Supplemental Materials, Methods and Data

Production of recombinant hERFE

Freestyle 293F cells (Life Technologies) were grown in shaking flask (250 rpm) at 37C in a 5% CO2 humidified incubator to cell density 10^6/ml in 100 ml of FreeStyle 293 Expression medium, then transfected per manufacturer's instructions (Invitrogen Catalog # K9000-01) using 100 µg of rhERFE1 or rhERFE2 plasmid DNA and 200 µl 293fectin (Life Technologies). The transfected cells were reincubated in the shaking flask (250 rpm) at 37C in a 5% CO2 humidified incubator for 3-5 days in 100 ml FreeStyle 293 Expression medium supplemented with Protease Inhibitor Cocktail (Sigma) and the medium was collected. rhERFE1 was purified from supernatant using ion-exchange columns (Macro-prep, Biorad) and eluted by stepwise increasing concentrations of NaCl/ Na_2HPO_4 buffer (0.1 to 1M, pH 7.5). rhERFE2 was purified using an anti-FLAG M2 affinity gel according to the manufacturer's protocol (Sigma), eluting with 100 µg/ml FLAG peptide (Sigma). NaCl/ Na_2HPO_4 buffer and FLAG peptide were removed by filtration through Amicon Ultra 30K device (Millipore) and recombinant ERFE resuspended in saline (0.9% NaCl). The purified protein was electrophoretically heterogeneous, indicating posttranslational processing and multimerization characteristic of the TNFα-C1q family of proteins[1]. Predominant bands on reducing SDS-PAGE were at 52 kD and 26 kD. Antigen concentration was estimated by absorbance (1 mg/ml) at 280 nm = 0.57.

Human ERFE Immunoassay

96-well high binding plates (Costar #3590) were coated with Mab#9 diluted to 1 µg/ml in sodium carbonate buffer (50 mM pH 9.6), overnight at 4C. Plates were washed 3-times with TBS-T (TBS + 0.05% Tween 20) then blocked for 1 h at RT with 200 µl/well Blocking Buffer (BB: PBS,0.2% Na casein, 0.05% Tween 20, 0.1M NaCl). Recombinant hERFE2 standard was first diluted to 1 µg/ml and then serially diluted in BB to 10, 5, 2.5, 1.25 and 0.625 ng/ml. After a 1 h incubation at 25C in a shaker at 300 rpm, the plate was washed 4-times with TBS-T for 10sec, 25C, 300 rpm per wash and incubated 1 h at 25C, 300 rpm in shaker with 100 µl/well biotinylated Mab#42 (1 µg/ml in BB). After incubation for 1 hour at 25C, 300 rpm in a shaker, the plate was washed 4-times as before, incubated for 45 min with Neutravidin-HRP conjugate (ThermoScientific #31030) 1/5000 (100 µl/well) washed again 3-times as before and developed with 100 µl TMB Substrate System for ELISA (ThermoScientific #34028) at RT in the dark for 10 min. The reaction was stopped by adding 50 µl of 2N sulfuric acid and the plates were read on a Spectramax 250 (Molecular Devices) at 450 nm.

Sample stability and assay performance under realistic conditions

Three patient sera were selected for serial assays and aliquoted and either kept at 4C or frozen at -80C. On days 0, 1, 7, 14, 21 and 28, the samples were brought to room temperature and assayed in triplicate. An aliquot of each sample defrosted from -80C was kept at 4C for serial retesting through the remaining time points. CV% under these conditions were estimated on all assays of each sample, and then separately for samples kept at 4C and -80C. These CV% values are overestimates of assay variability because they also include the effects of storage.

Table I: Sample stability and assay variability over 1 month

Serum erythroferrone	Low	n	Medium	n	High	n
All mean (regardless of storage)	24.6	21	46.4	21	99.5	21
All %CV (regardless of storage)	28	21	22	21	20	21
4C mean	23.5	6	46.4	6	92.8	6
4C %CV	32	6	31	6	25	6
-80C mean	27.9	6	51.3	6	107.3	6
-80C %CV	29	6	20	6	19	6

