University of Milan

Doctoral School in Molecular Medicine

A HEPCIDIN INHIBITOR MOBILIZES IRON FOR INCORPORATION INTO RED BLOOD CELLS IN AN ADENINE-INDUCED KIDNEY DISEASE MODEL IN RATS

PHD STUDENT: Valentina Vaja

TUTOR: Professor Maria Domenica Cappellini

CO-TUTOR: Doctor Jodie Babitt

PHD SCHOOL DIRECTOR: Professor Mario Clerici

ABSTRACT

Anemia is prevalent in patients with chronic kidney disease (CKD) and is primarily due to a complex interplay of relative erythropoietin deficiency, shortened red blood cell survival and abnormalities in iron homeostasis. A key feature in many patients with anemia of CKD is the limited iron availability for an efficient erythropoiesis despite adequate body iron stores. It is now well established that excess levels of the iron regulatory hormone hepcidin are responsible for downregulating the functional expression of the cellular iron exporter, ferroportin, thereby resulting in a blockade of iron absorption from the diet and iron retention in reticuloendothelial macrophage stores.

Adenine treatment in rats has been proposed as an animal model of anemia of CKD with high hepcidin levels that mirrors the condition in patients.

We developed a adenine-induced renal failure model in rats that simulates the renal failure and anemia condition in patients. We modified the Yokozawa et al. model by giving a diet supplemented with 0.75% adenine for 3 weeks followed by adenine free normal diet for another 3 weeks.

We then tested whether the small molecule bone morphogenetic protein (BMP) inhibitor LDN-193189, which has previously been shown to lower hepcidin levels, was able to mobilize iron into the plasma and improve iron-restricted erythropoiesis in adenine-treated rats.

The modified adenine model had a higher survival rate than previously reported models, while maintaining irreversible renal failure and anemia. We demonstrated that adenine rats had increased hepatic hepcidin mRNA levels, decreased serum iron concentration, increased spleen iron content, low hemoglobin levels and inappropriately low EPO levels relative to the degree of anemia, typical of the clinical condition in patients with anemia of CKD.

LDN-193189 lowered hepatic hepcidin mRNA and mobilized stored iron into plasma in adenine-treated rats. Moreover, the iron was efficiently incorporated into hemoglobin in reticulocytes. However, LDN-193189 alone did not prevent anemia progression in our model.

Lowering hepcidin improved iron availability, but did not improve anemia in an adenine-induced kidney disease model in rats. Co-administration of hepcidin lowering agents with erythropoiesis stimulating agents (ESAs) may be useful as a combination therapy to correct iron balance and thereby reduce the ESA dose needed to achieve target hemoglobin levels.

RIASSUNTO

L'anemia da insufficienza renale cronica è la conseguenza sia di una ridotta produzione di globuli rossi a livello del midollo osseo che di una minore sopravvivenza degli stessi e di una conseguente alterazione dell'omeostasi del ferro. L'eritropoiesi inefficace è prevalentemente correlata al deficit della sintesi dell'eritropoietina, ormone prodotto dal rene che stimola a livello del midollo osseo la produzione di globuli rossi. Una caratteristica fondamentale di molti pazienti con anemia da insufficienza renale cronica è la limitata disponibilità di ferro per un'efficiente eritropoiesi nonostante vi siano adeguati depositi di ferro nell'organismo. E' ormai ben noto che alti livelli di epcidina, il principale ormone regolatore dell'omeostasi del ferro, sono responsabili della down-regolazione dell'espressione funzionale della ferroportina, unico esportatore cellulare di ferro, che provoca un blocco dell' assorbimento di ferro dalla dieta e l'accumulo di ferro nei macrofagi reticoloendoteliali. Il principale pathway di regolazione dell'espressione dell'epcidina è la via delle BMPs (Bone Morphogenetic Proteins), che attraverso la fosforilazione e la conseguente attivazione delle proteine SMAD, attiva il promotore dell'epcidina e ne induce quindi la sintesi.

Un modello animale di anemia da insufficienza renale con un quadro che rispecchia la condizione clinica di pazienti caratterizzata da elevati livelli di epcidina è quello che prevede l'utilizzo di ratti con danni renali indotti da una dieta ricca di adenina.

In questo studio e' stato sviluppato un modello animale da insufficienza renale, che simula la condizione di insufficienza renale e anemia nei pazienti, modificando il modello di Yokozawa. E' stato poi testato se l'inibitore delle BMPs, LDN-193189, fosse in grado di mobilizzare il ferro e migliorare l'eritropoiesi nel modello animale. Questa molecola è nota per essere in grado di inibire in modo specifico la via di stimolazione BMP-dipendente interagendo con il recettore a livello extracellulare.

Il modello animale ha presentato un tasso di sopravvivenza più elevato rispetto ai modelli precedentemente riportati, pur mantenendo un' irreversibile insufficienza renale e una grave anemia. I ratti hanno presentato elevati livelli di epcidina epatica, diminuzione della concentrazione sierica di ferro, maggiore contenuto di ferro nella milza, bassi livelli di emoglobina e livelli impropriamente bassi di EPO rispetto al grado di anemia, tipica condizione clinica che si evidenzia in pazienti con anemia da insufficienza renale cronica.

Il trattamento con LDN-193189 ha indotto l'inibizione dell'espressione epatica di epcidina, attraverso il blocco della via BMPs, ed ha indotto una mobilizzazione del ferro accumulato con un suo riversamento nel plasma. Inoltre, il ferro è stato incorporato in modo efficiente nell'emoglobina dei reticolociti. Tuttavia, il composto da solo non ha impedito la progressione dell'anemia nel modello animale proposto. La diminuzione dei valori di epcidina ha portato ad un conseguente incremento di ferro disponibile, ma non ha permesso il miglioramento dell'anemia.

La co-somministrazione di agenti in grado di diminuire i livelli di epcidina e agenti stimolanti l'eritropoiesi (ESA) potrebbe essere un'utile terapia di combinazione per correggere l'equilibrio di ferro e, quindi, ridurre la dose di ESA necessario per raggiungere i livelli di emoglobina target.

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1. INTRODUCTION

1.1 *Anemia of CKD*

Chronic kidney disease (CKD) affects approximately 26 million adults in the United Sates and millions of others are at risk (1). CKD is associated with significant morbidity and mortality, and these patients face many other medical problems related to CKD. One of the major medical issues facing this population is anemia, which often develops early in the course of CKD and contributes to poor quality of life. It has been shown to be strongly predictive of adverse effects, including complications and death from cardiovascular causes.(2) Prior to the availability of human recombinant erythropoietin, patients receiving chronic dialysis treatment frequently required blood transfusions, exposing them to iron overload, viral hepatitis and HIV, and increasing production of antibodies to human antigens which can severely limit transplantation options.

The introduction of recombinant human erythropoietin in the late 1980s drastically changed the treatment of anemia in patients with CKD. The benefits of anemia treatment in this population reach far beyond the improvement of fatigue and decreased physical activity to a broad spectrum of physiologic functions. Thus the presence of anemia should be sought, diagnosed, and treated early in patients with CKD. The optimal hemoglobin (Hb) targets are still controversial and studies defining these goals are ongoing. The costs of anemia management in the chronic kidney disease population are considerable and need to be considered along with the risks and benefits.

