SUPPLEMENTARY APPENDIX

Residual erythropoiesis protects against myocardial hemosiderosis in transfusion-dependent thalassemia by lowering labile plasma iron via transient generation of apotransferrin

Maciej W. Garbowski,[1,2] Patricia Evans,[1] Evangelia Vlachodimitropoulou,[1] Robert Hider[3] and John B. Porter[1,2]

[1]Research Haematology Department, Cancer Institute, University College London; [2]University College London Hospitals and [3]Institute of Pharmaceutical Sciences, King's College London, UK

 doi:10.3324/haematol.2017.170605

Received: April 14, 2017.

Accepted: June 20, 2017.

Pre-published: June 22, 2017.

Correspondence: maciej.garbowski@ucl.ac.uk

Supplement to

Residual erythropoiesis protects against myocardial hemosiderosis in transfusion-dependent thalassemia by lowering labile plasma iron via transient generation of apotransferrin.

Maciej W. Garbowski, Patricia Evans, Evangelia Vlachodimitropoulou, Robert Hider, John B. Porter

Methods

In order to relate cardiac iron to biomarkers in patient blood samples, cardiac MRI and blood biomarkers were measured at baseline and ~1.5 years later, over which time the **transfusion iron load rate (ILR)** in mg/kg/day was calculated from the number of blood units transfused, assuming 200mg iron per unit, patients' mean weight and number of days between MRI scans[1]. The mean follow-up was 502.7 days (SD 189.5), baseline mean cardiac R2* 61.14±5.8s^{-1}, follow-up 56.96±5.19s^{-1}(SE). During the follow-up, the change in the mean cardiac iron level was significant (paired t-test p=0.011), although clinically trivial, of 4.17 s^{-1}. The cohort of 73 patients with transfusion dependent thalassaemia on deferasirox was divided into those with MH (n=24, cardiac T2*<20ms, R2*>50s^{-1}) and those without MH (n=49, T2*>20ms, R2* <50s^{-1})[2].

Plasma biomarkers

A range of serum and plasma biomarkers was used in the study to test for a relationship with cardiac iron.

Non-transferrin-bound iron (NTBI) was assayed using the HPLC-based nitrilotriacetate (NTA) method[3,4]. Briefly, 20µL of 800mM NTA (at pH=7) was added to 180µl serum and allowed to stand for 30min at 22^{o}C. The solution was ultra-filtered using the 30kDa Whatman Vectaspin ultracentrifugation devices at 12320***g*** and the ultra-filtrate (20µL) was injected directly onto an HPLC column (ChromSpher-ODS, 5µM, 100x3mm, glass column fitted with an appropriate guard column) equilibrated with 5% acetonitrile and 3mM deferiprone in 5mM MOPS (pH=7.8). The NTA-iron complex then exchanges to form the deferiprone–iron complex detected at 460nm by a Waters 996 photo-diode array. Injecting standard concentrations of iron prepared in 80mM NTA generated a standard curve. The 800mM NTA solution used to treat the samples and prepare the standards is treated with 20µM iron to normalize the background iron that contaminates reagents. This means that the zero standard gives a positive signal since it contains the added background iron as an NTA–complex (2µM). When unsaturated transferrin is present in sera, this additional background iron can be donated to vacant transferrin sites resulting in a loss of the background signal and yielding a negative NTBI value.

Labile plasma iron (LPI) assay was performed in a cell-free system (on patient plasma in Figure 1, Figure 2 and Table 1 or on buffered solutions in Figure 3) using the 2,3-dihydrorhodamine (DHR) method[5] with modifications as previously published[6], which were limited to the generation of standards in plasma-like

medium containing 20mg/mL human serum albumin. The assay was recently used in an international round robin[7]. Briefly, in a 96-well plate, quadruplicates of 20µL of serum or buffered solution were added to 180µL of plasma-like medium, containing 20mg/mL human serum albumin and freshly added 50µM DHR with 40µM ascorbate only for the first set of duplicates and 40µM ascorbate with 50µM deferiprone for the second set of duplicates. Fluorescence intensity measurements were taken in each well (excitation 485nm, emission 538nm) for 40 minutes, with readings every 2 minutes. Slopes of DHR fluorescence intensity were taken between 15 and 40 minutes, and expressed as fluorescence intensity units per minute. The duplicate values of the slopes in the presence and absence of deferiprone (r_1 and r_2, respectively) were averaged and subtracted (r_2-r_1) for both the standards and the unknowns, with the LPI value of the unknowns interpolated from the standard curve. Standards were prepared from Fe-NTA (1:7) solutions spiked into plasma-like-medium.

