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Journal

Haematologica, 100(2)

ISSN

1466-4860

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Publication Date

2015-02-01

DOI

10.3324/haematol.2014.116723

Peer reviewed

A competitive enzyme-linked immunosorbent assay specific for murine hepcidin-1: correlation with hepatic mRNA expression in established and novel models of dysregulated iron homeostasis

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ABSTRACT

Mice have been essential for distinguishing the role of hepcidin in iron homeostasis. Currently, investigators monitor levels of murine hepatic hepcidin-1 mRNA as a surrogate marker for the bioactive hepcidin protein itself. Here, we describe and validate a competitive, enzyme-linked immunosorbent assay that quantifies hepcidin-1 in mouse serum and urine. The assay exhibits a biologically relevant lower limit of detection, high precision, and excellent linearity and recovery. We also demonstrate correlation between serum and urine hepcidin-1 values and validate the competitive enzyme-linked immunosorbent assay by analyzing plasma hepcidin response of mice to physiological challenges, including iron deficiency, iron overload, acute blood loss, and inflammation. Furthermore, we analyze multiple murine genetic models of iron dysregulation, including β-thalassemia intermedia (*Hbb*$^{th3/+}$), hereditary hemochromatosis (*Hfe*$^{-/-}$, *Hjv*$^{-/-}$, and *Tfr2*$^{Y245X/Y245X}$), hypotransferrinemia (*Trf*$^{hpx/hpx}$), heterozygous transferrin receptor 1 deficiency (*Tfrc*$^{+/-}$) and iron refractory iron deficiency anemia (*Tmprss6*$^{-/-}$ and *Tmprss6*$^{hem8/hem8}$). Novel compound iron metabolism mutants were also phenotypically characterized here for the first time. We demonstrate that serum hepcidin concentrations correlate with liver hepcidin mRNA expression, transferrin saturation and non-heme liver iron. In some circumstances, serum hepcidin-1 more accurately predicts iron parameters than hepcidin mRNA, and distinguishes smaller, statistically significant differences between experimental groups.

Introduction

Hepcidin is a 25-amino-acid, cysteine-rich peptide first described in human blood and urine.[1,2] Hepcidin expression is induced by iron loading and inflammation and suppressed by anemia and hypoxia.[3-5] Iron-dependent regulation of hepatocyte hepcidin expression requires multiple proteins that together "sense" plasma transferrin-bound iron and transmit a bone morphogenetic protein-dependent signal to increase hepcidin gene transcription.[6] The predominant physiological role of hepcidin is to regulate cellular iron egress through the iron exporter ferroportin present on the surface of macrophages of the reticuloendothelial system, duodenal enterocytes and hepatocytes.[7-9] Hepcidin binds ferroportin, causing its internalization and degradation.[10] By this mechanism, hepcidin regulates the absorption of dietary iron via duodenal enterocytes and recycling of hemoglobin-derived iron in macrophages. While most mammals including humans, sheep,[11] dogs,[12] and horses[11] have a single hepcidin gene, mice have two: *Hepc-1* and *Hepc-2*.[3] Hepcidin-2 expression responds to iron,[13] but does not appear to regulate iron metabolism, in contrast to hepcidin-1, as transgenic overexpression of hepcidin-2 does not lead to an iron-related phenotype.[14,15]

Due to the small, compact structure and poor immunogenicity of hepcidin, quantitative immunoassays for this compound have been difficult to develop. The current standard method to evaluate hepcidin-1 expression in mice is quantitation of liver mRNA transcripts by reverse transcriptase polymerase chain reaction (qPCR). An assay for hepcidin-1 protein in small quantities of plasma and urine would permit a more dynamic understanding of its biology in research and pre-clinical murine models by accurately quantifying the biologically active hepcidin hormone itself and allowing serial measurements in a single animal over time. While mass spectrometry protocols for mouse hepcidin-1 quantitation have recently been described,[16,17] there is no assay that is both widely accessible and validated in diverse experimental models. Here we describe a competitive enzyme-linked immunosorbent assay (C-ELISA) that sensitively and specifically measures murine serum and urine hepcidin-1. We compared the hepcidin-1 C-ELISA to qPCR assays in three settings: (i) experimental manipulation of dietary iron, anemia and inflammation, (ii) previously described transgenic models of iron overload and iron deficiency, and (iii) novel compound mutant transgenic mice. We not only demonstrate that hepcidin-1 can be analyzed reliably in small volumes, enabling serial sampling of individual mice,

 doi:10.3324/haematol.2014.116723
The online version of this article has a Supplementary Appendix.
Manuscript received on September 2, 2014. Manuscript accepted November 18, 2014.
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but that the C-ELISA correlates better with iron-related phenotypes and, in some cases, its greater accuracy at lower concentrations permits statistically significant differences to be discerned in small datasets.

Methods

Mouse hepcidin-1 enzyme-linked immunosorbent assay

Antibodies to mouse hepcidin-1 were prepared similarly to a previously described approach (*Online Supplementary Materials and Methods*).[5]

Effect of dietary iron

Serum samples were collected from three groups of 5-week old C57BL/6 male and female mice (n=8 per gender, per diet; n=48 total) fed a low iron diet (4 ppm), normal iron diet (300 ppm), or high iron diet (30,000 ppm; Harlan Teklad Custom Diets) over the preceding 4 weeks. Serum samples were frozen at -80°C until analysis.

Effect of acute blood loss

Six-week old C57BL/6 male and female mice (n=6 per gender, n=12 total) were maintained on a low iron diet (20 ppm) for 12 days after introduction to the vivarium. Urine was collected and mice were bled at baseline via the mandibular vein (≤200 μL). Mice were returned to a 20 ppm diet until second urine and blood samples (50 μL) were collected 72 h later. Serum was prepared and stored at -80°C until analysis.

Effect of inflammation

The same group of C57BL/6 animals employed in the phlebotomy experiment described above (8 weeks old) were divided into two groups of male and female mice (n=3 per gender per group, n=12). The experimental group was treated with lipopolysaccharide (LPS, *Escherichia coli* O111:B4; Sigma) by intraperitoneal injection (1 μg LPS/g body weight).[18] Controls (n=3 per gender) were treated with phosphate-buffered saline. Blood and urine samples (50 μL) were collected after dietary acclimation at baseline before LPS injection and at 24 h after treatment.

Animal husbandry and transgenic mouse models

All procedures were performed in accordance with protocols approved by the respective Institutional Animal Care and Use Committees (see *Online Supplementary Materials and Methods*).

Statistics

For all datasets, two-tailed Student *t*-tests (Microsoft Excel) were employed to determine statistical significance, which was considered present when $P<0.05$.

Results

Antibody specificity

We produced high-titer polyclonal antisera against mouse hepcidin-1 and employed the protein A purified fraction to develop a C-ELISA. Western blot analysis demonstrated that the antibodies bind both synthetic hepcidin-1 and a novel synthetic hepcidin-1-biotin conjugate (Peptides International, Louisville, KY, USA) employed in the C-ELISA with equal affinity (*Online Supplementary Figure S1*).

The murine genome contains a second hepcidin gene, *Hepc-2*, which encodes a protein with 68% amino acid identity.[14] To determine the specificity of the antibodies, we tested the ability of hepcidin-1 and hepcidin-2 to compete in solution with the hepcidin-1-biotin conjugate in the C-ELISA. We detected no interaction, even at a hepcidin-2 concentration of 1000 ng/mL (Figure 1A). We also assessed the cross-reactivity of rat hepcidin, which is 80% identical to mouse hepcidin-1, and found no response at concentrations up to 250 ng/mL, and only a slight response beyond that, up to 1000 ng/mL. Anti-hepcidin-1 antibodies also did not bind to human hepcidin-25. This lack of cross-reactivity enabled us to employ human plasma or urine spiked with mouse hepcidin-1 to assess the performance of the assay.

