SUPPLEMENTARY APPENDIX

Intravenous iron preparations transiently generate non-transferrin-bound iron from two proposed pathways

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Supplement

Supplementary Methods: Bead NTBI assay

We used the previously published bead-NTBI method[1,2] with some modifications. The hexadentate CP851 chelator is covalently bound to a fluorescein moiety on the beads. Upon binding of iron to CP851 chelator on the beads, fluorescence is quenched proportionally to the amount of iron bound. This in turn is proportional to iron concentration in solution.

Standards preparation

All buffer solutions were made up from analytical grade chemicals and AnalaR NORMAPUR HPLC-grade water (VWR) and treated with Chelex® 100 sodium form (Sigma) to remove contaminating iron.

Ferric nitrilotriacetate (Fe-NTA, Sigma) standards were prepared from stock solutions and filtered using a 0.22µm syringe filter (Nylon P/N:FIL-S-PP-022-13-100 13mm x 0.22µm, Racing Green, Chromatography). 45µL of 20mM NTA stock solution was transferred to a 15mL Falcon tube to which 20µL of 18mM ferric nitrate atomic absorption iron standard solution (Fluka) was added; the tube was vortexed and 835µL of AnalaR water was added to a total of 900µL, whereupon the solution was left for 1h to equilibrate at room temperature. This generated 400µM Fe-NTA at 1:2.5 ratio. Adding a further 11.1mL of AnalaR water to this solution made up 12mL of the 30µM Fe-NTA standard, which was subsequently kept frozen at -20ºC as 500µL aliquots. The aliquots were used on the day of the assay to generate the serial dilution solutions for the standard curve (30, 10, 3, 1, 0.3, 0.1, 0.03, 0.01, 0µM).

Sample preparation

Serum samples were thawed at room temperature (bench-top) then vortexed and filtered using a 0.22µm syringe filter (as above) with the filtrates (250µL) centrifuged at 14,000 rpm for 10 minutes.

Assay procedure

The assay buffer 150mM NaCl 50mM 3-(N-morpholino)propanesulfonic acid (MOPS, VWR) buffer, pH 7.4, was chelexed overnight. 20µL of serum supernatant (diluted 5-fold in buffer solution, i.e. 10µL in 40µL buffer), was transferred to the labeled wells in an acid-washed (100mM HCL, Fisher) dry 96-well plate in duplicate. Freshly prepared Fe-NTA (1:2.5) standards, 20µL, were added in triplicate to the designated wells (without dilution). 5-Fold diluted control normal serum, 20µL, was added in triplicate to the designated wells. Next, the CP851 beads, suspended in freshly prepared buffer, were added. Stock solution of the beads was 4.8 x 10^7/mL (synthesized at Prof Yongmin Ma laboratory, School of Pharmaceutical Science, Zhejiang Chinese Medical University, Hangzhou, China). This was diluted 16-fold to 3.0 x 10^6/mL in buffer). Typically 18mL of the bead suspension in buffer was prepared and 180µL of the suspension aliquoted to each well. The plate was then transferred to a plate shaker at a low stirring level for 20h-incubation at 37ºC.

Flowcytometry procedure

We used the Beckman Coulter CytoFLEX flow-cytometer with CytExpert software, KCL. Beads were identified on a side-scatter-area vs. forward-scatter-area plot as a uniform population of events (gate P1, see Figure 1A). Bead doublets were identified and excluded on a side-scatter-area vs. side-scatter-height plot (gate P3 excluding doublets). On a histogram plot for the FITC fluorescence channel, a median of the distribution (gated as P1 AND P3) was taken as the value representing the bead fluorescence. Exclusion gating (P4) corrects for auto-fluorescence (not shown).

Data analysis

Data was gathered at 10,000 events per gate P1 on a fast (60µL/min) setting. Median fluorescence was extracted for samples, controls and standards and analyzed using a standard curve modeled in GraphPad Prism 6.0 software with a 4-parameter logistic function, see Figure 1DE, with iron concentration in logM units. The interpolated unknowns were transformed by exponentiation and multiplied by $1.0x10^6$ to obtain results in µmol/L, and corrected for the dilution factor.