Plasma hepcidin was assayed using mass-spectrometry[8], **transferrin saturation** was measured using the urea-gel method[9], **sTfR1** and **GDF-15** ELISAs were performed as before[10], according to manufacturers' specifications. Routine relevant haematological and biochemical biomarkers included automated **absolute reticulocyte count**, automated **nucleated red blood cell count** (NRBC), serum total **bilirubin**, **hemoglobin**, serum **ferritin**, and **total serum iron**.

Cell-line experiments on HL-1 cardiomyocytes

For cell-line experiments, murine HL-1 cardiomyocytes (ATCC number CRL-12197) were grown as previously published[11], on 80cm^2 flasks (SLS Ltd) or 48-well plates (Fisher Scientific, UK) coated with 0.5mL/100mL fibronectin (F-1141) in 0.02% gelatin (BD) in Claycomb medium supplemented with 300µM ascorbate, 100µM norepinephrine, 2mM L-glutamine, 100U/mL penicillin, 100µg/mL streptomycin and 10% fetal calf serum (serum batches for HL-1 cells as defined by SAFC). After confluence had been achieved (24-48h), monolayers were washed with Claycomb medium without FCS before subsequent 24h incubation with experimental media (culture media without FCS). Claycomb medium contains 1.76±0.03µM iron using ferrozine assay (see below) of which 0.8µM is transferrin iron, as per manufacturer's communication that typically 0.4µM holotransferrin is added during media production. PBS contains 2.57±0.19µM iron using ferrozine assay (see below), chelexed PBS contains 0.028±0.01µM iron.

Iron solutions for *in-vitro* work

In order for ferric iron to remain in solution at physiological pH=7.4, it needs to be liganded to protein, typically transferrin, or other ligands in order to prevent iron from being coordinated by water molecules – a process that eventually leads to precipitation as ferric hydroxide complexes. Citrate is the most relevant physiological ligand for ferric iron when transferrin is fully saturated[12]. As a model of NTBI, **ferric citrate buffered** in MOPS pH=7.4 was used following 24h incubation at room temperature after its preparation to allow for species stabilization[13,14]. During its preparation atomic absorption 18.036mM ferric chloride standard was mixed with 80mM sodium citrate at specified ratios and left for 30 minutes to equilibrate. Careful drop-wise addition of buffered MOPS pH=7.4, under

constant vortex-mixing, slowly (1-2min for each solution) increased the pH and then the solution was left to allow for species stabilisation. In the buffered solutions containing iron-to-citrate ratios of 1:3333, 1:1000, 1:333, 1:100, 1:33, 1:10, and 1:3.3, the ferric citrate species range from *predominantly* ferric dicitrate ($FeCit_2$, up to 1:100) through dimers (Fe_2Cit_2) and trimers (Fe_3Cit_3) and their stacks[13,14]. Because there exists a relationship between iron-to-citrate ratios in the solution and the predominant species present, i.e. that ratio determines the ferric citrate speciation, we also refer to the species by stating the relevant *causal* iron-to-citrate ratio, e.g. 1:100 species typically indicates a monomeric species such as the ferric dicitrate species $FeCit_2$, since under pH=7.4 it is the *predominant* form of ferric citrate present in 1:100 iron-to-citrate ratio solutions. We prepared ferric citrate solutions with constant citrate (at physiological concentration of 100μM). After species stabilization (i.e. at 24h from preparation), ferric citrate solutions were incubated in Claycomb medium with *confluent* HL-1 cardiomyocytes for 24 hours. When **ferric ammonium citrate (FAC)** was used, it was added as freshly prepared (from solids) to the experimental media. Freshly dissolved ferric ammonium citrate is a 1:2 Fe:citrate ratio ferric citrate (i.e. constant ratio ferric citrate) with inert ammonium cations in solution[15]. For the sake of simplicity we have not used albumin alongside ferric citrate to model NTBI.