Performance of the competitive enzyme-linked immunosorbent assay

Standard curves were generated with 12 or 8 calibration points beginning at 1000 ng/mL and proceeding lower by serial 1:2 or 1:3 dilutions, respectively. The 12-point curve was used to evaluate the initial assay's characteristics, including specificity (Figure 1), but all subsequent data utilized an 8-point curve. This standard was acceptable according to criteria outlined by DeSilva *et al.*,[19] insofar as the accumulated back-calculated values from all curves have an absolute mean relative error ≤10% and a coefficient of variation (CV) ≤15% (Table 1). The lower limit of detection (LLOD) of synthetic hepcidin in the standard curve [defined by two standard deviations (SD) above the zero standard] was 0.18 ng/mL, and the dynamic range of the assay extends to 333 ng/mL with an average half-maximal effective concentration (EC_{50}) of 3.3 ng/mL (Table 1). The LLOD for the C-ELISA is 3.6 ng/ml (1.3 nmol/L) and 1.8 ng/mL (0.065 nmol/L) and the lower limit of quantitation (LLOQ) is 10.3 ng/mL (3.7 nmol/L) and 5.1 ng/mL (1.86 nmol/L) for 5% and 10% serum dilutions, respectively. Similarly, five SD above the zero standard defines the derived LLOQ for 5% and 10% serum dilutions. The upper limit of quantitation of the C-ELISA was 6666 ng/mL (2418 nmol/L) and 3333 ng/mL (1209 nmol/L), for 5% and 10% serum dilutions, respectively.

Five-percent human serum spiked with murine hepcidin-1 gave an average recovery of 100% (range, 93-107%) across the range of the standard curve (n=4). Similarly, in hepcidin-1-spiked 10% human urine, we observed an average percent recovery of 99% (range, 93-105%) demonstrating minimal interference (n=3). Recovery in 10% serum gave slight interference, manifested only at <4 ng/mL, as evidenced by a 30-40% over-recovery of hepcidin-1 in spiked samples (*data not shown*).

Precision was determined by assaying human serum spiked with hepcidin-1. The average intra-assay CV was 3.1% (range, 1.3-5.2%; n=4). The average inter-assay precision of these samples (n=4 independent assays) was 5.9% (range, 4.7-7.7%). Similarly, human urine spiked with hepcidin-1 was analyzed, and yielded an average intra-assay CV of 5.5% (range, 1.0-11.7%; n=3), and an average inter-assay CV (n=3) of 3.5% (range, 1.5-5.9%). The linearity of the assay was also tested in human serum and urine spiked with 1000 ng/mL hepcidin-1 diluted 1:4, 1:8, or 1:16 in buffer (n=3 each). Average recovery was 94% (range, 92-95%) and 108% (range, 107-112%) in serum and urine, respectively.

Serum hepcidin-1 response to dietary iron correlates with hepatic hepcidin mRNA expression

Serum hepcidin-1, ferritin, and iron parameters were

determined in male and female mice fed low (4 ppm), normal (300 ppm), and high (30,000 ppm) iron diets (n=8 for each sex and diet). As expected, we found that serum hepcidin-1, iron, transferrin saturation and ferritin increased in parallel with increasing dietary iron (Figure 1B, Table 2). Only in mice fed the 300 ppm diet was there a statistically significant difference between serum hepcidin levels between male and female animals (Table 2, Figure 1B). Plasma iron and transferrin saturation were significantly different between genders in mice fed normal and high iron diets (Figure 1B, Online *Supplementary Figure S2B*), while ferritin was significantly different between genders in mice fed low and high iron diets (Figure 1B). Combining data for both genders and all iron diets, there was a strong ($r = 0.881$) linear correlation between $\log_{10}$[hepcidin-1] and transferrin saturation (*Online Supplementary Figure S2C*). In all mice subjected to dietary iron modulation, serum hepcidin-1 and liver mRNA expression were strongly correlated ($r = 0.889$, Figure 1C, *Online Supplementary Figure S1A*).

Correlation of serum hepcidin with urinary hepcidin

In the absence of a reliable assay for murine hepcidin-1, it has been impossible to determine whether in mice, as in humans,[20-22] urinary and serum hepcidin levels are correlated. To answer this question, serial serum and urine samples were collected from male and female mice on days 9, 12, 13, and 14 (n=6 per day; 14 males and 10 females; n=24 total) as they acclimated to a low iron diet (20 ppm). We found a moderate correlation ($r = 0.688$) between serum hepcidin and urinary hepcidin levels normalized to urinary creatinine (*Online Supplementary Figure S2D*).

The effects of acute blood loss and inflammation

The abundance of liver hepcidin-1 mRNA decreases in response to anemia.[4] Following acclimation to a low iron diet (20 ppm), we induced acute anemia by bleeding male and female mice (n=3 per gender). Hepcidin-1 was measured in serum and urine after the initial bleed and again 3 days later. As expected, we found that serum and urinary hepcidin were suppressed 2.3- to 3.6-fold, respectively (Figure 1D).

Hepcidin is a type II acute-phase protein induced by the inflammatory cytokine, interleukin-6.[5] In order to mimic acute inflammation, groups of male and female mice were