1.1.1. Pathophysiology of anemia in patients with CKD

Anemia is defined by the World Health Organization as a Hb concentration less than 13.0 g/dL in adult males and non-menstruating females and less than 12.0 g/dL in menstruating females.(3) Anemia is a common problem in patients with CKD, and its incidence increases as glomerular filtration rate declines. Population studies such as the National Health and Nutrition Examination Survey (NHANES) by the National Institutes of Health and the Prevalence of Anemia in Early Renal Insufficiency (PAERI) study suggest that the incidence of anemia is less than 10% in CKD stages 1 and 2, 20–40% in CKD stage 3, 50–60% in CKD stage 4 and more than 70% in CKD stage 5 (4,5).

The cause of anemia in patients with CKD is multifactorial. The most well-known cause is inadequate erythropoietin (EPO) production, which is often compounded by iron deficiency. As renal failure progresses, the contribution of EPO deficiency to anemia increases. The role of decreased renal EPO synthesis in CKD-associated anemia is supported by the severe anemia seen in anephric patients (6). However, the mechanisms impairing renal EPO production are not well understood. The production capacity of EPO remains significant even in end stage renal disease (ESRD) as these patients have been shown to respond with increased EPO synthesis in the setting of an additional hypoxic stimulus.(7) This

suggests that the decrease in EPO production in CKD is, in part, a physiologic response to achieve a chronically reduced Hb concentration.

Typically, EPO is produced in the peritubular capillary endothelial cells in the kidney relying on a feedback mechanism measuring total oxygen carrying capacity. Hypoxia inducible factor (HIF), which is produced in the kidney and other tissues, is a substance whose spontaneous degradation is inhibited in the presence of decreased oxygen delivery due to anemia or hypoxemia. The continued presence of HIF leads to signal transduction and the synthesis of EPO. Therefore the usual response is increased EPO production in the setting of anemia. The EPO then binds to receptors on erythroid progenitor cells in the bone marrow, specifically the burst-forming units (BFU-E) and colony-forming units (CFU-E). With EPO present, these erythroid progenitors differentiate into reticulocytes and red blood cells (RBCs). The absence of EPO leads to pre-programmed apoptosis. This is mediated by the Fas antigen. The decreased red blood cell production and continued loss of blood (by programmed red blood cell death) leads to worsening anemia.

There are other factors in chronic kidney disease which contribute to anemia. Acute and chronic inflammatory conditions have a significant impact on anemia in the CKD population by proinflammatory cytokines decreasing EPO production and inducing apoptosis in colony-forming unit-erythroid cells (CFU-E). The early induction of apoptosis in CFU-E cells stops the process of development into RBC. Inflammatory cytokines have also been found to induce the production of hepcidin, a recently discovered peptide generated in the liver, which interferes with RBC production by decreasing iron availability for incorporation into erythroblasts. This also impairs the production of RBC. Figure 1 illustrates the above-mentioned interactions.

Red blood cells also have a decreased life span in patients with CKD. While the normal life span of an RBC is about 120 days, it has been demonstrated that this is shortened to only 60–90 days in CKD patients. In patients without CKD, the bone marrow has significant capacity to increase red blood cell production and to correct for the shortened life span, but this response is blunted in patients with CKD by the relative EPO deficiency. Uremic toxins have been implicated as contributing to apoptosis as the anemia will often improve after initiation of dialysis. There have been a number of prospective and observational studies that have demonstrated improved Hb levels and decreased dose of erythropoiesis-stimulating agents (ESAs) with increased adequacy of dialysis (8-11).

It has been hypothesized that it is the middle molecules (molecular weight range of 500–2000 daltons) of uremia that contribute to bone marrow suppression (12).

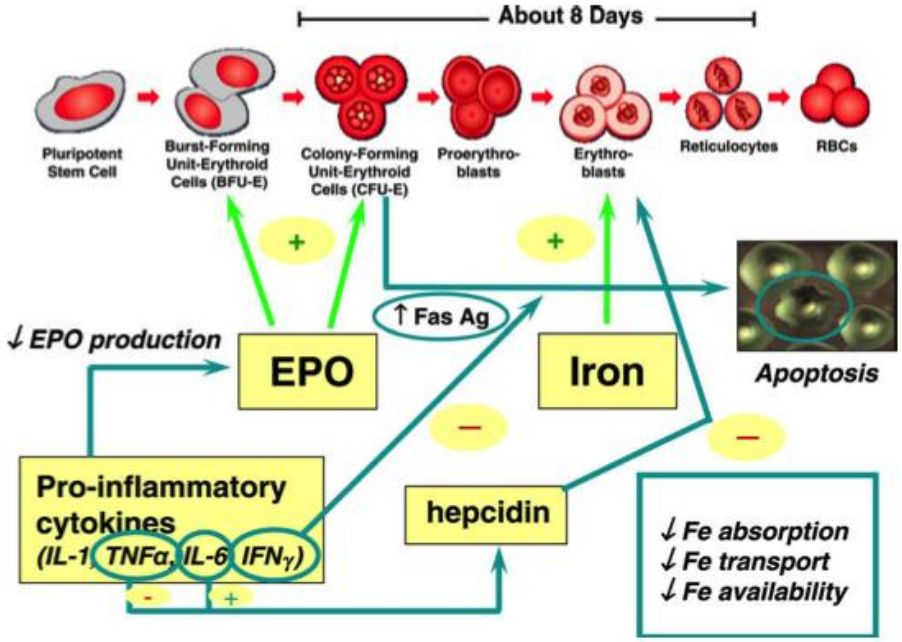


Fig. 1. Erythropoiesis in chronic kidney disease. Ag, antigen; EPO, erythropoietin; Fe, iron; IFN, interferon; IL, interleukin; RBCs, red blood cells; TNF, tumor necrosis factor.

Inadequate production of erythropoietin commonly is believed to be the most important factor in the pathogenesis of anemia in these patients, and many patients are treated with ESAs. However, approximately 10%-20% of patients are poorly responsive to ESA therapy (13). Prospective randomized controlled clinical trials, including the US Normal Hematocrit Study and the CHOIR (Correction of Hemoglobin and Outcomes in Renal Insufficiency) Study, raised concerns about the safety of ESAs when dosed to target higher hemoglobin levels, particularly when using higher doses and in patients who are poorly responsive to therapy (14-16). This has resulted in a US Food and Drug Administration black box warning on the product labeling of ESAs and significant controversy about the management of anemia in patients with CKD.

The cause of anemia in patients with CKD is multifactorial. In addition to relative erythropoietin deficiency, shortened erythrocyte survival and the erythropoiesis-inhibitory effects of accumulating uremic toxins also contribute to the anemia of CKD. Importantly, patients with CKD also have several abnormalities in systemic homeostasis of iron, an essential component in the production of red blood cells (17-18). First, hemodialysis patients in particular typically are in negative iron balance, losing approximately 1-3 g of iron per year, caused in part by blood trapping in the dialysis apparatus and repeated phlebotomy. Second, many patients are on ESA therapy to manage their anemia, which depletes iron stores by

driving increased production of red blood cells. Third, it has been recognized that patients with CKD also have impaired absorption of dietary iron. Randomized controlled trials have shown that oral iron is no better than placebo to treat iron deficiency in patients on hemodialysis therapy (19-21). These abnormalities of iron metabolism in patients with CKD may result in true iron deficiency, manifest as low serum transferrin saturation and ferritin level, which can be treated with supplemental iron. However, many patients also have a functional iron deficiency or reticuloendothelial cell iron blockade, characterized by low levels of circulating iron that limit erythropoiesis, even in the face of adequate or increased body iron stores. Management of these patients is less clear. This reticuloendothelial cell iron sequestration is characteristic of anemia of inflammation (also known as anemia of chronic disease), seen not only in patients with CKD, but also in patients with many other chronic diseases, including autoimmune disorders, chronic infections, and malignancy. Many patients with CKD have a chronic inflammatory state, which may be caused by an increased incidence of infections and/or induction of inflammatory cytokines by the hemodialysis procedure (22). Recent research suggests that the impaired intestinal iron absorption and impaired release of iron from body stores in patients with CKD, as in other patients with anemia of inflammation, may be caused by an excess of the key iron regulatory hormone hepcidin (23-25).