Quality control

Instrument validation was performed before each use. This involved system start-up procedure, Quality Control using CytoFLEX daily QC Fluorospheres (Beckman Coulter B53230) for laser calibration, and daily clean with a Beckman Coulter cleaning solution and distilled water.
Limit of Blank (95^{th} percentile of 8 replicates, LoB) 0.51nM, Limit of Detection ($LoB-1.654SD_{(low\ standard)}$, LoD) 14.6nM, Limit of Quantitation (LoQ) 30 nM, intra-assay and inter-assay precision CVs were 1.28% and 4.34% respectively (28 consecutively run assays over 82 days, with triplicate controls).

Supplementary Methods: Plasma Hepcidin measurement at KCL

Hepcidin standard was purchased from Peptides International and a working solution was prepared in methanol:water:formic acid (49:49:2) at a concentration of 100 µg/mL. The net peptide content was determined by amino acid analysis. This solution was aliquoted into a total recovery vials and then freeze-dried, stoppered and stored at -80ºC. The internal standard, $[^{13}C_9,^{15}N_1]Phe^{4,9}$-hepcidin(H-IS) was synthesized in-house and prepared similarly to hepcidin. All standards and QC samples were prepared in rabbit plasma (Sigma P4550). The samples and standards were allowed to thaw at room temperature and 200µL aliquot of each sample, standard or QC sample was transferred into a low binding multi-well plate, and 50µL of a 1.0µg/mL H-IS solution prepared in rabbit plasma added. Samples were mixed followed by the addition of 300µL of 4% phosphoric acid. Samples were mixed and then centrifuged at 5000 rcf for 10 minutes. Solid phase extraction wells were first conditioned with 200µL of methanol followed by equilibration with 200µL water. The supernatant was loaded onto the extraction plate. Samples were washed with 1% formic acid and hepcidin was eluted with 25µL of 40:50:10 of acetonitrile: 0.1% formic acid: trifluoroethanol (elution solvent).
Ten microliters of the eluent were chromatographed using an ACQUITY UPLC on an Acquity BEH 130 column, 1.7 µm, and 2.1 x 50 mm. The gradient elution solvents were 0.1% formic acid in water: acetonitrile: trifluoroethanol (90:5:5) (mobile phase A) and 0.1% formic acid in acetonitrile: water: trifluoroethanol (90:5:5) (mobile phase B), the sample was eluted on a linear gradient from 20% B to 60% B in 2

minutes at a flow rate of 200 µl/min. For hepcidin and H-IS, collision induced dissociation products of multiple charged precursors were detected in the positive ion selected reaction monitoring mode using a Waters Xevo TQS mass spectrometer. The electrospray voltage, source temperature, desolvation temperature and desolvation gas flow rate were 3.0 kV, 150 °C, 500 °C and 1000 L/hr, respectively. Selected reaction monitoring (SRM) transitions used for quantitation were for hepcidin 698.160 > 644.129 and the internal standard 702.777 > 648.741 both with a cone voltage of 20 and a collision energy of 16 eV. MassLynx version 4.1 was used for data acquisition. All peak area integration, regression analysis and sample quantitation was performed using TargetLynx. Specifically, peak area ratios of hepcidin and the internal standard were determined and calibration curves generated using a 1/concentration weighted linear regression model. All QC sample concentrations were then calculated from their peak area ratios against the calibration curves. Re-validated assay data is published previously (supplementary).[3]

Supplementary Methods: Inductively-Coupled Plasma Mass-Spectrometry (ICPMS) serum analyses (KCL)

Sample preparation

Samples were thawed at room temperature (bench-top) then vortexed and filtered using a 0.22µm syringe filter (Nylon P/N:FIL-S-PP-022-13-100 13mm x 0.22µm, Racing Green, Chromatography). Filtrates (250µL) were centrifuged at 14,000 rpm for 10 minutes and 90µL of the serum supernatant was transferred to an HPLC glass vial with a plastic insert, 10µL of 180µM ferrioxamine standard (from stock solution kept at 4 deg C throughout the study) was added, and the vials were capped. Final concentration of ferrioxamine in each sample was 18µM, which itself diluted the serum by 10%. Internal standard mean elution time across 16 runs over 158 days was 20.76±1.33min (range 18.34-22.52min) giving a between-run (inter-day) CV of 6.4%. The within-run (intra-day) CV was 0.74±0.43% (range 0.11-1.59%) and was below the 5% critical QC threshold for admissibility of run results.