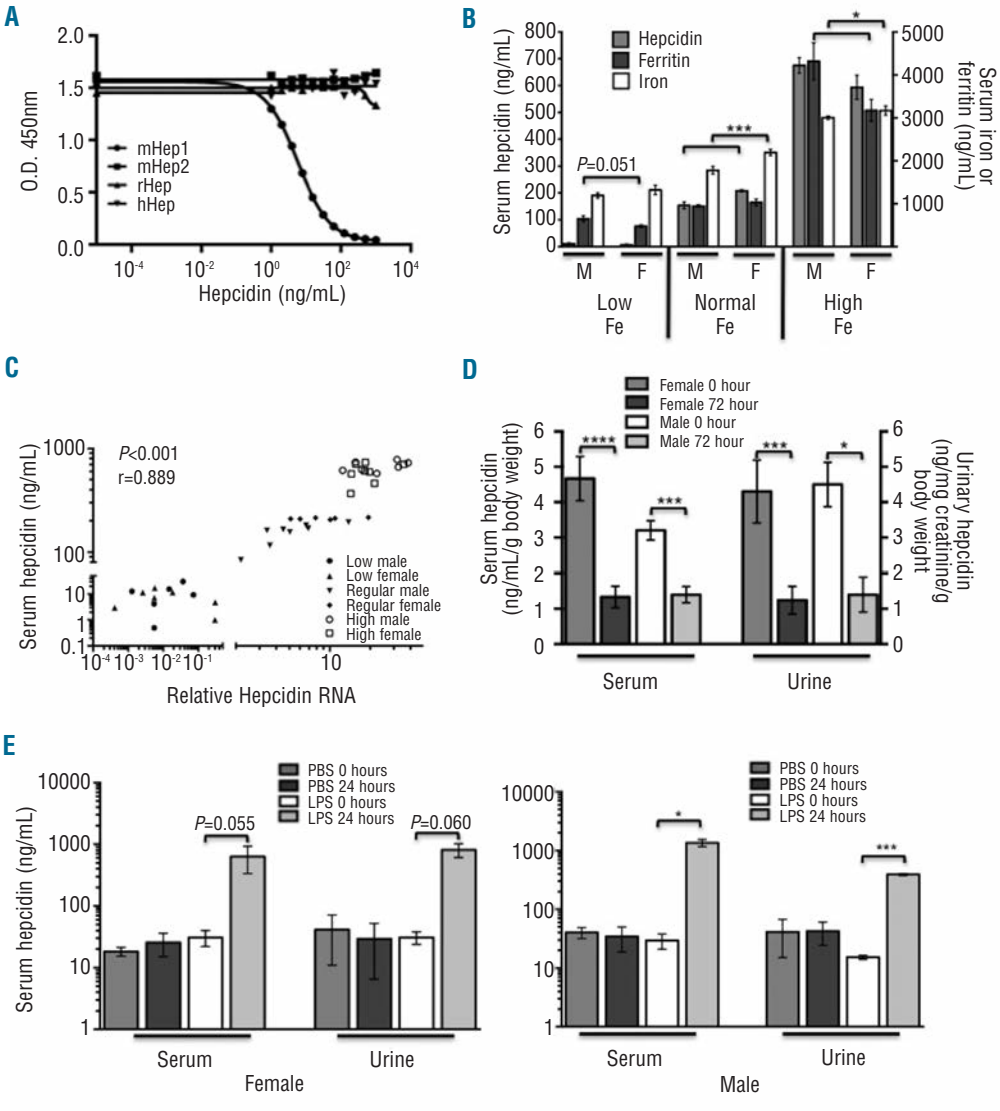

Figure 1. Characterization of a murine hepcidin-1 ELISA. (**A**) Specificity of murine immunoassay to hepcidin-1. A dose-response curve for mouse hepcidin-1 compared to conserved hepcidin peptides (mouse Hepcidin-2, rat hepcidin, and human hepcidin). The data depict 12-point standard curves serially diluted 1:1 starting from 1000 ng/mL. The X-axis is shown in log format. (**B**) Serum hepcidin-1 response to varying iron diets. Male and female mice were separately grouped and fed low (4 ppm Fe), normal (300 ppm Fe), or high (30,000 ppm Fe) iron diets (n=8). Serum was collected after 4 weeks and assessed for hepcidin-1, ferritin, and iron concentrations. (**C**) Correlation of serum hepcidin-1 by C-ELISA with hepcidin-1 mRNA from liver. Hepcidin-1 mRNA values were normalized to β-actin controls. (**D**) Serum and urine hepcidin-1 response to phlebotomy (n=6). (**E**) Serum and urinary hepcidin-1 response to inflammation. Female or male mice (n=3) were treated intraperitoneally with phosphate buffered saline (PBS) or 1 μg LPS/g body weight. Hepcidin-1 was unchanged in both serum and urine by PBS injections from time 0 to 24 h after injection. However, LPS challenge drastically increased hepcidin-1 in serum and urine from time 0 to 24 h after injection. Hepcidin (ng/mL) is represented on a log scale. Ratios are expressed ± SEM. *P* values were calculated using the Student t-test. ****$P<0.001$ ***$P<0.005$ and *$P<0.05$.

injected intraperitoneally with phosphate-buffered saline or LPS at a dose of 1 μg/g body weight.[18] Serum and urinary hepcidin-1 were measured immediately prior to injection and again 24 h later (Figure 1E). In both males and females, serum and urinary hepcidin-1 concentrations dramatically increased 24 h after exposure to LPS. For serum hepcidin, the average increase was 46-fold in males and 20-fold in females while the average increase in urinary hepcidin-1 was 26-fold in both genders.

Comprehensive re-analysis of genetic mouse models of dysregulated iron homeostasis with the hepcidin-1 competitive enzyme-linked immunosorbent assay

In order to further validate the murine hepcidin-1 C-ELISA assay, we compared its performance to that of qPCR of liver RNA by re-analyzing previously published cohorts of animals with genetic iron disorders. We evaluated serum hepcidin in the *Trf*$^{hpx/hpx}$ mouse model of hypotransferrinemia, which has profound iron overload due to a near complete absence of transferrin and severely suppressed hepcidin.[23,24] We also studied animals lacking the transmembrane serine protease Tmprss6, which are iron-deficient due to overexpression of hepcidin.[25-30] In *Trf*$^{hpx/hpx}$ animals we found serum concentrations of hepcidin (14.98 ± 2.0 ng/mL) at least 10-fold lower than in wild-type animals (Figure 2A). In contrast, despite their iron deficiency, *Tmprss6*$^{-/-}$ mice have hepcidin concentrations 2-fold higher than those of normal mice (Figure 2C). Animals heterozygous for a *Tmprss6* null allele (*Tmprss6*$^{+/-}$) demonstrate a liver iron phenotype intermediate between that of *Tmprss6*$^{-/-}$ and wild-type mice.[29] Here we demonstrate that these mice do not exhibit an intermediate level of hepcidin peptide or mRNA (Figure 2C,D), presumably as a consequence of compensatory regulatory events.[31] The hepcidin C-ELISA also enabled us to evaluate the effect of a *Tmprss6* missense mutation, *Tmprss6*^{hem8} (*Tmprss6*^{I286F}), more precisely. *Tmprss6*$^{hem8/hem8}$ mice have a slightly less severe phenotype than that of *Tmprss6*$^{-/-}$ animals.[32] Whereas there is no difference in expression of *Tmprss6* mRNA in these models,[32] serum hepcidin-1 concentrations were increased only about half as much in *Tmprss6*$^{hem8/hem8}$ animals as they were in *Tmprss6*$^{-/-}$ null mice (Figure 2C,E). This observation is concordant with activity of the mutant Tmprss6^{hem8} protein *in vitro*, where it has nearly half the enzymatic activity of the wild-type protein.[32] The *Tmprss6*$^{hem8/-}$ compound mutant mice have approximately the same hepcidin expression as *Tmprss6*$^{-/-}$ animals (Figure 2G).[32]

We evaluated serum hepcidin in the *Hbb*$^{th3/+}$ model of thalassemia intermedia and the *Hfe*$^{-/-}$ model of hereditary hemochromatosis. In both cases, hepcidin level measured by qPCR is suppressed relative to the extent of systemic iron overload.[33] We also examined serum hepcidin in each of these mutant strains following treatment with a lipid nanoparticle encapsulated short interfering RNA (siRNA) directed against *Tmprss6*, which ameliorates the thalassemia and hemochromatosis iron and anemia phenotypes.[33] We confirmed inappropriately low levels of serum hepcidin in iron-loaded mutants and induction of hepcidin by *Tmprss6* siRNA treatment (Figure 3A,C). By and large, in these and previous analyses of *Trf*$^{hpx/hpx}$ and *Tmprss6*$^{-/-}$ animals, the correlation between qPCR and serum hepcidin-1 analyses is strong (Figures 2B,D,H, and 3B,D). The Pearson correlation coefficients for each genetic cohort analyzed individually are listed in *Online Supplementary Table S1*.

We also re-examined hepcidin in a *Tfr2*$^{-/-}$ model of hereditary hemochromatosis,[34] in wild-type animals overexpressing a liver-specific *Hfe*-transgene,[35] and in *Tfr2*$^{-/-}$ animals carrying the same transgene. The latter two models are iron deficient, owing to *Hfe*-induced hepcidin overexpression. In contrast to experiments performed on animals with inbred genetic backgrounds, in these crosses, the serum hepcidin-mRNA correlation was not as robust (Figure 3E,F). Importantly, correlation of transferrin saturation (Figure 3G) and non-heme liver iron (Figure 3H) with hepcidin measured by C-ELISA was far better than with hepcidin measured by qPCR. This suggests that serum hepcidin may be more indicative of iron-related biological outcomes.