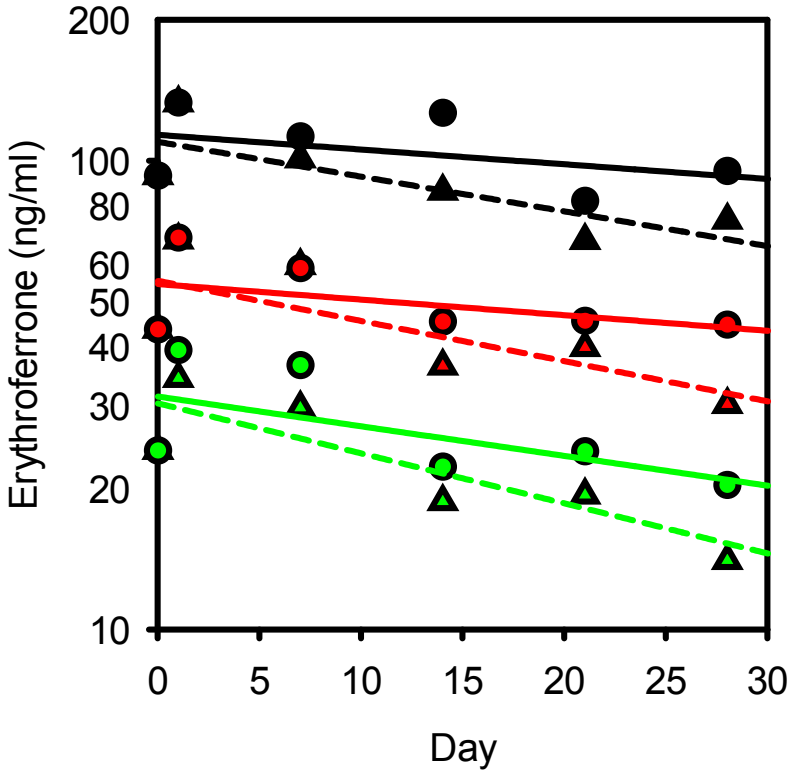

Figure S1: Time plot of measured ERFE values in aliquots of three samples stored at -80C (circles and solid lines) vs 4C (triangles and dashed lines). Lines represent exponential decay regression curves (note log scale on the vertical axis). Although 1 month is an insufficient period to robustly quantitate sample stability, samples stored at -80C are consistently more stable than those stored at 4C.

Interference

In selecting the monoclonal antibodies for the assay, we included a screen of potential interference by recombinantly produced and bioactive mouse ERFE (amino acid sequence 71% identical to human ERFE) immobilized on ELISA plates. Neither of the selected antibodies bound to mouse ERFE. In comparison, the closest human match to erythroferrone, adipolin, encoded by Fam132a, is only 37% identical, including a very low homology in the area of the known epitope of Mab #9. Moreover, adipolin is present in human sera at very low concentrations[2] (around 1

ng/ml) so clinically important interference is highly unlikely. More experience with the human ERFE ELISA will be needed to specifically identify potential sources of interference.

One example of interference has been noted after analyzing samples from more than 200 individuals. In this case, all samples from a single normal male blood donor gave high out–of–range readings even after dilution at 1:50. The addition of 2 mg/ml of rabbit IgG (Sigma) to all dilution buffers brought the values into normal range and revealed the same pattern of response to blood donation as in other blood donors (see Figure 1). The addition of rabbit IgG did not affect the readings on a previously tested control donor sample set. We attribute the interference in this donor's serum to circulating heterophilic human antibodies present in the donor's serum and directed against rabbit immunoglobulins, an uncommon cause of interference reported previously[3]. Such antibodies crosslink the two rabbit Mabs used in our ELISA regardless of the concentration of ERFE, giving a falsely increased reading but the interference can be eliminated by the addition of excess rabbit IgG.

Characteristics of blood donors of 2u of erythrocytes

Generally, only repeat donors whose blood passed all screening previously are accepted for this largest FDA-approved 2u erythrocyte apheresis. In the year prior to enrollment, the donors (all male) had an average of 2.4±0.2 whole blood equivalent donations. Baseline serum erythropoietin values were 14±1 vs 13±1 U/L at 112 days (P=0.43). These levels are normal at baseline and return to normal after donation, suggesting that the influence of the blood donation on erythropoiesis wears off by the time of the next donation, even if iron stores may or may not recover. The single first-time donor enrolled had similar baseline and 112 day serum erythropoietin as the repeat donors.

Supplemental References

1. Wang Y, Lam Karen SL, Yau M-h, Xu A. Post-translational modifications of adiponectin: mechanisms and functional implications. *Biochem J*. 2008;409(3):623-633.
2. Tan BK, Chen J, Hu J, et al. Circulatory changes of the novel adipokine adipolin/CTRP12 in response to metformin treatment and an oral glucose challenge in humans. *Clin Endocrinol (Oxf)*. 2014;81(6):841-846.
3. Hennig C, Rink L, Fagin U, Jabs WJ, Kirchner H. The influence of naturally occurring heterophilic anti-immunoglobulin antibodies on direct measurement of serum proteins using sandwich ELISAs. *J Immunol Methods*. 2000;235(1-2):71-80.