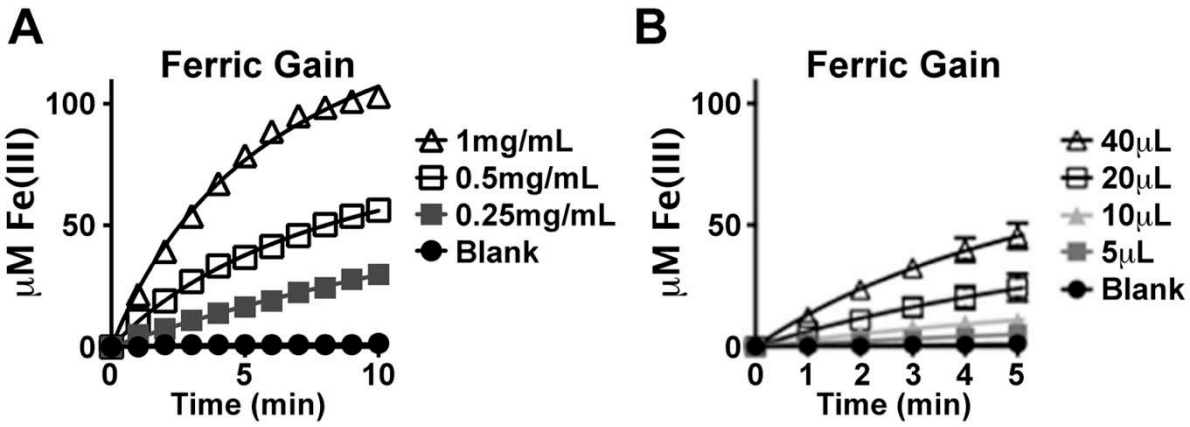

Figure S2: Optimization of ferroxidase activity for serum and CSF in HBS pH 7.2. A. Measuring the rate of velocity of Fe^{3+} production over 10 min for a serum concentration range (0-1mg/ml) identified 0.25mg/ml as the optimal concentration to produce a linear rate of increase. **B.** In similar conditions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