Serum hepcidin in novel compound mutant genetic mouse models of dysregulated iron metabolism

Previously, we and others proposed a model whereby HFE bound to transferrin receptor (TFRC) in human hepatocytes participates in regulating hepcidin production in response to plasma iron.[36] In this model, as saturation rises, and total body iron burden increases, diferric transferrin (Tf-Fe_2) competes HFE from TFRC, signaling for increased hepcidin expression. Conversely, as transferrin saturation diminishes, more HFE binds to TFRC and hepcidin production is reduced. To better understand the importance of the stoichiometry of the HFE/TFRC/Tf-Fe_2 complex, we bred mice lacking *Hfe* to animals heterozygous for a null mutation in the transferrin receptor (*Tfrc*$^{+/-}$). *Tfrc*$^{+/-}$ animals, regardless of *Hfe* status, were relatively anemic (*Online Supplementary Figure S3A,B*) and had smaller red blood cells (*Online Supplementary Figure S3C*), consis-

Table 1. Curve characteristics of the murine hepcidin-1 C-ELISA.

	N	Assay range, ng/mL	Average, %	Range, %
CV	6	1.4-330	6.7	3.7 - 11.9
RE	6	1.4-330	1.9	-1.3 - 4

CV: coefficient of variance; RE: relative error.

Table 2. Serum concentrations of hepcidin-1, ferritin and iron in male and female mice fed varying iron diets.

	Hepcidin ng/mL			Ferritin ng/mL				Serum Fe μM			
Iron diet	Male	Female	*P*	Male	Female	*P*	r*	Male	Female	*P*	r*
Low	10.4	7.1	ns	651.0	475.4	0.05	0.90	21.3	23.6	ns	0.92
Normal	153.6	208.0	0.003	944.0	1030.6	ns		31.8	39.3	0.003	
High	675.3	593.6	ns	4317.8	3174.8	0.05		53.8	59.1	0.02	

*n = 8 per gender per diet; ns = non-significant; Low diet (4 ppm Fe), Normal diet (300 ppm Fe), High diet (30,000 ppm Fe). *Correlation coefficient compared to hepcidin-1 values for both genders across all iron diets.*

tent with reduced erythroid iron availability. We found that animals lacking Hfe have elevated transferrin saturation and non-heme liver iron and diminished non-heme spleen iron concentrations, whether or not they are heterozygous for a *Tfrc* null allele (*Hfe*$^{-/-}$ or *Hfe*$^{-/-}$ *Tfrc*$^{+/-}$ genotypes, Figure 4A-C). Accordingly, liver hepcidin mRNA measured by qPCR and serum hepcidin levels measured by C-ELISA were decreased (Figure 4D,E). Loss of one allele of *Tfrc* promoted hepcidin expression, but there was no corresponding change in iron parameters (Figure 4A-E). In *Tfrc*$^{+/-}$ mutants, liver hepcidin mRNA and serum hepcidin concentrations were concordant (Figure 4F). Furthermore, transferrin saturation and non-heme liver iron measurements correlated better with serum hepcidin than with hepcidin-1 mRNA expression (Figure 4G,H).

It has been postulated that HFE and TFR2 also function in a complex with hemojuvelin (HJV), a bone morphogenetic protein co-receptor, to modulate hepcidin expression in relation to serum transferrin saturation.[37] To determine whether loss of either HFE or TFR2 protein had an additive effect on iron overload in the absence of HJV, we bred *Tfr2*$^{-/-}$ and *Hfe*$^{-/-}$ animals to *Hjv*$^{-/-}$ animals. Mice lacking either *Hjv* or *Tfr2*, or both genes had significantly elevated transferrin saturation and non-heme liver iron, as well as decreased non-heme spleen iron (Figures 5A-C), congruent with the low hepcidin expression that we observed in both qPCR and C-ELISA assays (Figures 5D-F). Importantly, a statistically significant difference between animals lacking Tfr2, Hjv or both proteins was detected only with the serum assay, possibly, once again, due to inherent variability in the qPCR-based assay. Furthermore, serum hepcidin correlated better than qPCR with transferrin saturation (Figure 5G) and non-heme liver iron (Figure 5H). Likely reflective of their fully replete iron status, mice lacking Tfr2, Hjv or both proteins had significantly elevated hemoglobin, hematocrit, and mean corpuscular volume compared to wild-type animals (Online Supplementary Figure 4A-C). Interestingly, the mean corpuscular volume was disproportionately increased in *Tfr2*$^{-/-}$ mice, possibly relating to its proposed intrinsic effect on erythropoiesis.[6]

As expected, in animals lacking Hfe or Hjv or both proteins, transferrin saturation and liver non-heme iron were greatly increased (Figure 6A,B), while non-heme spleen iron was decreased (Figure 6C). Commensurate with the smaller decrease in serum hepcidin (Figure 6D,E), in general, *Hfe*$^{-/-}$ mice had a less severe phenotype than that of either the *Hjv*$^{-/-}$ null or *Hjv*$^{-/-}$ *Hfe*$^{-/-}$ compound null mutants. The two analytical methods correlated well (Figure 6F),