Instrument

Perkin Elmer Flexar HPLC (LC Autosampler, LC Pump, Solvent Manager, UV/VIS Detector, Column Oven) coupled to a Perkin Elmer NexION 350 D Inductively-Coupled Plasma Mass Spectrometer (ICP-MS) was used with Syngistix and Chromera operating software (KCL). All instruments were operated strictly according to manufacturers' instructions. The LC UV/Vis detector was set at 254nm. Sample manager was set to a single injection volume of 10µL. A size exclusion column Bio SEC-5, 300Å, 7.8x300mm (Agilent Technologies) equilibrated from storage, was used with a guard column (Agilent Bio SEC-5, guard, 5µm, 500Å, 7.8x50mm) and cleaned every 32 injection-runs, as per manufacturer's instructions.
Isocratic flow was set at 0.75mL/min at room temperature using a mobile phase (50mM ammonium acetate pH 7.4 based on AnalaR NORMAPUR HPLC grade water) with each run lasting 45 minutes, without washing between sample runs but with wash after the last sample-run.
All media solutions were vacuum-filtered using an assembled manifold (Vacuum filtration manifold phenomenex, Millipore) with a 0.22µm Millipore filter and de-gassed in a sonicator for 30 minutes at room temperature before HPLC use.
The following ICP-MS conditions were used: dynamic reaction cell (DRC) mode: nebuliser flow 0.95-1.00 L/min, auxilliary gas flow 1.2 L/min, plasma gas flow

18L/min, RF power 1600 W, ammonia gas flow 0.6mL/min, RPq 0.8, analyte Fe56 (55.9349) dwell time 200ms.
The results were reviewed and integrated within Chromera software (Figure 2A), data analysis was performed on GraphPad Prism Ver 6.0 and custom MS Excel templates.

IVIP iron in plasma

IVIP iron (IVIP-Fe) co-eluted with the endogenous pre-Tf protein-associated iron, which is a cluster of typically 3 peaks that run before transferrin (13.17min), Figure 2A. IVIP-Fe was integrated and quantitated against the internal standard (FO). Where IVIP-Fe co-eluted with the transferrin peak itself, the transferrin peak was tangentially skimmed and not included in the integration. In order to correct for the endogenous pre-Tf protein-associated iron, the baseline value of the latter (-10min from the IVIP injection) was subtracted from every subsequent chromatographic profile thus providing the estimation of IVIP-Fe. This subtraction represents changes in plasma IVIP-Fe, where the subtracted baseline value (of pre-Tf protein-associated iron) is typically <0.1% of the peak value. Alternatively, IVIP-Fe was also calculated by subtracting TBI from TSI. Plasma half-life of the IVIP-Fe was derived from fitting mono-exponential models of the decay of the IVIP-Fe levels and from non-compartmental analysis (alongside other PK parameters).

Supplementary Results: Serum ferritin iron content

The pre-Tf protein-associated iron at baseline elutes typically with 3 peaks between 10 and 11.5 minutes (Figure 2A, blue trace in Figure S1A, Figure S1B, Figure S1C). As evident from the placebo profile in Figure S1D, these peaks are changing dynamically over the 312h of follow up. In principle, they represent 3 separate protein species that each carry iron. Potential candidates at that molecular weight range include haptoglobin-Hb complex, and ferritin. However, the baseline of the pre-Tf protein-associated iron may be compared to its end-of-study level, and assuming all else being equal (e.g. haptoglobin-Hb complex), the difference in pre-Tf iron in that comparison (EOS-baseline) should represent the change in ferritin iron content on study. At baseline (-10 min), no IVIPs are present and at 312h sufficient number of half-lives have elapsed for the IVIPs to be completely removed. On that assumption, the change in serum ferritin iron content from 312 hours back to baseline was negative on average but highly variable and the change was not statistically significant (Figure S2D).

Supplementary Results: Erythropoietic response

All patients were non-anaemic at baseline, and there was no difference in Hb between treatment groups at baseline (Table 1). As a result of frequent blood sampling throughout the study (total volume 424mL), patient Hb levels sustained a downward trend; this needs to be considered in the context of low dose of IVIP being administered. The degree of Hb reduction is significant between baseline and EOS (ANOVA time effect $p<0.0001$, mean reduction 7.2±1.1 g/L at 312h). Nevertheless, there was no difference between treatment groups in the degree to which Hb level was reduced at the end of study due to the cumulative phlebotomies on study (Figure S3 left). Essentially, what was administered as IVIP iron was removed as RBC iron. Reticulocyte percentage count increased with time and across treatment modalities (data not shown). Whether that was as a result of multiple blood sampling, loading with iron, or both, cannot be definitively established in this study. Similar behaviour

was observed for sTfR, however the increases in sTfR were only significant for IS and placebo (Figure S3 right).