Ferrotransferrin preparation

In order to produce diferric transferrin, apotransferrin was saturated using ferric nitrilotriacetate (Fe-NTA) in excess of transferrin binding (or to 95%) with subsequent 24h dialysis against 2L of PBS using 10,000 MWCO Slide-A-Lyzer Dialysis Cassette (Thermo Scientific) to remove excess iron and NTA. Transferrin saturation solutions were prepared by mixing thus obtained diferric transferrin with calculated amounts of MOPS-buffered apotransferrin in 25mM bicarbonate to obtain the nominal % saturation of transferrin. In this model of transferrin saturation, the monoferric transferrin species are not present, which may be considered a limitation of this model, however in our experiments we wished to address the nascence of apotransferrin which, unlike the monoferric species, is the consequence of uptake and recirculation of diferric (and monoferric) transferrin by the erythron[16].

The **viability** of HL-1 cardiomyocytes, in the presence of 0-30μM buffered ferric citrate ± 5μM apotransferrin, was 96.6±0.7%, as assessed by the electric current exclusion method on CASY Model TT Cell Counter and Analyzer (Roche).

Cellular iron assays

Total cellular iron was assayed by **ferrozine** using previously published methods[17]. Briefly, following incubation of cell monolayers in experimental media, cells were washed 3-4 times with PBS (including one wash with DFO- or CP40-containing PBS to remove membrane associated iron). Washed monolayers were then lysed with 200μL of 200mM NaOH and left overnight on a plate shaker. A 100μL aliquot of the lysate was added to 100μL of 100μM HCl (standard solution matrix) and treated with 100μL of a freshly prepared iron-extracting solution (1:1 vol/vol 5% $KMnO_4$/1.4M HCl) for 2h at 60°C under fume hood extraction. This was followed by the addition of 30μL of a freshly prepared iron-detecting solution, containing

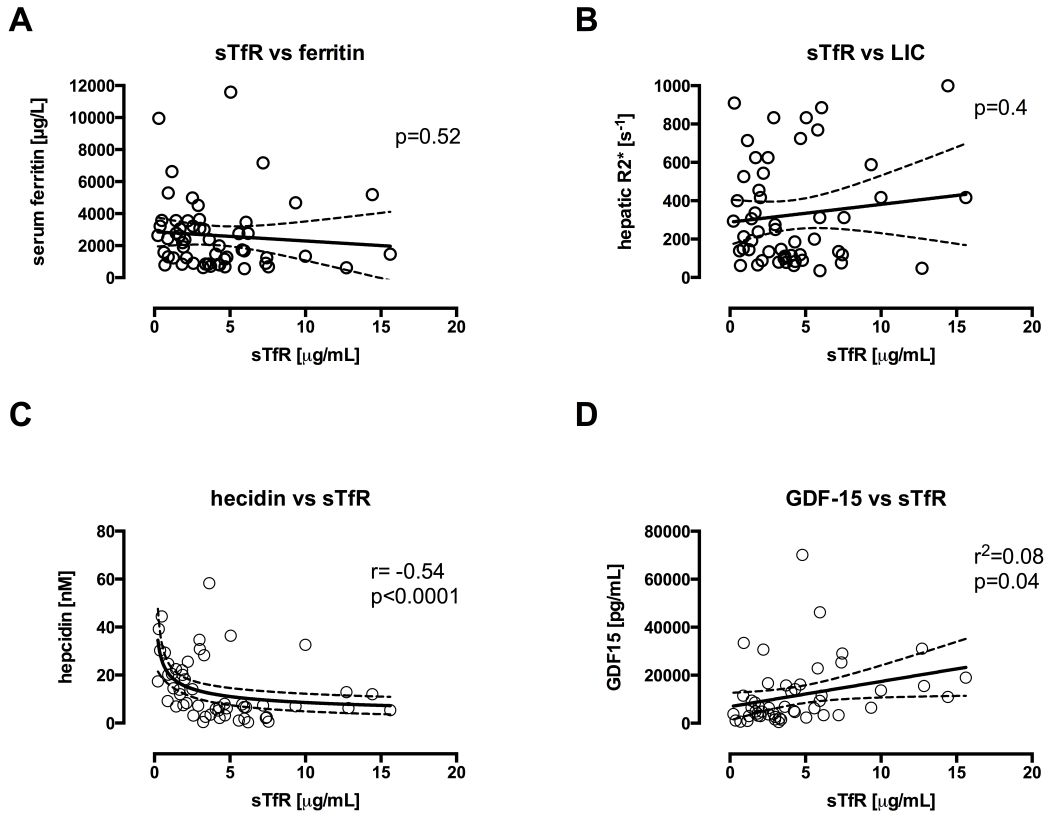
6.5mM ferrozine, 6.5mM neocuproine, and 1M ascorbic acid in 2.5M ammonium acetate, and the mixture was incubated for 30 minutes at room temperature on an orbital plate shaker. The absorbance of the ferrozine-iron complex was measured at 562nm using a plate-reader. Results were interpolated from a standard curve obtained using atomic absorption ferric chloride standards and 100µL of 200mM NaOH (lysate matrix) and reported as cell lysate iron concentration (µM).