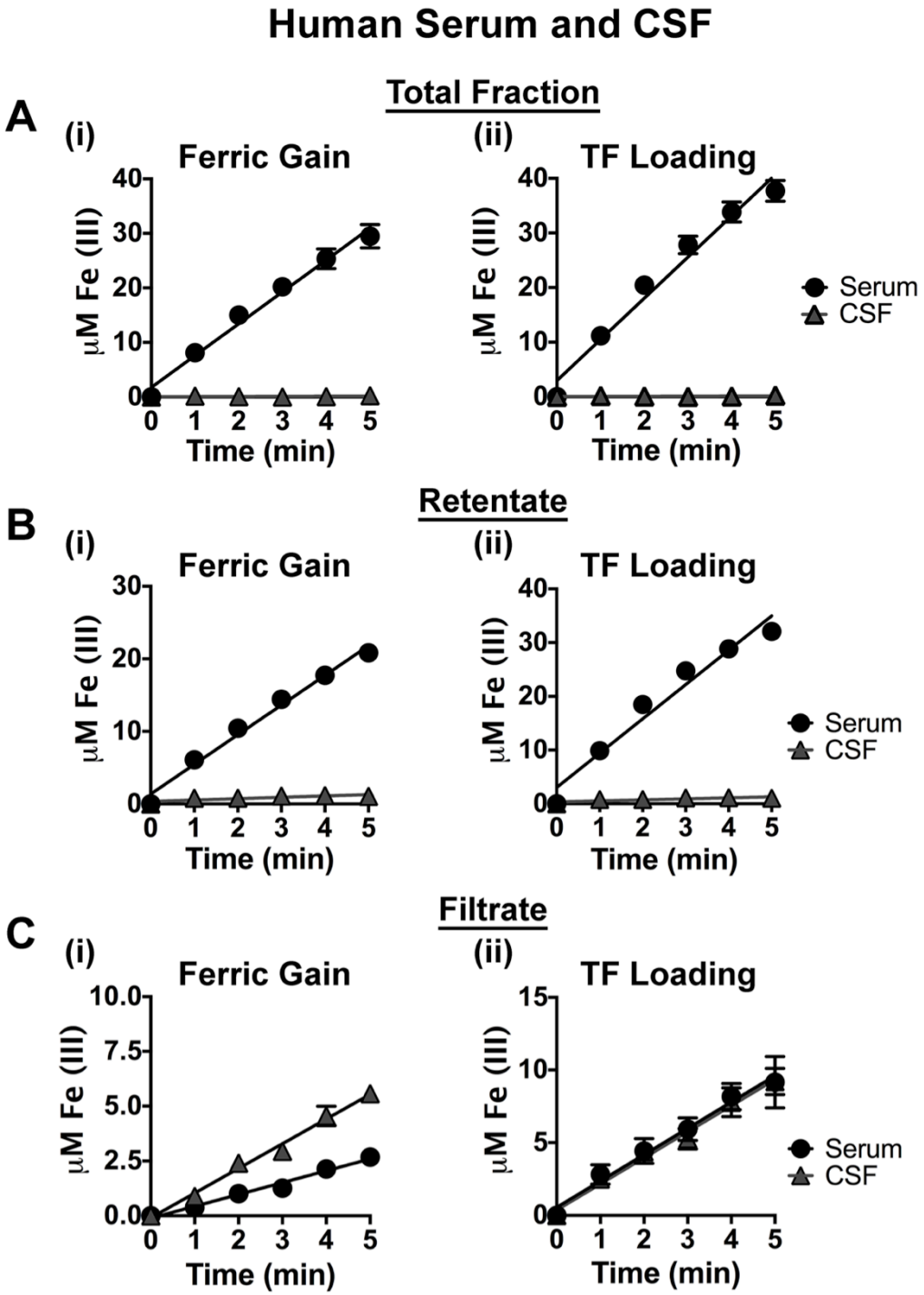

Figure S3. Ferroxidase activity in comparable volumes of serum and CSF. A. 5µl of either total human serum or CSF were kinetically quantified for Fe^{3+} production (Ferric Gain) (**i**) and apo-TF loading (**ii**) over 5min. **B**. As in **A**, 5µl of retentate fraction from serum or CSF were kinetically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

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); $FeSO_4$ (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= 3 read in duplicates.