1.2 Iron biology and homeostasis

1.2.1 Iron Intake

Iron is the most abundant element on Earth by mass and the fourth most abundant in the Earth's crust but it readily oxidizes into insoluble compounds with poor bioavailability. In this environment, biological organisms evolved to conserve iron. Quantitative analysis of tissue iron distribution and fluxes in humans illustrates how this is accomplished (26).
The typical adult human male contains about 4 g of iron of which about 2.5 g is in hemoglobin, 1 g is stored predominantly in hepatocytes and hepatic and splenic macrophages, and most of the rest is distributed in myoglobin, cytochromes, and other ferroproteins. Only about 1–2 mg/day is lost from the body predominantly through desquamation and minor blood loss. In the steady state, this amount is replaced through intestinal iron absorption. Although the loss of iron may increase slightly with increasing iron stores, these changes do not significantly contribute to homeostasis; intestinal iron absorption is by far the predominant determinant of the iron content of the body.
Recovery from blood loss causes an increase in iron absorption up to 20-fold, indicating that the duodenum where iron absorption takes place has a large reserve capacity for iron absorption.

1.2.2 Iron Recycling

Under normal circumstances, the reutilization of iron recycled from senescent cells accounts for most of the iron flux in humans. With the erythrocyte lifespan of 120 day, 20 – 25 mg of iron is required to replace the 20 – 25 mL of erythrocytes that must be produced every day to maintain a steady state. Other cell types also turn over but their much lower iron content contributes relatively little to the iron flux. Macrophages in the liver, spleen, and marrow (formerly called the reticuloendothelial system) phagocytose senescent or damaged erythrocytes, degrade their hemoglobin to release heme, extract iron from heme using heme oxygenase (27) and recycle the iron to the extracellular fluid and plasma. Steady-state iron flux from recycling can increase up to 150 mg/d in conditions with ineffective erythropoiesis in which the number of erythroid precursors is increased and accompanied by the apoptosis of hemoglobinized erythrocyte precursors in the marrow and shortened erythrocyte survival (28).

1.2.3 Iron Distribution and Storage

Free iron is highly reactive and causes cell and tissue injury through its ability to catalyze the production of reactive oxygen species. In living organisms, iron is complexed with proteins or small organic molecules (citrate, acetate), which mitigate its reactivity. Transferrin is the physiological carrier of iron in plasma. Normally, only 20% – 40% of the available binding sites on transferrin molecules are occupied by ferric iron. The iron content of plasma is only 2 – 3 mg so this

compartment must turn over every few hours. Erythrocyte precursors take up iron almost exclusively through transferrin receptors (TfR1) so the iron supply to erythrocyte precursors is completely dependent on plasma transferrin. In contrast, hepatocytes and other non-erythroid cells can also take up iron that is not bound to transferrin (nontransferrin-bound iron or NTBI), a process that becomes important during iron overload when plasma transferrin saturation reaches 100% (29). The predominant cellular storage form of iron is the hollow spherical protein ferritin whose cavity contains iron in ferric form complexed with hydroxide and phosphate anions.

1.2.4 Regulation of Plasma Iron Concentrations

Despite varying dietary iron intake and changes in erythropoietic activity owing to occasional or periodic blood loss, iron concentrations in plasma normally remain in the 10 – 30 mM range. Chronically low concentrations decrease iron supply to erythropoiesis and other processes leading to anemia and dysfunction of other cell types sensitive to iron deprivation. Chronically high iron concentrations lead to intermittent or steady-state saturation of transferrin with iron and the generation of NTBI with consequent deposition of excess iron in the liver, endocrine glands, cardiac myocytes, and other tissues. Excess cellular iron may cause tissue injury by catalyzing the generation of reactive oxygen species, which can cause DNA damage, lipid peroxidation, and oxidation of proteins.

1.2.5 Systemic Iron Homeostasis

Phenomenological description of systemic iron homeostasis was developed starting in the 1930s (26). Homeostatic mechanisms regulate dietary iron absorption and iron deposition into or withdrawal from stores depending on the amount of stored iron (“stores regulator”) and the requirements of erythropoiesis (“erythropoietic regulator”). The description of the molecular processes that underlie iron homeostasis has progressed rapidly in the last two decades but is still not complete.

1.3 Cellular iron regulation

1.3.1 Cellular Iron

Cells require iron predominantly for incorporation into various ferroproteins, where iron exists in iron – sulfur clusters, in heme or hemelike prosthetic moieties, or in other more loosely associated forms. It now appears that most cell types in the body autonomously regulate their iron uptake solely to meet their individual requirements for iron. These cells do not export appreciable amounts of iron and are presumed to give up their iron only when they undergo cell death and are recycled by macrophages. In contrast, several specialized cell types supply or store iron to meet the needs of the entire organism, and are therefore equipped to export iron into extracellular fluid and plasma. Iron-exporting cells include duodenal enterocytes that absorb dietary iron, macrophages that recycle iron from senescent or dead cells, and macrophages and hepatocytes that store iron and release it to meet systemic demand. During pregnancy, the placental syncytiotrophoblast must transport maternal iron into the fetal circulation to meet the iron requirements of fetal growth and development. The endothelial cells that form the blood – brain barrier must also selectively transport iron as it now appears that the iron concentrations in the brain are not appreciably increased in systemic iron overload disorders. Finally, erythroid precursors need much more iron than any other cell type as each cell synthesizes more than a billion heme molecules, therefore facing greater iron-homeostatic challenges.

1.3.2 Cellular Iron Uptake

Transferrin-mediated iron uptake is the best understood mechanism of cellular iron import. Although the transferrin receptor (TfR1) is expressed in many cell types, erythrocyte precursors contain most of the TfR1 molecules and take up the great majority of iron-transferrin in the organism. Iron-transferrin is endocytosed via the cell membrane TfR1 and internalized into endosomal recycling vesicles. As the vesicle acidifies, the low pH releases the transferrin- bound ferric iron and the iron-free (apo)transferrin-TfR1 complex returns to the cell membrane (Fig. 2)