Reactive Oxygen Species assay

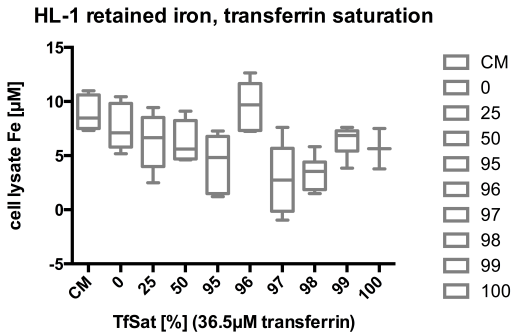
The intracellular reactive oxygen species (ROS) assay was performed using 2,7-dichlorofluorescein diacetate (H_2DCF-DA), which is de-esterified intracellularly by live cells to 2,7-dichlorofluorescein (DCF). 9µM H_2DCF-DA in DMSO/PBS was added to confluent HL-1 monolayers after PBS rinse and incubated for 30 min in 48-well plates at 37°C and 5% CO_2. Following three PBS rinses, 500µL PBS aliquot was added with 100µM CP40 or 5µM apotransferrin (final concentration). CP40 chelator was used as a control for extra-cellular chelation[18]. Immediately afterwards, ferric citrate, buffered in MOPS pH=7.4, was added to a final concentration of 0-30µM. Fluorescence was monitored for 60 minutes (excitation 493nm, emission 523nm). Slopes, shown as mean±SD, were calculated from fluorescence-versus-time data to represent the cytosolic ROS formation over 1h using linear regression.

The extracellular chelator CP40 was a kind gift from Professor Robert Hider, KCL. All chemicals were from Sigma-Aldrich apart from 0.05% trypsin-EDTA, L-glutamine, streptomycin and penicillin (Life Technologies), soybean trypsin inhibitor (Invitrogen), atomic absorption ferric chloride standard (Aldrich), ammonium acetate (Fisher) or if otherwise stated.