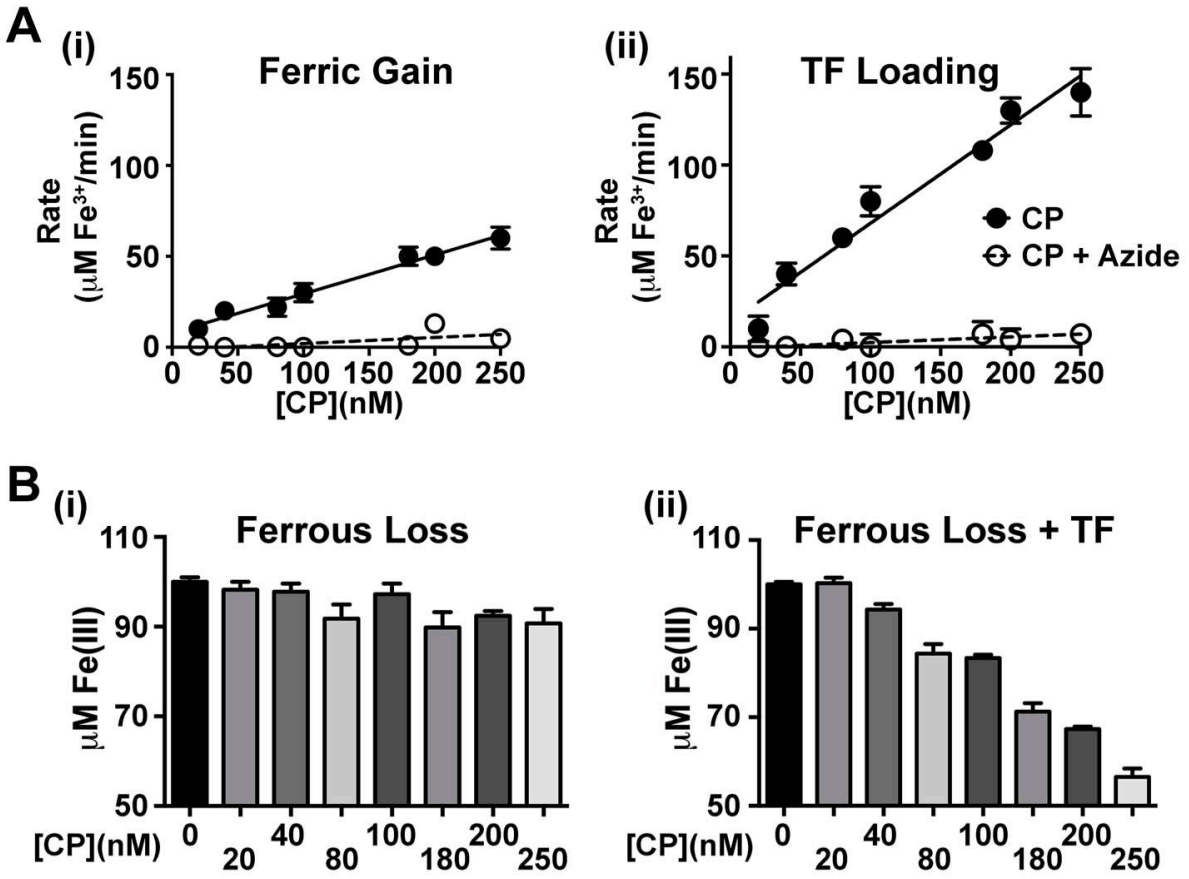

Figure S4: Determining the sensitivity of the multiplex assay to CP. A. Ferroxidase activity of purified CP at varying concentrations (0 – 250nM) was calculated kinetically using Ferric gain (**i**) and apo-TF loading (**ii**) over 10min. **B**. After 10min, remaining Fe^{2+} post CP conversion was measured at endpoint with the addition of a 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); $FeSO_4$ (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.M, n= 2 read in duplicates.