Supplementary Figures

Figure S1

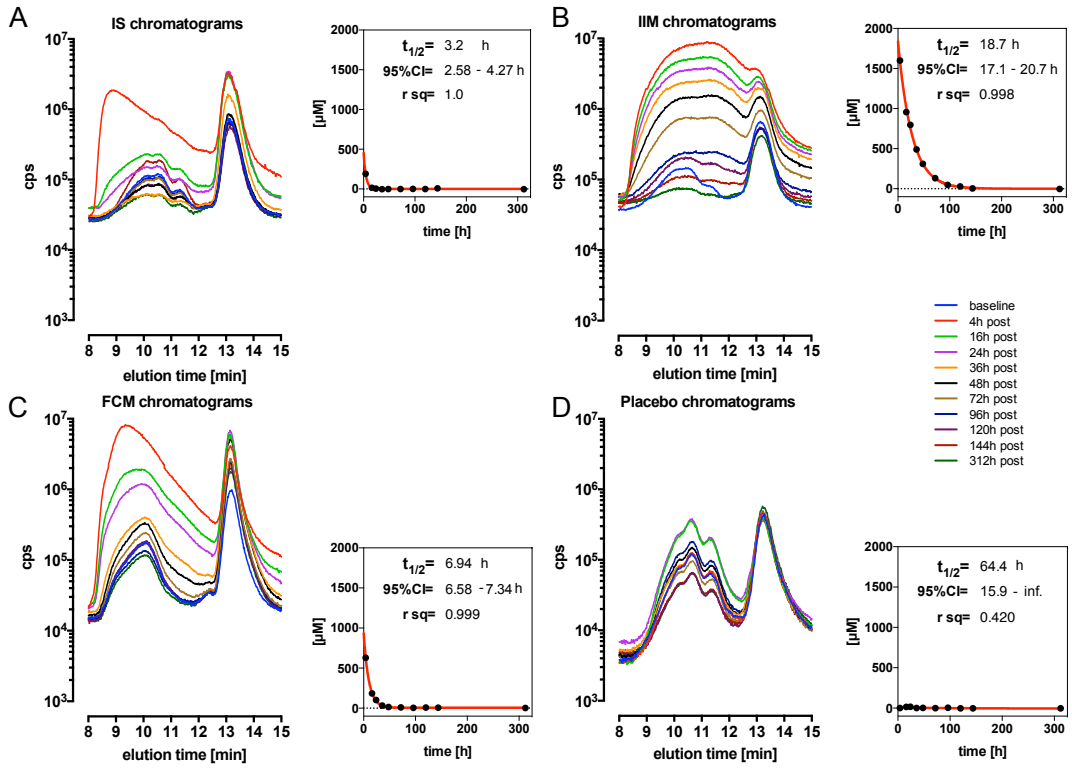

Figure S1. Overlay of chromatograms from all time-points within an example patient from each study arm. Chromatograms, with counts-per-second (cps) on a log scale to enhance resolution, were shown between 8 and 15 min elution time for 11 consecutive samples from baseline to 312h, see legend. Respective insets show mono-exponential decay fits of the post-C_{max} data derived from pre-Tf peak AUCs corrected for baseline AUC, with fitted values of the half-life of the IVIP shown. (A) iron sucrose (IS) chromatograms, (B) iron isomaltoside 1000 (IIM) chromatograms, (C) ferric carboxymaltose (FCM) chromatograms, and (D) Placebo chromatograms.