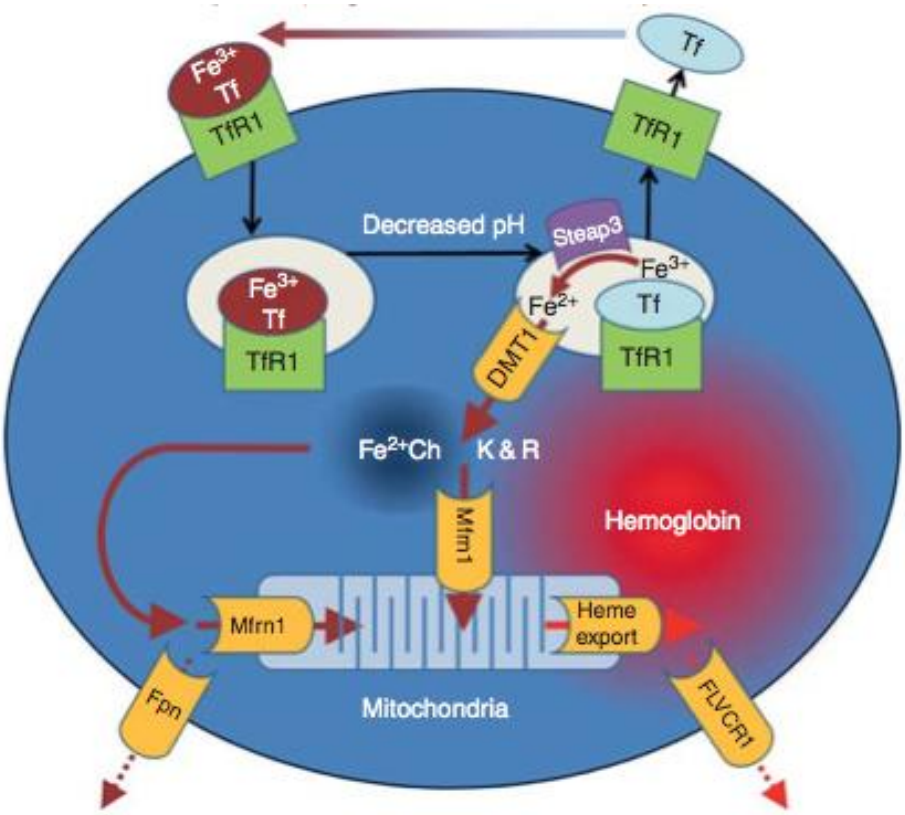


Fig. 2. Iron traffic in erythrocyte precursors synthesizing hemoglobin. Iron is taken up as diferric transferrin by the transferrin receptor (TfR1). Acidification of the endocytic vesicle releases ferric iron from transferrin, and the membrane ferrireductase Steap3 reduces it to ferrous iron, which is then exported to the cytoplasm by DMT1. The complex of iron-free apotransferrin (Tf) and TfR1 is returned to the plasma membrane where the neutral pH causes Tf to dissociate from its receptor. The transferrin cycle is completed when Tf is reloaded with ferric iron by duodenal enterocytes or iron-recycling macrophages. Ferrous iron exported by DMT1 may be delivered to mitochondrial mitoferrin-1 (Mfrn1) by direct contact (the kiss-and-run mechanism, K&R) or through intermediate transport by as-yet uncharacterized cytoplasmic chaperones (Fe2þCh). Mitoferrin-1 imports iron into mitochondria where iron is incorporated into newly synthesized heme. Heme is exported via an unknown exporter (Heme export) and incorporated into globin chains to generate hemoglobin. Under some circumstances, iron is exported as ferrous iron via ferroportin (Fpn) or as heme via feline leukemia virus C receptor (FLVCR1).

The neutral pH at the membrane causes the apotransferrin to dissociate from TfR1, whereupon apotransferrin diffuses away to be loaded with iron again, repeating the cycle. From the vesicle, iron is delivered to mitochondria where it is incorporated into protoporphyrin IX to form heme, or incorporated into nascent iron – sulfur clusters. Alternatively, iron can be exported from the vesicle into the cytoplasm where it is incorporated into cytoplasmic ferroproteins or stored in cytoplasmic ferritin.

Nontransferrin-bound iron (NTBI) (29) usually accumulates when the iron- binding capacity of transferrin is exceeded and then circulates complexed mostly with citrate or acetate. Hemoglobinopathies such as β-thalassemia major and intermedia are associated with particularly high levels of NTBI. Some cells, including hepatocytes, cardiomyocytes, and cells of endocrine glands can take up NTBI, although the precise mechanism is not well understood. Candidate NTBI transporters include L-type voltage-gated calcium channels, DMT-1 and Zip14.

1.3.3 Intracellular Iron Transport

To undergo transport to the cytoplasm or mitochondria, ferric iron must be reduced to its ferrous form through the action of ferrireductases. Recent studies indicate that Steap (sixtransmembrane epithelial antigen of the prostate) proteins 1 – 4 are among the relevant ferrireductases, with Steap3 having a particular importance in erythroid precursors (Fig. 2), assisted perhaps by Steap2 and Steap4 (30). To reach the cytoplasm, ferrous iron must cross the membrane of the vesicle. In many cells, the proton-dependent ferrous iron transporter divalent metal transporter-1 (DMT1) appears essential for iron transport from the vacuole into the cytoplasm but in macrophages its homolog natural resistance-associated macrophage protein (Nramp1) may also contribute (31). Because of its chemical reactivity, iron is chaperoned in the cytoplasm, at least in part by multifunctional poly(RC)- binding proteins (PCBPs) (32). In particular, PCBP1 mediates the delivery of iron to cytoplasmic ferritin and both PCBP1 and 2 are involved in the delivery of iron to cytoplasmic iron-dependent prolyl and asparaginyl hydroxylases that mediate oxygen sensing (33). It is not known how iron is transported to mitochondria. In erythroid cells, there is evidence for a “kiss-and-run” mechanism (Fig. 2) whereby iron could be transferred from endosomal vesicles directly to mitochondria (34) but it is not clear how much this mechanism contributes to the iron flux into mitochondria and whether it also functions in nonerythroid cell types.

1.3.4 Mitochondria and Iron

Consistent with their autonomous evolutionary origin, mitochondria are equipped with distinct iron transporters. Iron uptake into mitochondria depends on the inner mitochondrial membrane proteins mitoferrin 1 and 2, with the former predominantly expressed in erythroid cells and the latter ubiquitously (35). In erythroid cells, mitoferrin 1 interacts with the ATP-binding transporter Abcb10 and with ferrochelatase to form a plausible pathway for the delivery of iron for heme formation (36). How heme is exported from mitochondria for incorporation into

hemoglobin and other hemoproteins is not known. Mitochondria also contain a distinct ferritin, mitochondrial ferritin, for local iron storage.