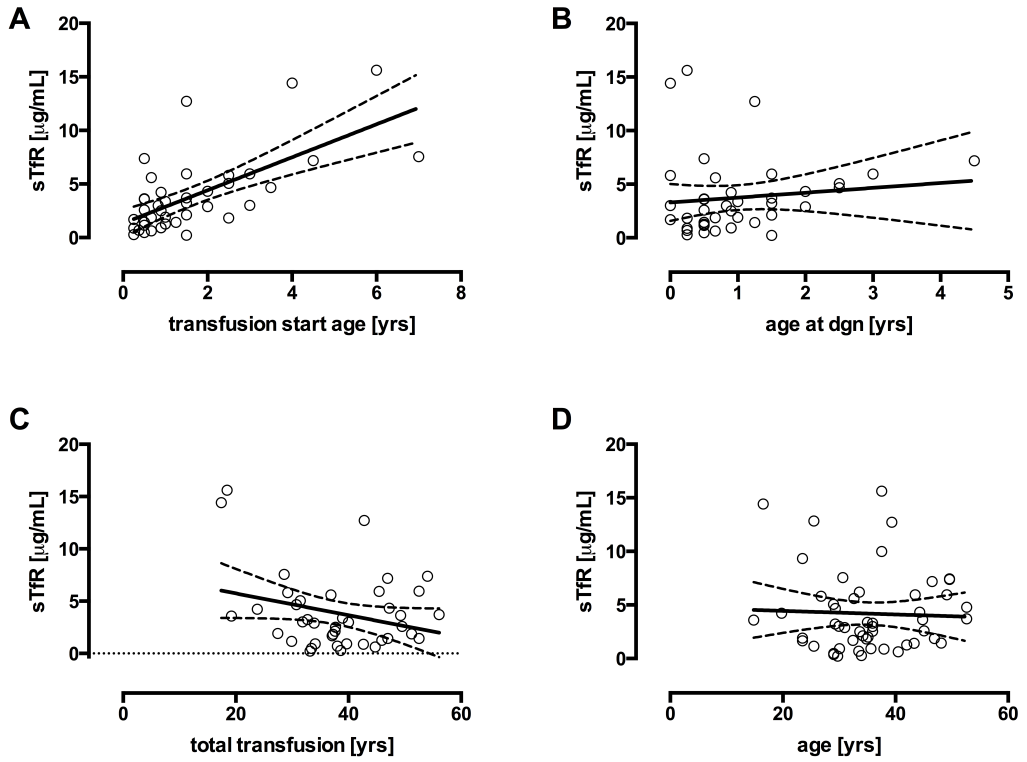
Supplemental Figures:



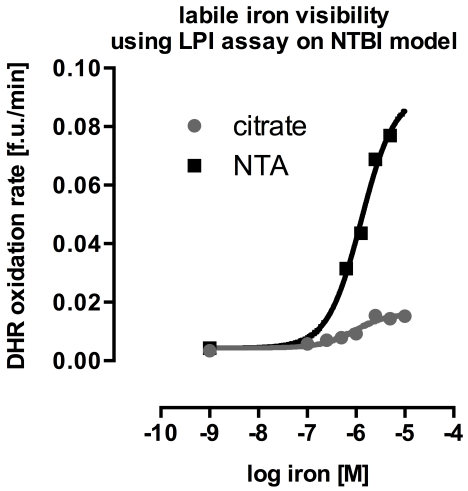
Supplement Figure 1. The correlations and regressions of soluble transferrin receptor-1 (sTfR1) with markers of iron overload, hepcidin and GDF-15.
(A) Relationship of serum ferritin and sTfR1, linear regression slope p=0.52. (B) Relationship of hepatic R2* with sTfR1, linear regression slope p=0.4. (C) Relationship of plasma hepcidin and sTfR1, Spearman correlation coefficient r= -0.54, p<0.0001; power series regression $y=20.06[\pm 2.09]x^{-0.37[\pm 0.09]}$. (D) Relationship of GDF-15 and sTfR1, linear regression slope p=0.04.



Supplement Figure 2. Dose response of iron retention in HL-1 cells to transferrin saturation.
Confluent HL-1 cardiomyocytes were incubated for 24h with Claycomb medium, containing 36.5µM transferrin concentration, with transferrin saturation obtained by mixing diferric transferrin to apotransferrin at specified ratios, mean±SD, n=6; see methods.



Supplemental Figure 3. The relationship of patient history factors to sTfR1 in TDT. (A) Regression of sTfR on the age when chronic transfusion was started, slope p<0.0001. (B) Regression of sTfR on age at diagnosis, slope p=0.47. (C) Regression of sTfR on the total duration of transfusion dependence, slope p=0.07. (D) Regression of sTfR on patient's age, slope p=0.77.