Figure S2

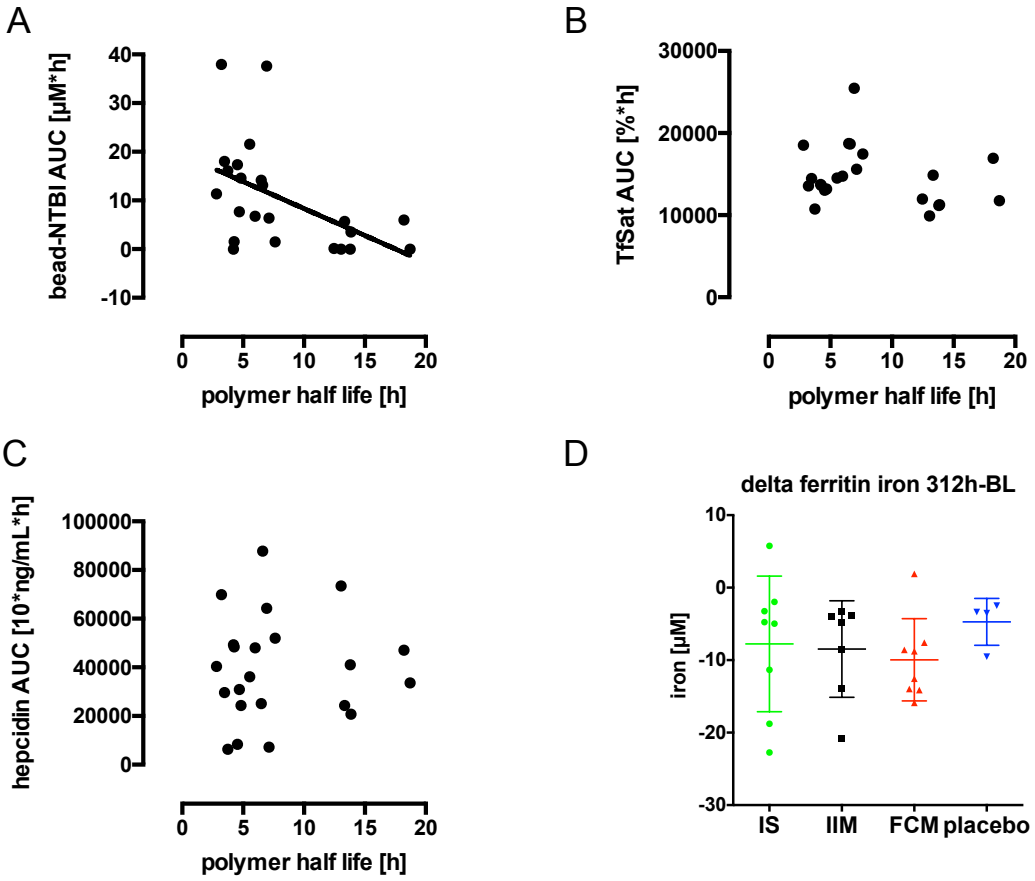

Figure S2. Relationships of plasma half-life of the iron polymers with (A) NTBI AUC, Spearman correlation -0.58, p=0.0041 (B) TSAT AUC, p>0.05 (ns) (C) hepcidin AUC, p>0.05 (ns). (D) Change in serum ferritin iron content on study, estimated from the difference in the pre-Tf peaks (312h minus baseline), shown as replicates with mean±SD for all treatment groups; n=8 for each IVIP, n=4 for placebo.