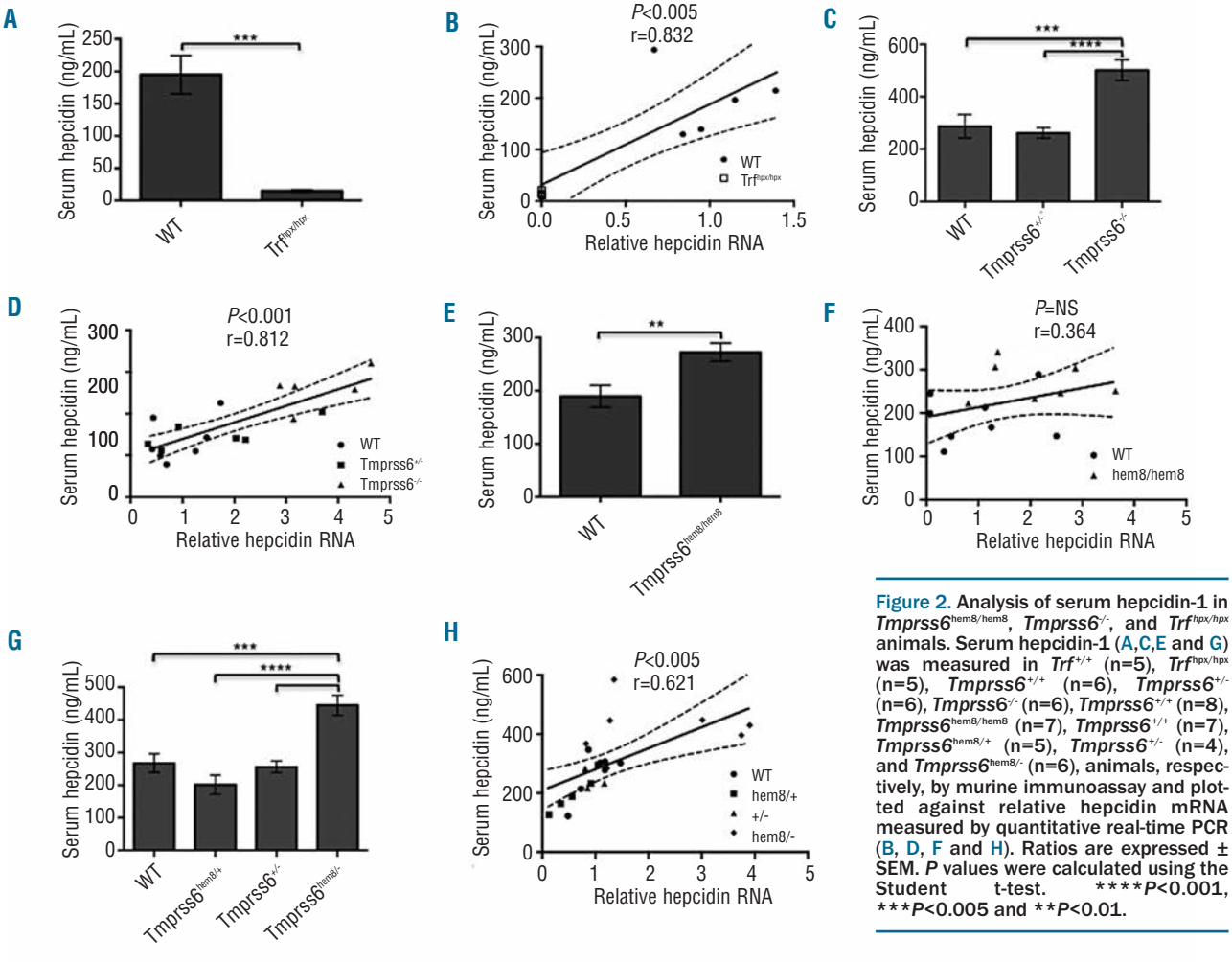

Figure 2. Analysis of serum hepcidin-1 in *Tmprss6*$^{hem8/hem8}$, *Tmprss6*$^{-/-}$, and *Trf*$^{hpx/hpx}$ animals. Serum hepcidin-1 (A,C,E and G) was measured in *Trf*$^{+/+}$ (n=5), *Trf*$^{hpx/hpx}$ (n=5), *Tmprss6*$^{+/+}$ (n=6), *Tmprss6*$^{+/-}$ (n=6), *Tmprss6*$^{-/-}$ (n=6), *Tmprss6*$^{+/+}$ (n=8), *Tmprss6*$^{hem8/hem8}$ (n=7), *Tmprss6*$^{+/+}$ (n=7), *Tmprss6*$^{hem8/+}$ (n=5), *Tmprss6*$^{+/-}$ (n=4), and *Tmprss6*$^{hem8/-}$ (n=6), animals, respectively, by murine immunoassay and plotted against relative hepcidin mRNA measured by quantitative real-time PCR (B, D, F and H). Ratios are expressed ± SEM. *P* values were calculated using the Student t-test. ****$P<0.001$, ***$P<0.005$ and **$P<0.01$.

but C-ELISA measurements correlated better than qPCR with both transferrin saturation and non-heme liver iron (Figure 6G,H). As in all murine hereditary hemochromatosis models, hemoglobin and hematocrit were elevated in mice lacking either Hfe or Hjv or both proteins (*Online Supplementary Figure S5A-C*).

Discussion

We have developed and comprehensively validated a sensitive, robust C-ELISA to accurately measure serum hepcidin-1 in 2.5-10.0 μL of murine serum and urine with no cross-reactivity with mouse hepcidin-2. The assay has high sensitivity with a LLOD of 3.6 ng/mL and a wide dynamic range – up to 6666 ng/mL using a 5 μL serum sample (5% serum). With 5% serum and urine, mean recovery of spiked synthetic hepcidin-1 peptide was 100% and 99% (range, 93-107%), respectively. Spike recovery of synthetic hepcidin-1 in 5% serum yielded an average recovery of 93% at a serum hepcidin concentration of 1.4 ng/mL. Decreasing sample volume to 2.5% decreased the sensitivity of the assay at the lowest concentrations (LLOD 7.2 ng/mL), while 10% serum greatly increased the assay's sensitivity (LLOD 1.8 ng/mL) but introduced some interference that affected spike recovery at concentrations below 4 ng/mL. The C-ELISA allows accurate, linear hepcidin-1 quantitation in a range of sample volumes with 5% serum being optimal.