Supplemental Figure 4. Labile plasma iron for ferric nitrilotriacetate and ferric citrate species is shown.
Labile plasma iron (LPI) assay performed using 0-10µM ferric nitrilotriacetate and 0-10µM ferric citrate at constant citrate (100µM), mean±SD, n=2; see methods.

References

1. Cohen AR, Glimm E, Porter JB. Effect of transfusional iron intake on response to chelation therapy in β-thalassemia major. *Blood*. 2008;111(2):583–587.
2. Anderson LJ, Holden S, Davis B, et al. Cardiovascular T2-star (T2*) magnetic resonance for the early diagnosis of myocardial iron overload. *Eur. Heart J.* 2001;22(23):2171–2179.
3. Singh S, Hider RC, Porter JB. A direct method for quantification of non-transferrin-bound iron. *Anal. Biochem.* 1990;186(2):320–323.
4. Porter JB, Abeysinghe RD, Marshall L, Hider RC, Singh S. Kinetics of removal and reappearance of non-transferrin-bound plasma iron with deferoxamine therapy. *Blood*. 1996;88(2):705–13.
5. Esposito BP, Breuer W, Sirankapracha P, et al. Labile plasma iron in iron overload: Redox activity and susceptibility to chelation. *Blood*. 2003;102(7):2670–2677.
6. Lal A, Porter J, Sweeters N, et al. Combined chelation therapy with deferasirox and deferoxamine in thalassemia. *Blood Cells, Mol. Dis.* 2013;50(2):99–104.
7. de Swart L, Hendriks JCM, van der Vorm LN, et al. Second international round robin for the quantification of serum non-transferrin-bound iron and labile plasma iron in patients with iron-overload disorders. *Haematologica*. 2015;101(1):38–45.
8. Bansal SS, Halket JM, Fusova J, et al. Quantification of hepcidin using matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* 2009;23(11):1531–1542.
9. Evans RW, Williams J. The electrophoresis of transferrins in urea/polyacrylamide gels. *Biochem. J.* 1980;189(3):541–546.
10. Porter JB, Walter PB, Neumayr LD, et al. Mechanisms of plasma non-transferrin bound iron generation: Insights from comparing transfused diamond blackfan anaemia with sickle cell and thalassaemia patients. *Br. J. Haematol.* 2014;167(5):692–696.
11. Claycomb WC, Lanson N a, Stallworth BS, et al. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc. Natl. Acad. Sci. U. S. A.* 1998;95(6):2979–2984.
12. Grootveld M, Bell JD, Halliwell B, et al. Non-transferrin-bound iron in plasma or serum from patients with idiopathic hemochromatosis. Characterization by high performance liquid chromatography and nuclear magnetic resonance spectroscopy. *J. Biol. Chem.* 1989;264(8):4417–4422.
13. Evans RW, Rafique R, Zarea A, et al. Nature of non-transferrin-bound iron: Studies on iron citrate complexes and thalassemic sera. *J. Biol. Inorg. Chem.* 2008;13(1):57–74.
14. Silva AMN, Kong X, Parkin MC, Cammack R, Hider RC. Iron(III) citrate

speciation in aqueous solution. *Dalton Trans.* 2009;(40):8616–8625.

15. Matzapetakis M, Raptopoulou CP, Tsohos A, et al. Synthesis, Spectroscopic and Structural Characterization of the First Mononuclear, Water Soluble Iron−Citrate Complex, (NH4)5Fe(C6H4O7)2·2H2O. *J. Am. Chem. Soc.* 1998;120(10):13266–13267.

16. Huebers HA, Finch CA. The physiology of transferrin and transferrin receptors. *Physiol. Rev.* 1987;67(2):520–582.

17. Riemer J, Hoepken HH, Czerwinska H, Robinson SR, Dringen R. Colorimetric ferrozine-based assay for the quantitation of iron in cultured cells. *Anal. Biochem.* 2004;331(2):370–375.

18. Vlachodimitropoulou Koumoutsea E, Garbowski M, Porter J. Synergistic intracellular iron chelation combinations: Mechanisms and conditions for optimizing iron mobilization. *Br. J. Haematol.* 2015;170(6):874–883.