1.3.5 Cellular Iron Homeostasis

Cellular, as opposed to systemic, iron homeostasis assures that sufficient but not excessive amounts of iron are taken up by each cell to meet its individual requirement for ferroprotein synthesis. The system that has evolved relies on posttranscriptional regulation through the interaction of iron-regulatory proteins (IRP1 and IRP2) with iron-regulatory elements (IREs) in messenger RNAs (mRNAs) that encode key iron transporters, ferroproteins, and enzymes involved in iron-utilizing pathways. The IRE/ IRP system effectively regulates iron uptake, provides for the storage of excess iron in ferritin, and coordinates the synthesis of heme, iron – sulfur clusters, and ferroproteins with the availability of iron. The system in effect acts to decrease wasted expenditure of synthetic energy and substrates, and to prevent accumulation of toxic forms of iron. Target mRNAs contain IREs that form characteristic stem-loop structures either in the 50 region, where IRP binding represses translation and decreases protein synthesis, or in the 30 region where IRP binding prevents endonucleases from cleaving sensitive regions of the mRNA, thus stabilizing mRNA and increasing protein synthesis (37). IRP1 and IRP2 are structurally related but interact with iron in distinct ways. Both proteins bind to IREs when cellular iron levels are low. In the presence of iron, IRP1 incorporates an iron–sulfur cluster, does not bind IREs, and acts as an aconitase enzyme converting citrate to isocitrate in the Krebs cycle. In contrast, IRP2 is ubiquitinated by a complex iron-dependent process and then degraded in proteasomes (38,39). The dual specificity of IRP1/aconitase may have a functional role in the regulation of erythropoiesis by iron availability, as the provision of the aconitase product isocitrate reverses some of the suppressive effect of iron deprivation on erythropoiesis and the enzymatic inhibition of aconitase has the opposite effect (40,41). Many mRNAs are regulated by the IRP/IRE system (42) and fall into three classes: (1) iron acquisition, generally with IREs in the 30 region resulting in increased protein synthesis during cellular iron deprivation; (2) iron utilization and storage, with IREs in the 50 region, resulting in repressed protein synthesis during iron deprivation; and (3) iron export, with IREs also in the 50 region and protein synthesis repressed during iron deprivation. Proteins subject to IRE/IRP regulation include TfR1 and DMT1 involved in cellular iron uptake, aminolevulinic acid synthase 2, which catalyzes the first step of the heme synthesis pathway in erythroid cells, the heavy and light subunits of ferritin involved in iron storage, and ferroportin, the iron exporter expressed in tissues and cells involved in iron export to plasma. The net effect of the IRE/IRP response during cellular iron deficiency is to increase cellular iron uptake, mobilize iron from cellular storage, decrease iron utilization, and, when iron becomes sufficient or excessive to reverse these responses and direct more iron into cellular storage and export. Further fine-tuning of iron import and export is achieved by differential splicing of target mRNAs in different tissues to either include or exclude IREs. As an example, systemic adaptation to iron deficiency may be facilitated by a ferroportin mRNA isoform that

lacks IRE, which may allow iron-transporting duodenal enterocytes to deliver iron to plasma for systemic needs even if the enterocyte is sensing iron deficiency, and may transfer iron from erythroid cells to other tissues more critically dependent on iron (43).

1.3.6 Generalized Regulation of Protein Synthesis by Iron in Erythroid Cells

In addition to the regulation of the synthesis of individual proteins by iron, erythroid cells also contain a mechanism for a generalized adaptive response to iron deficiency. This response is affected by the heme-regulated inhibitor kinase (HRI) belonging to a class of kinases activated by cellular stress, including nutrient deprivation, viral infection, and endoplasmic reticulum stress (44). During iron deficiency as heme concentrations drop, heme dissociates from HRI, causing it to undergo specific autophosphorylation to become a catalytically active kinase targeting the a subunit of eukaryotic translational initiation factor 2 (eIF2a). Activated HRI inhibits translational initiation by phoshorylating eIF2a. Not all protein synthesis is inhibited however, as activated HRI may promote the synthesis of transcription factors that are protective during iron-deficient erythropoiesis (45). A priori, it is not obvious how iron deficiency results in the production of smaller, less-hemoglobinized erythrocytes rather than fewer normally sized and hemoglobinized cells. Studies with HRI-deficient mice showed that HRI protects erythroid precursors from apoptosis induced by excessive production of globin chains and contributes to the microcytosis and hypochromia seen in iron deficiency, erythropoietic protoporphyria, and β-thalassemia.

1.3.7 Iron and Hypoxia Sensing

The hypoxia-sensing pathway may also contribute to cellular iron homeostasis. Prolyl and asparaginyl hydroxylases, which inactivate the HIF transcription factors, are not only sensitive to oxygen tension but also to iron concentrations because they use iron as a catalytic cofactor. In support of the potential role of HIF in iron regulation, tissue-specific deletion of HIF2a in mouse enterocytes decreased intestinal iron absorption as well as the expression of DMT1 in enterocytes (46). HIF2a bound to the DMT1 promoter and transactivated it. The broader physiologic function of HIF in cellular iron homeostasis still remains to be established and may vary in different tissues depending on oxygen tension and other factors.

1.4 Systemic iron homeostasis

1.4.1 The Central Role of Hepcidin

Systemic iron homeostasis encompasses the regulatory circuitry that controls the absorption of dietary iron, the concentration of iron in extra-cellular fluid and blood plasma, and the release of iron from macrophages involved in iron recycling and from iron-storing hepatocytes. It now appears that there is a single systemic regulator of iron, the hepatic peptide hormone hepcidin.

1.4.2 Hepcidin Synthesis and Structure

Human hepcidin is predominantly produced by hepatocytes as a 25 amino acid peptide (2789.4 Da) (47,48), that is secreted into the circulation. Subsequent amino-terminal processing of the 25 amino acid form can result in the appearance of 2 smaller hepcidin forms of 22 and 20 amino acids. These hepcidin peptides form a hairpin structure, with 4 intramolecular disulfide bridges (Fig. 3) (49). On the basis of some studies, investigators have reported that hepcidin binds divalent metals (Cu2+, Fe2+, Zn2+, and Ni2+), but these findings are inconsistent, e.g., some studies have suggested the presence of iron in the core of the peptide in a tetrahedral sulfur coordination (50-52), whereas others provide evidence for an amino-terminal Cu(2+)– Ni(2+)-binding (ATCUN) motif (53,54). The ability of hepcidin to bind iron and other divalent metals suggests there may be a nonhormonal role for hepcidin in iron metabolism or a conformational mechanism for uptake of divalent metals as part of hepcidin's hormonal role in regulating ferroportin degradation.

Much is still unknown on the origin of the smaller isoforms of hepcidin, although data suggest that calcium-independent tissue activity in pancreas extracts might lead to the systemic N-terminal truncation of hepcidin-25 to hepcidin-22, and that dipeptidylpeptidase 4 is involved in the processing of hepcidin-22 into hepcidin-20 (55,56). Under physiological conditions hepcidin-20 and hepcidin-22 are present in the urine, but not, or at very low concentrations, in the serum (57-59). Interestingly, these smaller hepcidin isoforms occur only in serum of patients with diseases that are associated with increased concentrations of hepcidin-25, such as acute myocardial infarction (AMI), sepsis, anemia of chronic disease (ACD), metabolic syndrome, and chronic kidney disease (CKD) (60-63). In vivo studies in mice have demonstrated that only full-length 25 amino acid hepcidin induces significant hypoferremia when injected intra-eritoneally (64). These findings are corroborated by in vitro studies that showed that the truncated 22 amino acid and 20 amino acid forms have greatly diminished and almost complete loss of ferroportin regulatory activity, respectively, compared with 25 amino acid hepcidin (65).

Recent studies have demonstrated hepcidin expression by cells other than hepatocytes, although at much lower levels by comparison. These include kidney tubule, heart, retina, monocytes, neutrophils, fat cells, alveolar cells, pancreatic β-cells, and cardiomyocardal cells (66-72). The hepcidin produced by these cells,

however, is unlikely to make a significant contribution to systemic circulating concentrations, but may exert local effects in these tissues.