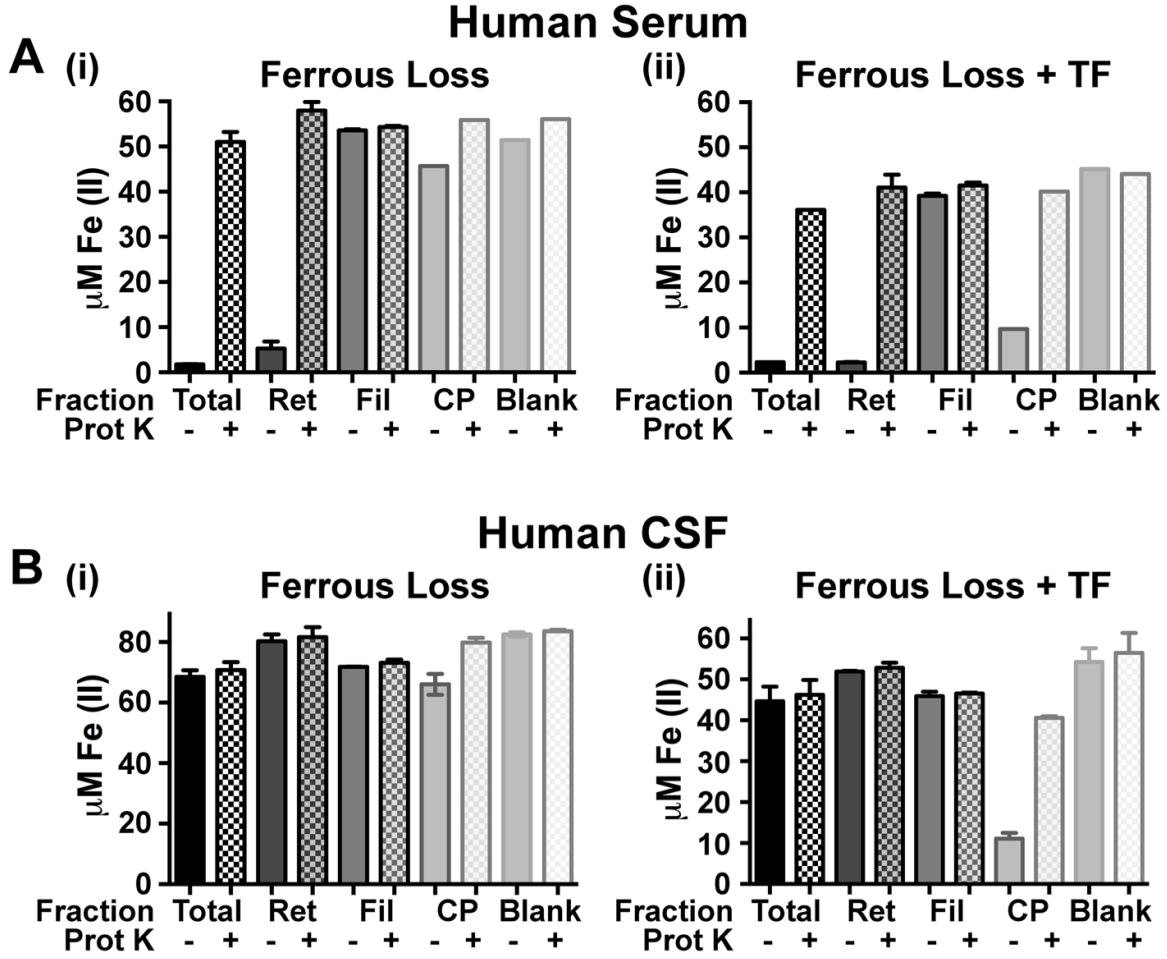

Figure S5: Determining enzymatic activity in serum and CSF. In support of Figure 3 that demonstrates Ferric Gain and apo-TF loading components to the triplex assay, ferrous loss was also measured. **A.** Total, retentate and filtrate fractions of human serum was incubated with proteinase K (50µg/ml) overnight at 37^{o}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^{2+} 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^{o}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^{2+} loss caused by auto-oxidation within the assay conditions. The individual data points shown are means ± S.E., n= 3 read in duplicates.

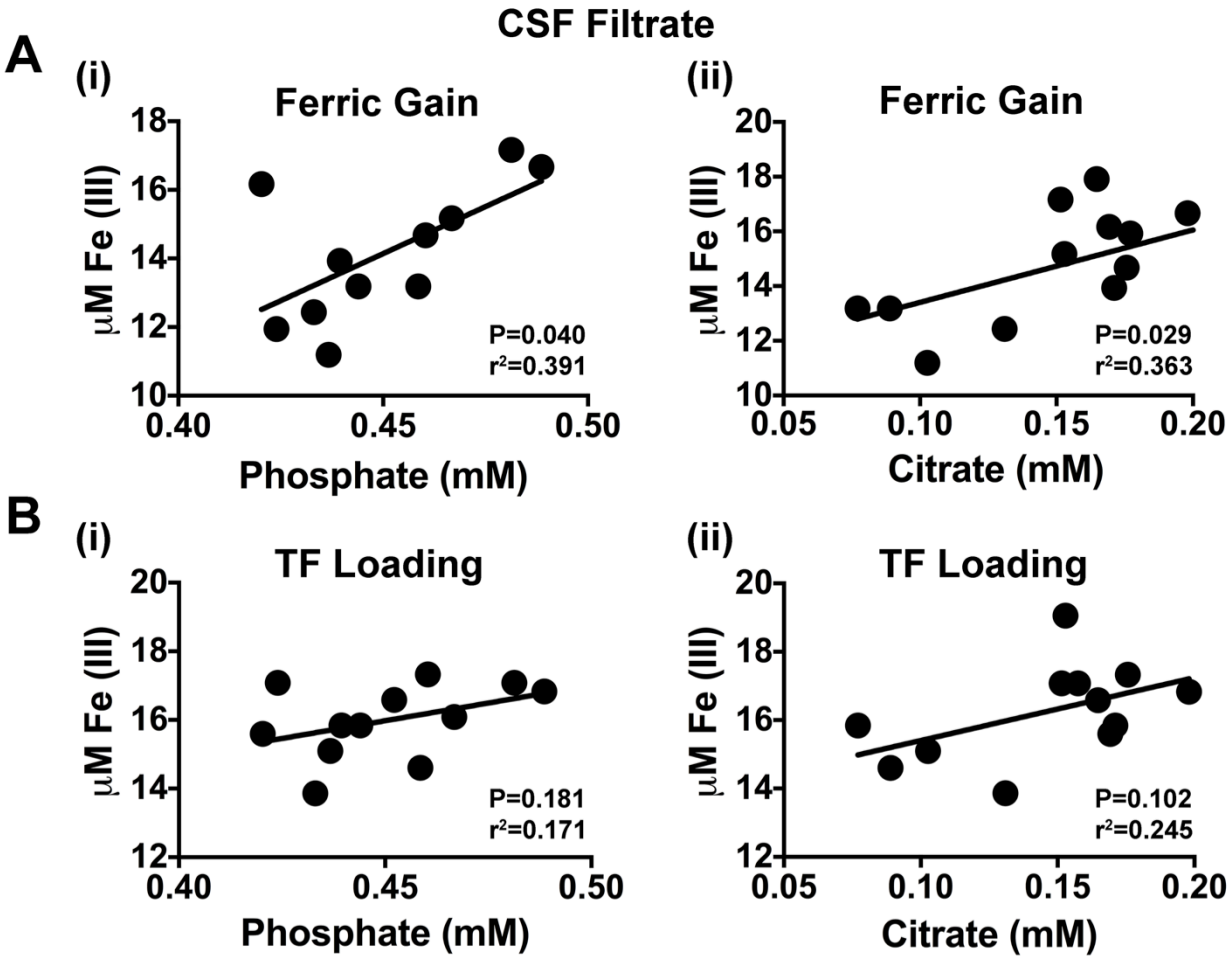

Figure S6: Separate Phosphate and Citrate analysis in CSF filtrate. A. In support of Figure 4B, activity obtained by Ferric Gain (A) and TF loading (B) was correlated with phosphate (**i**) or citrate (**ii**) concentration for CSF filtrate. Samples were the same as used for the combined polyanion correlation 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.