Figure S3

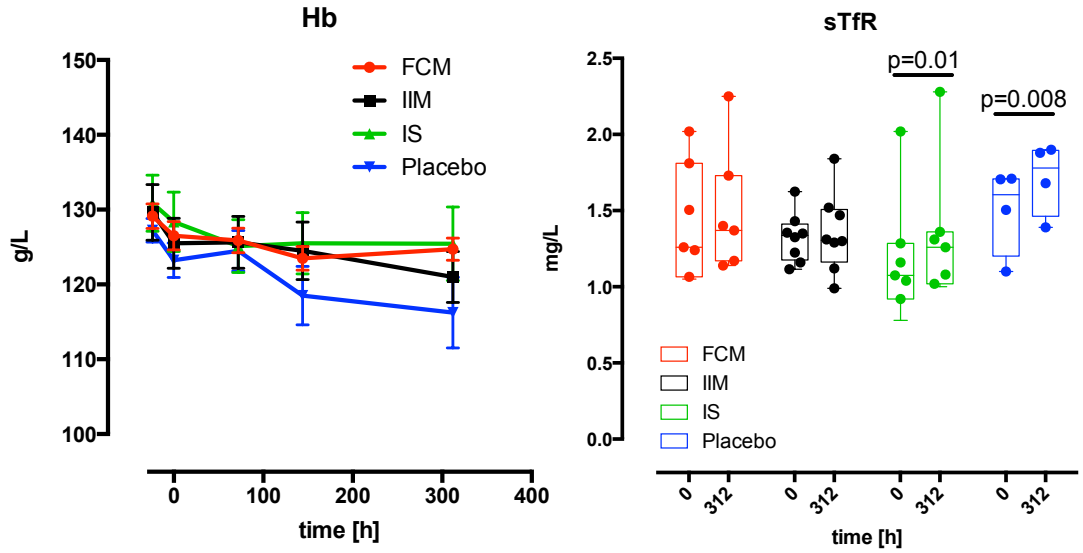

Figure S3. Erythropoietic response on study. Haemoglobin trend on study (left panel); sTfR comparison 0 vs. 312h within treatment groups and placebo, Two-way ANOVA with respect to time and treatment and Holm-Sidak's multiple comparison test p values shown where significant (right panel).

Figure S4

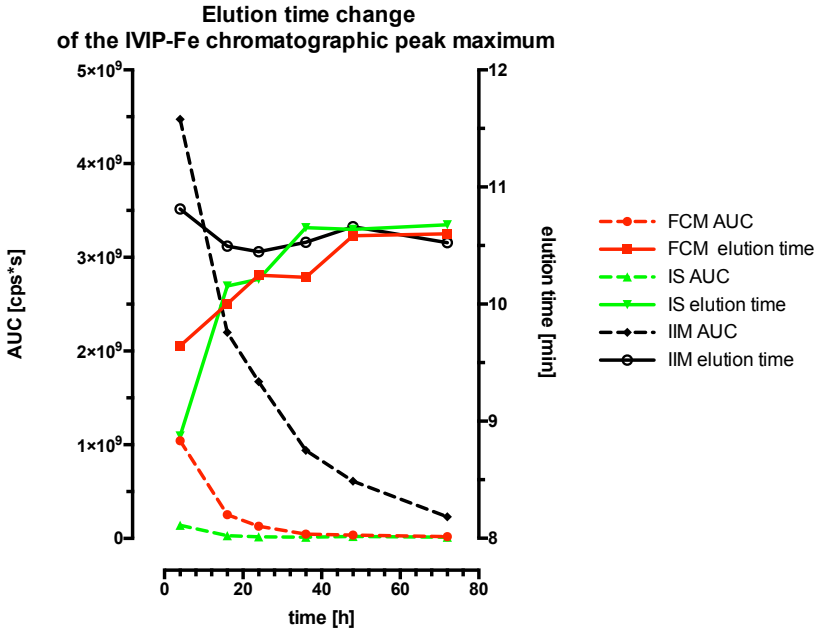

Figure S4. Change in the IVIP-Fe chromatographic peak elution time with respect to time on study. Chromatographic AUC values for each IVIP-Fe peak (left axis) are paired with the respective elution time (right axis) for each peak at the respective time-point and regressed on the study time (4-72h from baseline). Example date shown for IIM, FCM and IS.

Figure S5

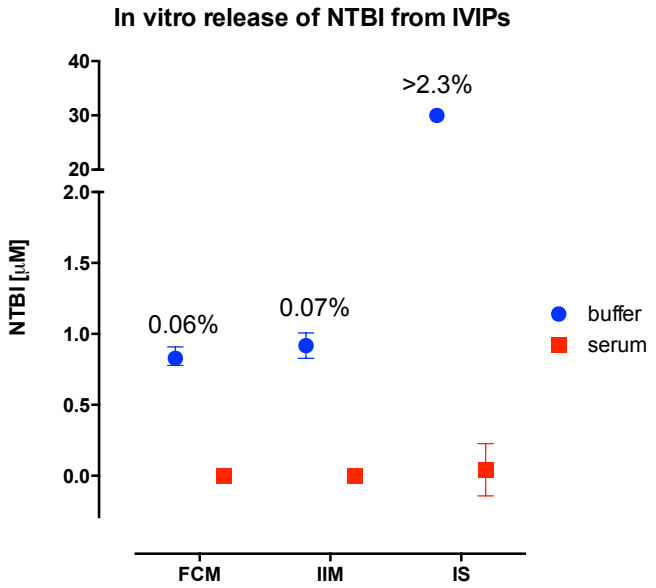

Figure S5. The in vitro NTBI release from IVIPs using the CP851-NTBI assay. 1.3mM IVIP iron was incubated for 1h at room temperature with 150mM NaCl 50mM MOPS pH 7.4 buffer or normal serum before being assayed by the NTBI assay, data shown as median±range, n=3. NTBI as a percentage of total IVIP iron (1.3mM) is shown. Disappearance of NTBI during incubation with serum confirms the NTBI as exchangeable with transferrin. The IS buffer NTBI value of 30µM is at the upper limit of the assay, so should be interpreted as >30µM.