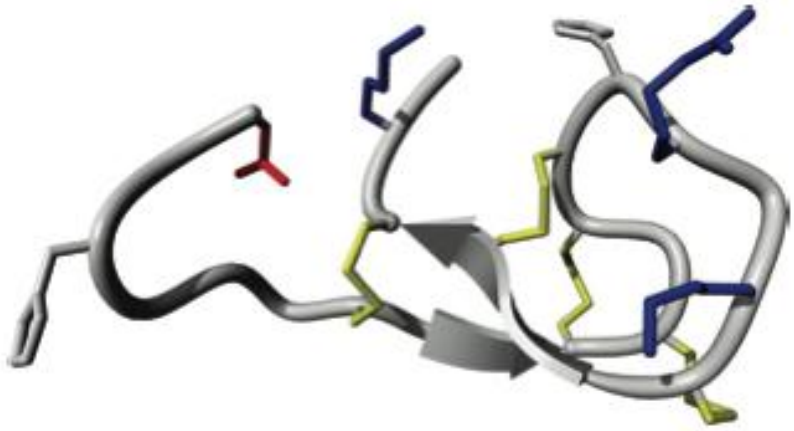


Fig. 3. Molecular structure of hepcidin. A model of the structure of hepcidin-25 according to Jordan et al. (3) is shown, with the β-sheets (grey arrows), the peptide backbone (grey), the disulfide bonds (1–8, 2–4, 3–6, and 5–7) (yellow), positively charged arginine and lysine residues (blue), and the negatively charged aspartic acid residue (red).

1.4.3 Hepcidin Kinetics

Circulating hepcidin was recently found to be bound to α2-macroglobulin with relatively high affinity and to albumin with relatively low affinity. On the basis of theoretical calculations, 11% of hepcidin was estimated to be freely circulating (28). Whether binding to these carrier molecules influences the functional properties of hepcidin is uncertain.

Hepcidin clearance is assumed to occur via cellular codegradation with ferroportin at its sites of action, and via excretion by the kidneys. Because of its low molecular weight and small radius, unbound hepcidin is likely to freely pass into the glomerular filtrate. In small studies in humans the fractional excretion of hepcidin has been calculated to be as low as 0%–5% (73,74), either because it is reabsorbed, similarly to other small peptides, or because it is not freely filtered. Evidence for the latter explanation comes from the finding of increases of only 1- to 6-fold of serum hepcidin concentrations in patients with glomerular dysfunction (61, 62, 24, 75) compared with the 20- to 30-fold increase of serum β2-microglobulin. The excretion of the latter low molecular weight protein is known to be almost completely governed by glomerular filtration. It is possible that binding to α2-macroglobulin or other carrier proteins prevents circulating hepcidin from being freely filtered. Alternatively, the otherwise expected increased circulating

concentrations in patients with decreased renal filtration may be offset by a compensatory feedback decrease in hepatic hepcidin production.
It is speculative, but conceivable, that under certain conditions hepcidin escapes renal tubular reabsorption. This lack of reabsorption may play a role in several disorders of iron metabolism that are associated with tubular dysfunction and increased concentrations of urine hepcidin, such as inflammation, iron overload, and malaria (77,78). Possible local tubular production of hepcidin must also be taken into account in the interpretation of urine as a mirror of serum hepcidin concentrations in such studies (79) as well as possible defective tubular reabsorption of hepcidin. Additional studies to investigate the possibility that local urinary infections or tubular dysfunction can contribute to hepcidinuria are warranted.

1.4.4 Hepcidin Function

Hepcidin-25 is thought to be the major regulator of dietary iron absorption and cellular iron release. It exerts its regulatory function by counteracting the function of ferroportin, the major cellular iron exporter in the membrane of macrophages, hepatocytes, and the basolateral site of enterocytes. Hepcidin-25 induces the internalization and degradation of ferroportin (80-83), resulting in increased intracellular iron stores, decreased dietary iron absorption, and decreased circulating iron concentrations (Fig. 4).

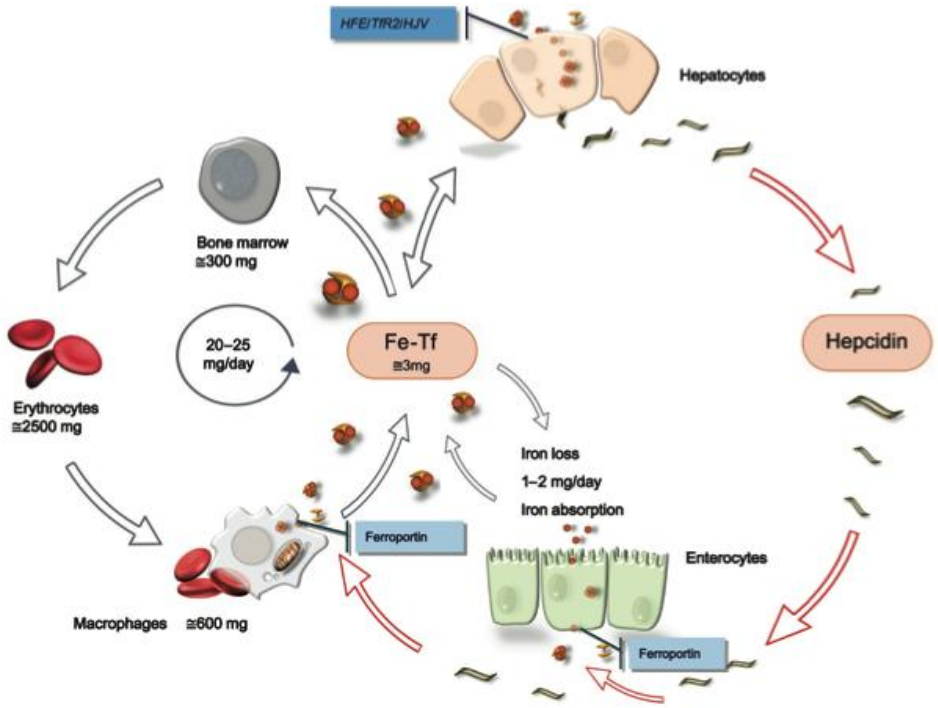


Fig. 4. Iron uptake and recycling. Most of the used body iron is recycled from senescent erythrocytes by macrophages, and returned to the bone marrow for incorporation in erythroid precursors. The liver and RE macrophages function as major iron stores. Importantly, the total amount of iron in the body can be regulated only by absorption, whereas iron loss occurs only passively from sloughing of skin and mucosal cells as well as from blood loss. This results in the absorption and loss of 1–2 mg iron every day. Hepcidin, a peptide produced in the liver, controls the plasma iron concentration by inhibiting iron export by ferroportin from enterocytes and macrophages. As a consequence, an increase in hepcidin production leads to a decrease in plasma iron concentrations. Hepcidin expression is regulated by body iron status, inflammation, erythroid iron demand, and hypoxia via regulation pathways involving the expression of the genes HFE, transferrin receptor 2 (TFR2), and hemochromatosis type 2 (juvenile) (HFE2) (also known as HJV). Adapted from (142, 223).

In addition to its role in regulating systemic iron metabolism, hepcidin may also contribute to host defense. Hepcidin was originally identified as an antimicrobial peptide (58). Although in vitro studies suggest bacteriocidal effects of hepcidin, these effects would require concentrations higher than those observed in the circulation. Such concentrations may be achieved locally, for instance in phagosomes of infected macrophages (84). Hepcidin might also contribute indirectly to host defense by reducing plasma iron concentrations. Iron is necessary

for microbial growth, and reductions in plasma iron are bacteriostatic. Moreover, hepcidin was found to modulate lipopolysaccharide-induced transcription in both cultured macrophages and in vivo mouse models (85). This latter observation suggests a role for hepcidin in modulating acute inflammatory responses to bacterial infection.