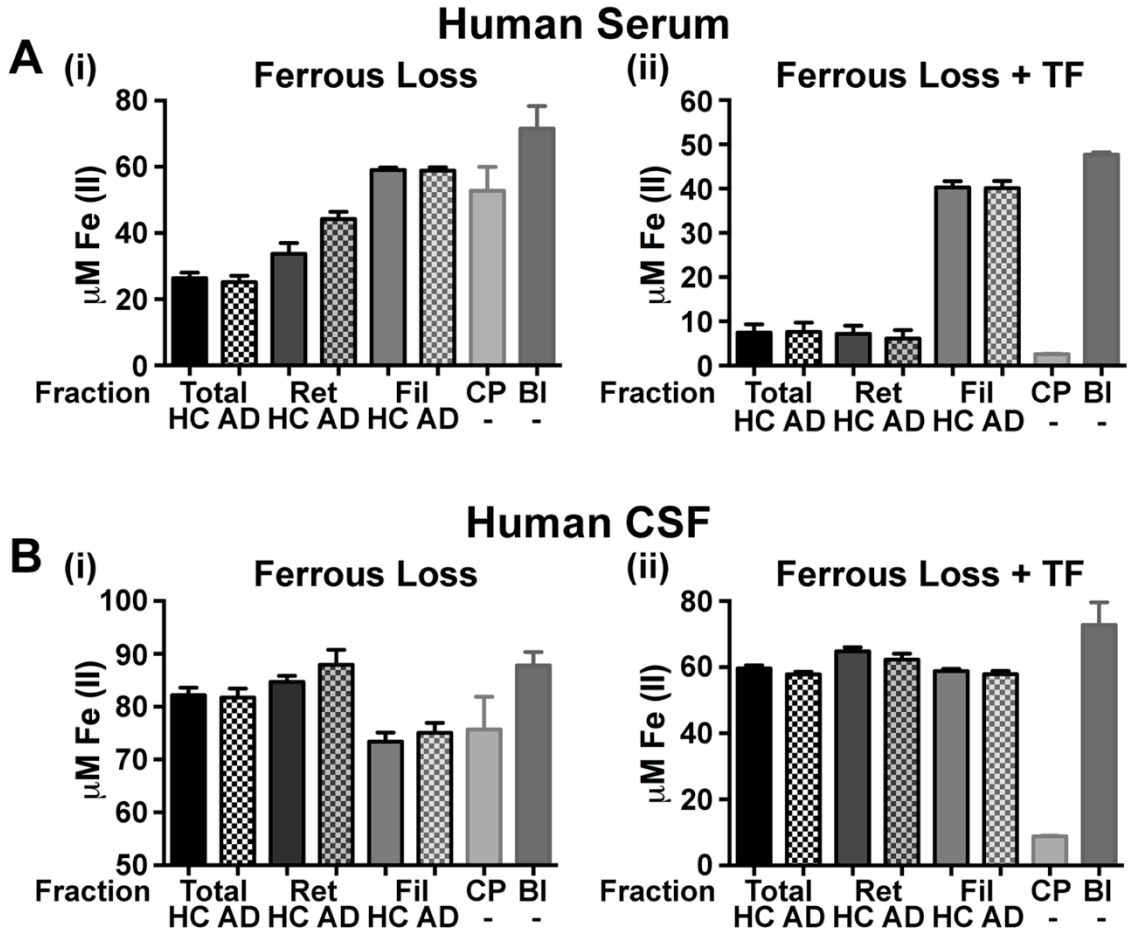

Figure S7. Determining ferrous loss by ferroxidase activity in human control and Alzheimer's disease biological fluid. In support of Figure 5 that demonstrates Ferric Gain and apo-TF loading components to the triplex assay, ferrous loss was also measured. **A.** Total, retentate and filtrate fractions 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 Fe^{2+} loss +/- apo-TF. 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. CP (250nM) was used as a positive control whereas the blank (Bl) indicated Fe^{2+} loss caused by auto-oxidation within the assay conditions. The individual data points shown are means ± S.E., with each group containing n= 10 read in duplicates.