In both serum and urine, the average values of the CV of

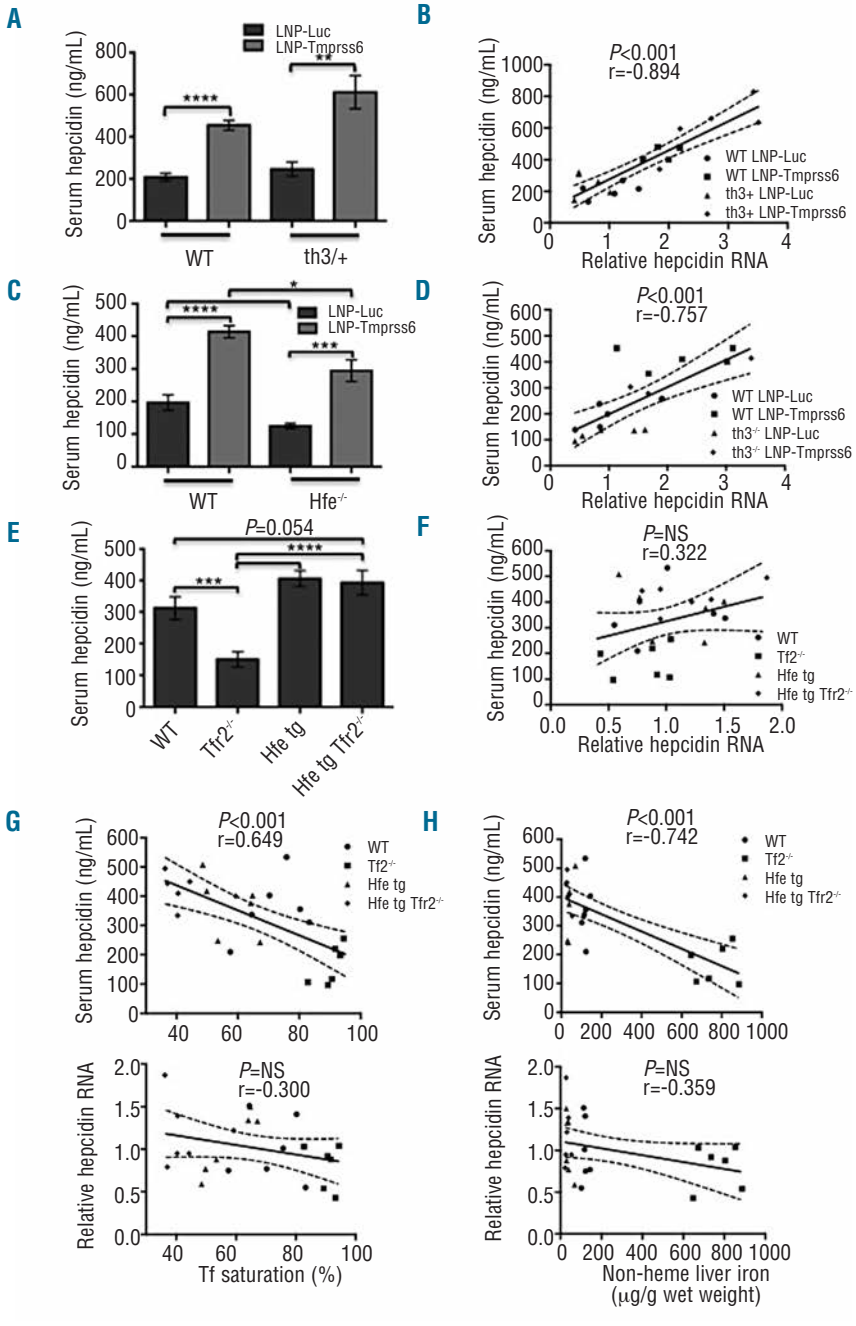

Figure 3. Comparison of hepcidin measured by C-ELISA and qPCR in mouse models of thalassemia intermedia and hereditary hemochromatosis. (A-D) Mouse models of thalassemia-intermedia (*Hbb*$^{th3/+}$) and hereditary hemochromatosis (*Hfe*$^{-/-}$) were treated with lipid nanoparticle formulated Tmprss6 siRNA (LNP-Tmprss6) or a luciferase control (LNP-Luc) as published previously (n=5 for each treatment group).[33] (A,C) Serum hepcidin-1 was measured with the murine immunoassay. (B,D) Total mRNA was harvested from liver and hepcidin-1 assessed by quantitative real-time PCR, normalized to β-actin (*Actb*) and plotted *versus* serum hepcidin. (E) Serum hepcidin-1 was measured in wild-type (WT), *Tfr2*$^{-/-}$, *Hfe*^{WT} transgenic or *Tfr2*$^{-/-}$ *Hfe*^{WT} transgenic livers (n=6 for each genotype)[35] and (F) total mRNA was harvested from liver and compared as above. (G) Serum and quantitative real-time PCR measured hepcidin-1 levels were compared to % serum transferrin saturation and (H) non-heme liver iron (μg/g wet weight). Ratios are expressed ± SEM. *P*-values were calculated using the Student *t*-test. ****$P<0.001$, ***$P<0.005$ and **$P<0.01$.

precision were under 6%, an accepted norm for diagnostic assays.[19] Although we did not systematically assess plasma as a substrate, based on past experience with comparable human C-ELISA data and limited preliminary data, the assay should perform well in lithium heparin prepared plasma (TBB, *unpublished data*). Nevertheless, we will have to evaluate this biological matrix more systematically. The results from a broad range of murine models exhibiting dysregulated iron homeostasis demonstrate that the C-ELISA is sensitive and robust with a broad reportable range in singleton assays employing 5% serum or urine (*i.e.*, 1:20 dilutions). The small sample volumes of serum or urine required for hepcidin measurement using the C-ELISA permits serial sampling with minimal secondary physiological effects, enabling true longitudinal assessment of individual mice, and reducing the numbers of animals required to perform experiments examining serum hepcidin concentrations over hours or months. This also has the important added benefit for pre-clinical research of mitigating the biological variability inherent in current "longitudinal" PCR experiments involving cohorts of animals killed at serial time points for determination of liver mRNA.

At present, the standard method to detect changes in hepcidin expression relies on qPCR of liver extracts.[13] Like the C-ELISA, mass spectrometry measurements from mouse[17] and human tissue culture cells[38] have shown high degrees of correlation with liver transcripts. However, contrary to our observations, in these mouse studies, there was no correlation with serum iron. Furthermore, one

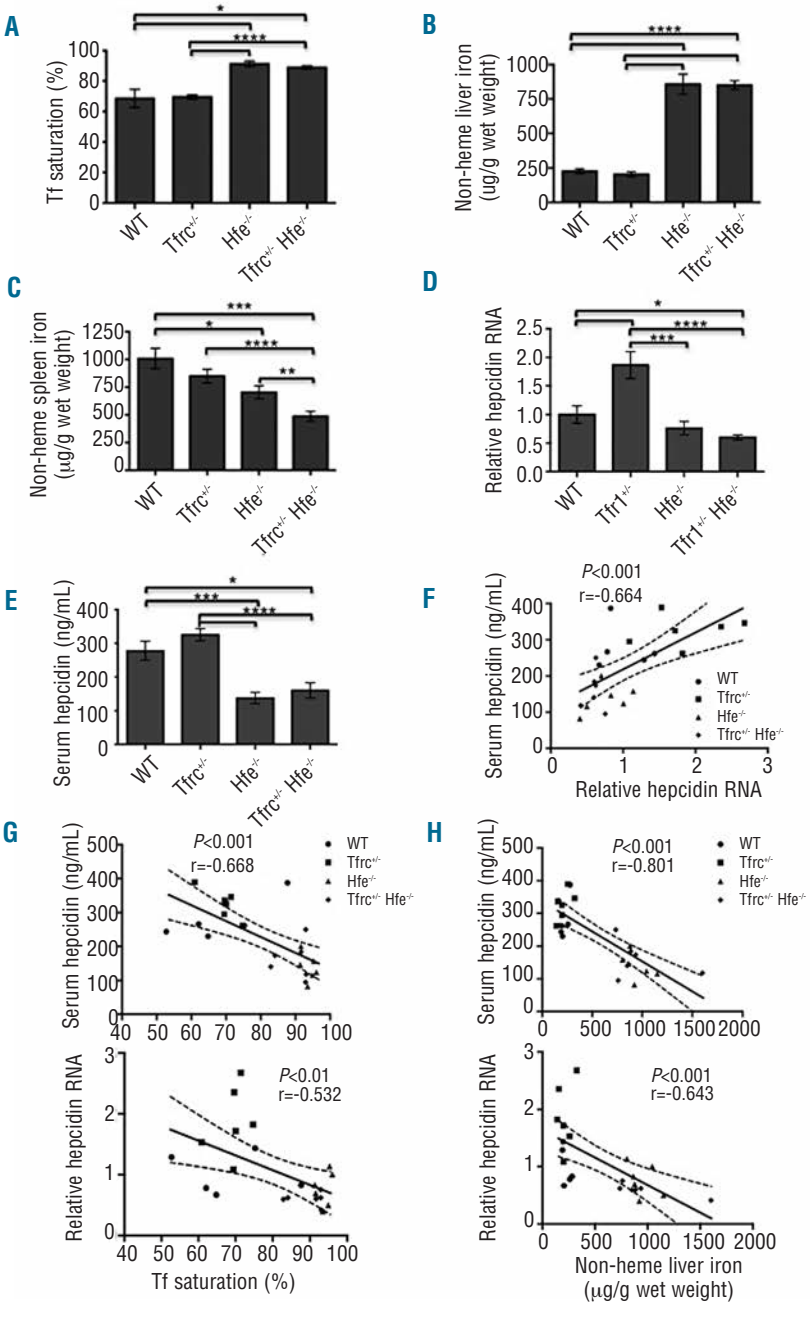

Figure 4. Phenotypic analysis of *Tfrc*$^{+/-}$ *Hfe*$^{-/-}$ mice. Analysis of (A) serum transferrin (Tf) saturation (%), (B) non-heme liver iron and (C) spleen iron (µg/g wet weight). Results for WT (*n*=5), *Tfrc*$^{+/-}$ (*n*=9), *Hfe*$^{-/-}$ (*n*=11), and *Tfrc*$^{+/-}$ *Hfe*$^{-/-}$ (*n*=11) animals are depicted. Total mRNA was harvested from livers (n=5 or 6 for each genotype) and hepcidin (*Hamp*) mRNA (D) was assessed by quantitative real-time PCR, and normalized to β-actin (*Actb*). Serum hepcidin-1 (E) was measured by murine immunoassay and plotted against (F) relative hepcidin mRNA measured by quantitative real-time PCR. (G) Serum and quantitative real-time PCR measured hepcidin-1 levels were compared to % serum transferrin saturation and (H) non-heme liver iron (µg/g wet weight). Ratios are expressed ± SEM. *P* values were calculated using the Student *t*-test. ****$P<0.001$, ***$P<0.005$, **$P<0.01$ and *$P<0.05$.

mass spectrometry assay failed to detect hepcidin-1 in mouse urine.[39] In contrast, our C-ELISA clearly demonstrates the presence of hepcidin-1 present in murine urine under steady state and pro-inflammatory conditions. It is possible that pre-analytical peptide enrichment steps required for the mass spectrometry assay may selectively isolate hepcidin-1 or hepcidin-2 in a matrix-dependent manner.[17] As such, this is the first report of concordance between murine serum and urinary hepcidin-1 levels, comparable to that observed using C-ELISA[20] or mass spectrometry methodologies in humans.[21,22] Overall, we observed a correlation coefficient of $r = 0.69$ for serum and urinary hepcidin-1 from combined gender sample sets. Taken separately, male and female mice also had similar degrees of correlation (*data not shown*). However, a more complete understanding of effects of gender and genetic background is required to fully appreciate the interplay between serum and urinary hepcidin, an avenue of inquiry now easily permitted by the availability of this assay.