Hepcidin produced by various cell types other than hepatocytes may have local effects in these tissues. Through an autocrine interaction with ferroportin, local hepcidin may protect the nearby cells from iron deficiency, prevent extracellular oxidative stress, affect inflammatory reponses, and/or deplete extracellular iron pools that are available for extracellular pathogens (86,87). Although the smaller forms of hepcidin do not elicit a hypoferremic response, it is currently unknown whether they retain other identified biological functions of hepcidin-25 (e.g., in host defense or in metal binding) (88).

1.4.5 Hepcidin Regulation

Several physiologic and pathologic processes regulate the synthesis of hepcidin (Figure 5) [reviewed in (89)]. Situations in which demand for circulating iron is increased (particularly erythropoietic activity) elicit a decrease in hepatocellular hepcidin synthesis. These conditions include iron deficiency, hypoxia, anemia, and conditions characterized by increased erythropoietic activity. A decrease in hepcidin results in the release of stored iron and an increase in dietary iron absorption. On the other hand, infection and inflammation cause an increase in hepcidin synthesis. This increased synthesis leads to a deficiency of iron available for erythropoiesis, and is considered to be the mechanism underlying reticuloendothelial (RE) iron sequestration, intestinal iron absorption impairment, and low serum iron concentrations characteristic of anemia of chronic disease.

The functional signaling routes by which (a) iron status, (b) erythropoietic activity, (c) hypoxia, and (d) inflammation affect hepcidin expression are increasingly being investigated. These routes comprise 4 highly interconnected regulatory pathways (Figure 5).

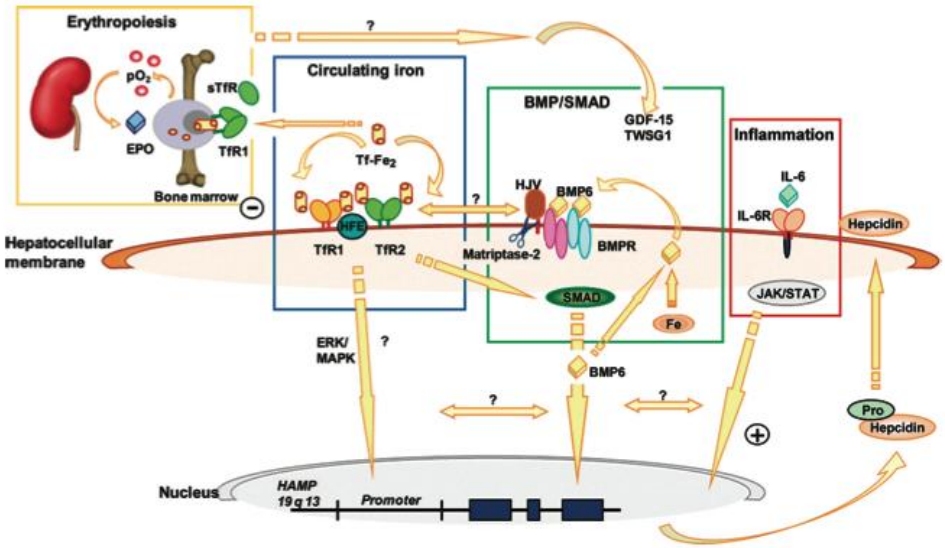


Fig. 5. Molecular and functional pathways of hepatocyte hepcidin synthesis. Three molecular pathways can be distinguished: the HFE/TfR2, BMP/SMAD, and JAK/STAT. This proposed model depicts 2 iron signals to hepcidin, 1 mediated by intracellular iron stores (Fe) and the other by circulating iron (Tf-Fe2). Hepatocellular iron stores increase the expression of BMP-6, which serves as an autocrine factor by interacting with surface BMP receptors. HJV is a BMP coreceptor that augments BMP binding. The consequent activation of intracellular SMAD proteins transduces a signal to increase hepcidin transcription. HJV is subject to cleavage by furin, which is regulated by iron and hypoxia, to form a soluble component (sHJV) (61). sHJV can subsequently act as a decoy coreceptor and antagonist of BMP-6 induced hepcidin synthesis. Under low iron conditions membrane bound HJV is also cleaved by matriptase-2 (scissors) again weakening the BMP-6 signal. Extracellular Tf-Fe2 mediates a second iron signal. In this scheme, Tf-Fe2 displaces HFE from TfR1. HFE is then liberated to interact with TfR2. The HFE–TfR2 complex activates hepcidin transcription via BMP/SMAD signaling. Several studies have provided indirect evidence for the involvement of the hepatic ERK/MAPK signaling pathway in hepcidin regulation by iron through TfR2 and/or HFE [reviewed in (44)]. Recent studies in mice, however, demonstrate that acute and chronic enteral iron administration did not activate the ERK/MAPK pathway, which suggests that this route may not be of physiological relevance for iron homeostasis in vivo (46). Hypoxia influences liver-specific stabilization of HIF-1, which induces matriptase-2 and the subsequent cleavage of HJV (62). The latter pathway may be synergistic to the increased release of sHJV upon its cleavage by furin under hypoxic conditions. Erythropoiesis is controlled in part by EPO production in the kidney and communicates with the hepatocyte by the proteins GDF15 and TWSG1, which inhibit the BMP/SMAD signaling to hepcidin. Inflammatory stimuli, such as IL-6, induce hepcidin synthesis through the janus

kinase/signal transducer and activator of transcription-3 (JAK/STAT) pathway. These pathways have recently been reviewed (44). Adapted from (133, 224). pO2, partial oxygen pressure; sTfR: soluble TfR; BMPR, BMP receptor; IL-6R: IL-6 receptor.

1.4.5.1 Regulation by Systemic Iron Availability

HFE has been suggested to act as a bimodal switch between two sensors of the concentration of Tf-Fe2, TfR1, and TfR2, on the plasma membrane of hepatocytes (90). This model is supported by the following findings: HFE binds the ubiquitously expressed TfR1 at a site that overlaps the transferrin binding domain, and Tf-Fe2 thus competes with HFE binding to TfR1. By contrast, TfR2 can bind both HFE and Tf-Fe2 simultaneously (91). Mice bearing an engineered TfR1 mutation with increased HFE binding show low hepcidin expression and systemic iron overload similar to HFE-deficient mice, suggesting that the TfR1 sequesters HFE to prevent its participation in hepcidin activation. Conversely, mutations that abolish the HFE-TfR1 interaction or mice with increased HFE levels display elevated hepcidin expression and succumb to iron deficiency (92). Hepcidin activation by holotransferrin requires both HFE and TfR2 (93). These observations support a model in which high concentrations of Tf-Fe2 displace HFE from TfR1 to promote its interaction with TfR2, which is further stabilized by increased Tf-Fe2 binding to the lower-affinity TfR2. The HFE- TfR2 complex then activates hepcidin transcription. Future research is needed to establish the stoichiometry of the proteins involved in this “Tf-Fe2-sensing complex” and to clarify whether HJV is part of it.

Although HFE and TfR2 clearly contribute to hepcidin activation, the BMP signaling pathway is quantitatively the most critical. By as yet only partially understood mechanisms, it integrates signals from the “Tf-Fe2-sensing complex” and the hepatocytic iron stores. Central to the latter is BMP6, which is positively regulated by iron.

BMP are a subfamily of cytokines that belong to the transforming growth factor-β superfamily (94).