Figure S6

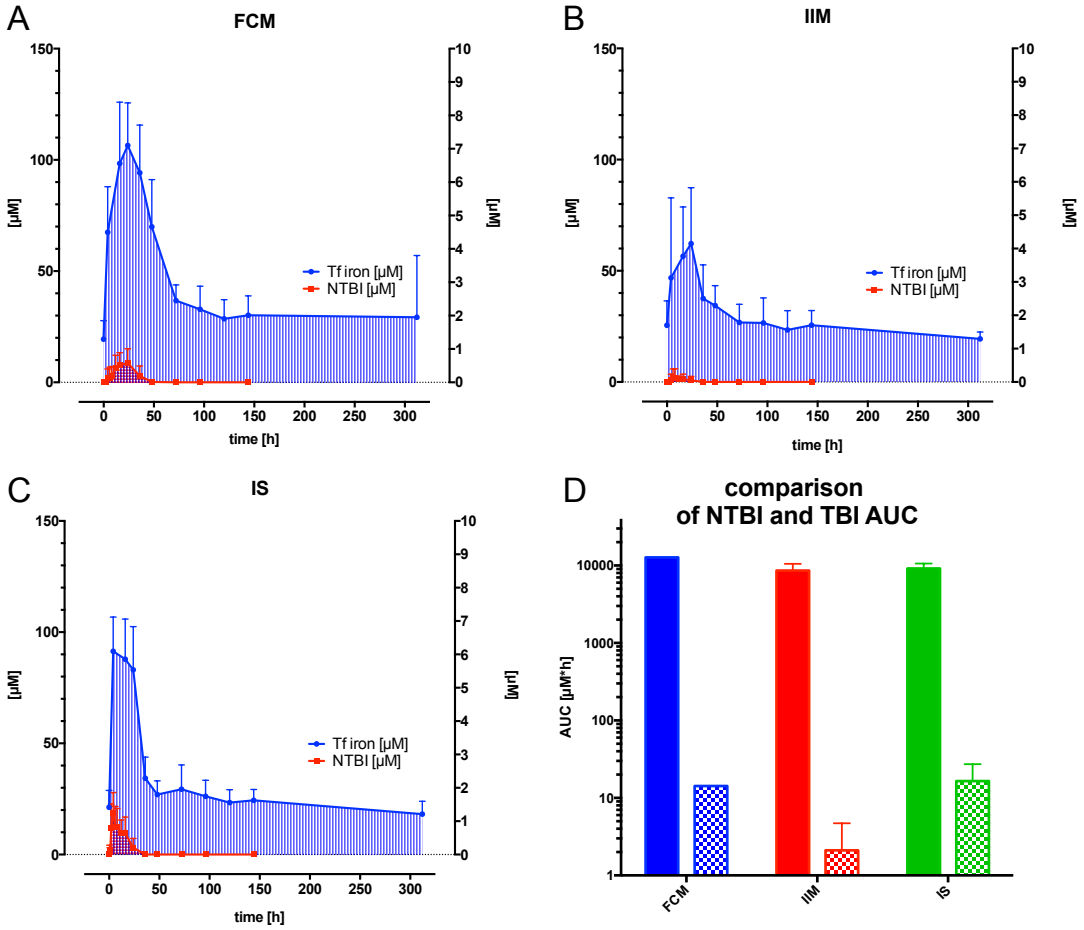

Figure S6. Comparison of TBI and NTBI exposure as AUC between IVIP treatments. TBI AUC is shown on the left axis while NTBI AUC on the right axis is exaggerated 15-fold for visual clarity. (A) FCM, (B) IIM, (C) IS, (D) comparison of TBI and NTBI AUCs in selfsame units (µM*h), shows that NTBI AUC is ≤0.1% of TBI AUC.

References

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