Using this assay, we have reaffirmed several key principles of hepcidin regulation in mice based purely on mRNA expression data. For example, changes in erythropoietic activity have been shown to modulate liver hepcidin mRNA expression during anemia in an erythropoietin- and bone marrow activity-dependent manner.[40-42] Phlebotomy-induced acute anemia reduced hepcidin-1 liver mRNA.[4] Concordant with these data, our immunoassay measured a 2.3- to 3.6-fold decrease in hepcidin-1 in males and females, in serum and urine 3 days after blood collection. Furthermore, as a type II acute-phase protein

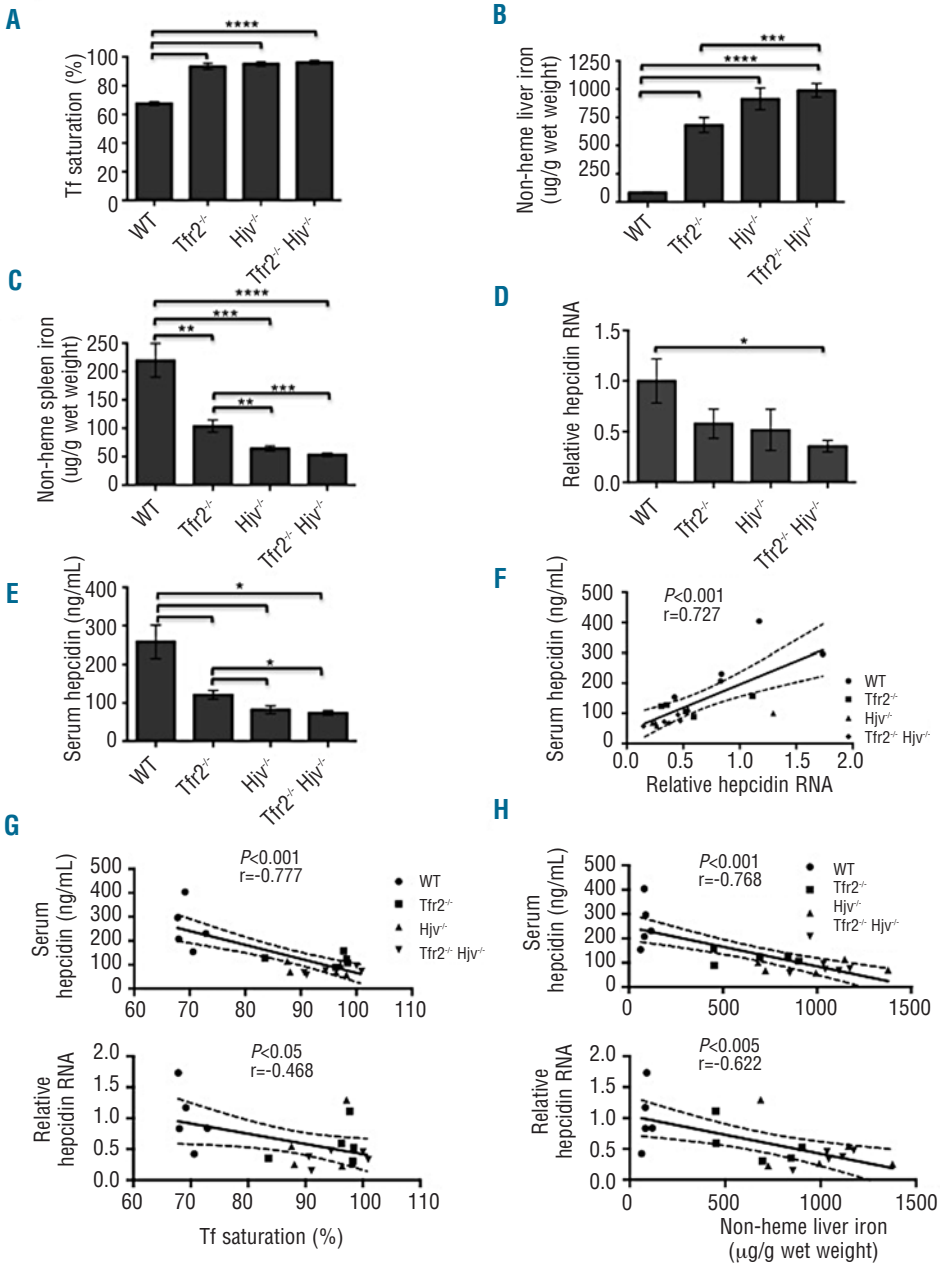

Figure 5. Phenotypic analysis of *Tfr2*$^{-/-}$ *Hjv*$^{-/-}$ mice. Analysis of (A) serum transferrin saturation (%), (B) non-heme liver iron and (C) spleen iron (µg/g wet weight). WT (n=8), *Tfr2*$^{-/-}$ (n=7), *Hjv*$^{-/-}$ (n=8) and *Tfr2*$^{-/-}$ *Hjv*$^{-/-}$ (n=8) are depicted. Total mRNA was harvested from livers (n=5 for each genotype) and hepcidin (*Hamp*) mRNA (D) was assessed by quantitative real-time PCR, and normalized to β-actin (*Actb*). Serum hepcidin-1 (E) was measured by murine immunoassay and plotted (F) against relative hepcidin mRNA measured by quantitative real-time PCR. (G) Serum and quantitative real-time PCR measured hepcidin-1 levels were compared to % serum transferrin saturation and (H) non-heme liver iron (µg/g wet weight). Ratios are expressed ± SEM. *P* values were calculated using the Student *t*-test. . ****P<0.001, ***P<0.005, **P<0.01 and *P<0.05.

responsive to interleukin-6,[5,43] hepcidin increased 4-fold following LPS injection as measured by northern blot analysis of liver RNA.[3] With 10-times the LPS dosage, we observed a hepcidin-1 increase of 46-fold in serum from males and 20-fold in serum from females and urine from animals of both genders.

We previously demonstrated that suppression of *Tmprss6* mRNA in rodent models of β-thalassemia intermediate (*Hbb*$^{th3/+}$) and classical hereditary hemochromatosis (*Hfe*$^{-/-}$) enhances hepatic hepcidin expression and ameliorates the thalassemia and hemochromatosis phenotypes.[33] Although qPCR analysis of liver mRNA demonstrated a significant increase in hepcidin expression in both models, there was considerable variability, especially at the lower values measured in *Hfe*$^{-/-}$ animals. However, C-ELISA analysis of hepcidin in *Hfe*$^{-/-}$ serum samples demonstrated a greatly increased significance between treatment groups. We also showed that transgenic overexpression of Hfe, even in animals with a truncated, non-functional Tfr2,[35] leads to overexpression of hepcidin and hypochromic, microcytic anemia. In this case, qPCR analysis of liver hepcidin showed a downward trend of expression in *Tfr2*$^{-/-}$ mice and a trend toward increased expression in Hfe transgenic animals. By contrast, C-ELISA analysis of these same cohorts of animals revealed unequivocally diminished hepcidin expression in animals lacking Tfr2 and elevated serum hepcidin in transgenic Hfe mice. Furthermore, comparison of serum hepcidin concentration to both transferrin saturation and non-heme liver iron revealed a close correlation between hepcidin production and metrics of iron metabolism; correlations between qPCR of liver hepcidin mRNA and these parameters are less informative. Taken together,

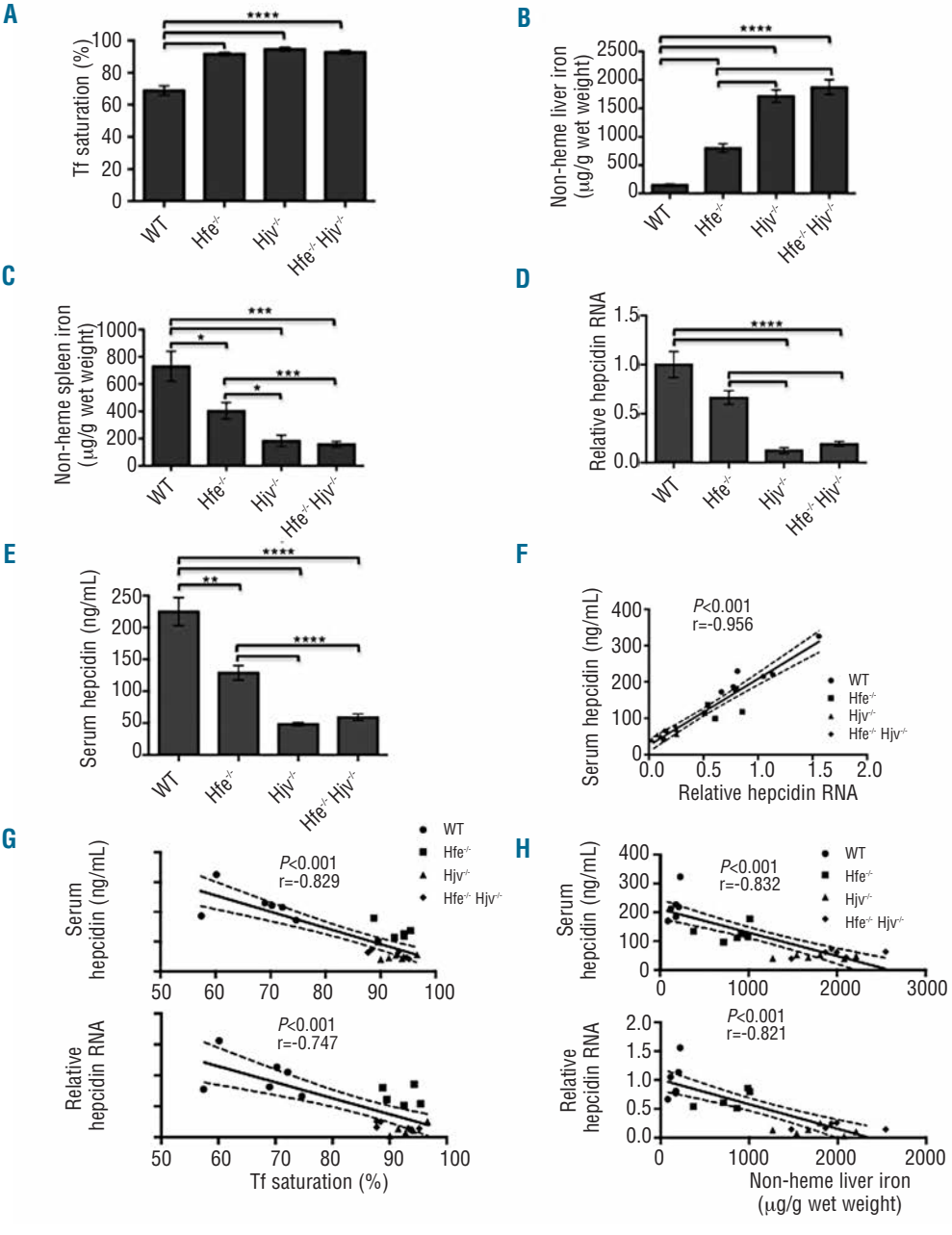

Figure 6. Phenotypic analysis of *Hfe*$^{-/-}$ *Hjv*$^{-/-}$ mice. Analysis of (A) serum transferrin (Tf) saturation (%), (B) non-heme liver iron and (C) spleen iron (μg/g wet weight). WT (*n*=7), *Hfe*$^{-/-}$ (*n*=8), *Hjv*$^{-/-}$ (*n*=8) and *Hfe*$^{-/-}$ *Hjv*$^{-/-}$ (*n*=10) animals are depicted. Total mRNA was harvested from livers (*n*=6 for each genotype) and hepcidin (*Hamp*) mRNA (D) was assessed by quantitative real-time PCR, and normalized to β-actin (*Actb*). Serum Hepc-1 (E) was measured by murine immunoassay and plotted against (F) relative hepcidin mRNA measured by quantitative real-time PCR. Serum and quantitative real-time PCR measured hepcidin-1 levels were compared to serum transferrin saturation (G,%) and non-heme liver iron (H, μg/g wet weight). Ratios are expressed ± SEM. *P* values were calculated using the Student *t*-test ****P<0.001, ***P<0.005, **P<0.01 and *P<0.05.

these retrospective analyses demonstrate that this hepcidin C-ELISA is a more sensitive, accurate and precise method that permits the distinction of finer differences in serum hepcidin concentrations otherwise not detected by mRNA expression analyses.

In support of the prevailing hypothesis that Tf-Fe_2 displaces HFE from TFRC to positively regulate hepcidin expression, we previously demonstrated that mutations in TFRC leading to constitutive association with or disassociation from HFE lead to hepcidin-related iron overload and anemia, due to hepcidin suppression or expression, respectively.[36] Similarly, we demonstrate that loss of one allele of *Tfrc* leads to elevated serum hepcidin and red blood cell parameters compatible with cellular iron deficiency. Whether this is a primary defect in hepatocellular hepcidin regulation or a secondary effect from defective red blood cell precursor iron uptake (or a combination of both) is uncertain. However, since concomitant homozygous loss of HFE and heterozygous loss of TFRC (*Hfe*$^{-/-}$ *Tfrc*$^{+/-}$ genotype) leads to iron overload and similarly iron-deficient red blood cell parameters and anemia, at least part of the effect is intrinsic to the red cell precursor.

Furthermore, it has been postulated that HFE and TFR2 function in a complex with HJV to modulate hepcidin expression.[37] To explore the interrelationship of HJV, HFE and TFR2 with hepatic hepcidin expression, we bred *Tfr2*$^{-/-}$ and *Hfe*$^{-/-}$ lines to *Hjv*$^{-/-}$ animals. We reaffirmed the finding that loss of HJV leads to greatly diminished hepcidin expression and iron overload.[44] More importantly, we show here that loss of Hfe or Tfr2 in combination with loss of Hjv does not make the iron-loading phenotype more severe, further cementing the hypothesis that HFE and TFR2 function upstream of HJV to regulate hepcidin expression and, subsequently, iron metabolism. We clearly showed a significant decrease of serum hepcidin in *Tfr2*$^{-/-}$ *Hjv*$^{-/-}$ double nulls compared to *Tfr2*$^{-/-}$ single null animals using this C-ELISA assay, an effect that was not observed by qPCR. These data collectively demonstrate the value of assessing serum hepcidin with this C-ELISA compared to qPCR. The C-ELISA can distinguish small differences in hepcidin mRNA expression by detecting low nanogram concentrations and changes in hepcidin-1, permitting more subtle interpretations of results in mouse models of hepcidin dysregulation.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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