Bmp6 knockout mice show hepcidin deficiency and tissue iron overload (95,96), although BMP2 and BMP4 can also bind to HJV. BMP6 is thought to act in an autocrine manner analogous to its role in chondrocyte differentiation (97) to induce signaling via Hemojuvelin (HJV), a glycophosphatidylinositol (GPI)-linked membrane protein (98), the BMP coreceptor that adapts BMP receptors for iron regulation (99). The BMP/HJV complex joins the type I (Alk2 and Alk3) and the type II (ACTRIIA) BMP receptors to induce phosphorylation of receptor activated SMAD (R-SMAD) proteins, Smad1, Smad5, and Smad8 in the cytoplasm, and subsequent formation of active transcriptional complexes involving the co-SMAD factor, SMAD4 (100).

Two sequence motifs (the proximal BMP-RE1 and the distal BMP-RE2) of the hepcidin promoter are critical for transcription via HJV, BMP6, and SMAD4 (101), and the promoter region that contains BMP-RE2 confers iron responsiveness to the hepcidin promoter (102). Multiple lines of evidence highlight the importance of HJV/

BMP/SMAD signaling for hepcidin activation: (1) mice lacking HJV show attenuated R-SMAD phosphorylation in the liver (99), (2) administration of BMP2 and BMP6 to mice induces hepcidin mRNA and decreases serum iron levels, (3) BMP antagonists (such as dorsomorphin) inhibit hepcidin mRNA expression and increase serum iron levels (103), (4) liver-specific disruption of the co-SMAD4 causes severe iron overload with diminished hepcidin transcription (100), and (5) the inhibitory iSMAD7 potently suppresses hepcidin transcription in cellular models (104). Interestingly, R-SMAD phosphorylation is also attenuated in mice lacking HFE, suggesting that HJV and HFE act together to activate hepcidin transcription (105,106). Crosstalk between the BMP/SMAD and p38-MAPK signaling pathways activates hepcidin mRNA expression in response to Tf-Fe2 in primary hepatocytes. Activation of p38-MAPK and Erk1/2 depends on both HFE and TfR2, as this pathway is attenuated in mice lacking HFE or TFR2 and in double-knockout mice (107).

Apart from mutations of the hepcidin gene itself, only HJV mutations lead to a near absence of hepcidin expression and the most severe form of hereditary hemochromatosis. Thus, HJV is central for hepcidin expression, and the point of convergence of multiple regulatory inputs.

The membrane-associated protease TMPRSS6 that is mutated in IRIDA physically interacts with HJV and cleaves HJV when both proteins are expressed on the cell surface, suggesting that HJV is the major TMPRSS6 target for iron regulation (108). Genetically, the combined deficiency of HJV and TMPRSS6 causes iron overload, suggesting that TMPRSS6 acts upstream of HJV (109,110). Increased HJV surface expression has however yet to be confirmed in Tmprss6-deficient mice or IRIDA patients.

Furin-mediated cleavage releases HJV from cells to generate soluble HJV (sHJV), which antagonizes BMP-dependent hepcidin activation. Furin mRNA expression is regulated by iron and hypoxia, conferring another level of control (108). Because of the high HJV expression in skeletal muscle, it is tempting to speculate that sHJV is released as a muscle signal in iron deficiency. Importantly, cleavage of HJV by other proteases does not seem to be redundant with that by TMPRSS6, as lack of TMPRSS6 activity causes iron deficiency in humans and mice. Future work needs to address how TMPRSS6 expression and activity are regulated, and the relative contributions of TMPRSS6 and furin to the regulation of HJV and systemic iron homeostasis need to be further defined.

Neogenin, a DCC (deleted in colorectal cancer) family member, appears to stabilize HJV to enhance BMP signaling and hepcidin expression. Consistently, mice lacking neogenin exhibit hepatic iron overload, low hepcidin levels, and reduced BMP signaling (111).

Another molecule that may negatively regulate hepatic BMP signaling for hepcidin production is SMAD7, an inhibitory SMAD protein that is induced by TGF-β signaling. SMAD7 appears to antagonize TGF-β signaling by acting in a negative-feedback loop. Similar to the expression of hepcidin and *Bmp6*, hepatic mRNA levels of *Smad7* increase with chronic dietary iron loading (112). In primary murine hepatocytes, *SMAD7* expression is induced by BMP6, whereas *SMAD7* overexpression greatly reduces hepcidin mRNA levels and abolishes the hepcidin

response to BMP6 (104). These findings suggest that SMAD7 may function to provide negative feedback to dampen BMP signaling for hepcidin production. The relative contribution of SMAD7 activity to hepcidin regulation in vivo has yet to be established.

1.4.5.2 Regulation by Hypoxia

Liver-specific stabilization of the hypoxia-inducible factor 1 (HIF1) and HIF2 decreases hepcidin expression, and chemical HIF stabilizers can suppress hepcidin mRNA expression in hepatoma cells (113). These findings have raised the possibility that iron-dependent prolyl hydroxylases involved in HIF degradation may act as hepatic iron sensors. Whether or not HIFs directly bind to the hepcidin promoter is currently controversial.
In vivo, hypoxia induces erythropoietin (EPO) synthesis, which in turn stimulates erythropoiesis. EPO injection into mice reduces hepcidin levels in a dose-dependent manner and can override signals that activate hepcidin expression. Even low dose EPO injections in human volunteers promptly decrease urinary excretion of hepcidin (114). Because experimental blockade of erythropoietic activity prevents its effect, EPO likely suppresses hepcidin by stimulation of erythropoiesis rather than more directly (115).

1.4.5.3 Regulation by Inflammatory and Stress Signals

The inflammatory cytokines IL1 and IL6 are both potent inducers of hepcidin expression, a response whose clinical importance for ACD has been discussed above. IL6 activates the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway, which activates the hepcidin promoter via a STAT-binding motif close to the transcription start site (116). The BMP signaling pathway also contributes to the inflammatory response via SMAD4 (100,101). Mice injected with lipopolysaccharide (LPS) augment hepcidin transcription even in the context of iron overload; likewise, LPS counteracts the diminished hepcidin expression in response to iron deficiency, suggesting that the two signals are integrated at the hepcidin promoter and that inflammatory and iron stores regulators operate independently rather than following a strict hierarchy (117). Hepcidin expression is also increased by endoplasmic reticulum (ER) stress. This stress response can be controlled by the transcription factor cyclic AMP response element-binding protein H (CREBH) (118) or by the stress-inducible transcription factors CHOP and C/EBPalpha (119). It has also been suggested that increased hepcidin transcription and iron deprivation may represent defense mechanisms against excessive cell proliferation and cancer, possibly by binding of the p53 tumor suppressor protein to a response element in the hepcidin promoter (120).

1.4.5.4 Hepcidin Regulation by Anemia

Anemia and ESA administration are potent inhibitors of hepcidin expression, allowing greater iron availability for erythropoiesis (121,122). The dominance of the inhibitory effect of anemia and erythropoietic drive over the stimulatory effects of iron on hepcidin regulation is evidenced in β-thalassemias. In this disease, hepcidin levels remain low because of very high erythropoietic activity, even in the face of increasing serum iron and tissue iron deposition that ultimately lead to fatal iron overload (123,124). Although ESAs have been shown to inhibit hepcidin transcription in isolated liver cells in vitro through erythropoietin receptor signaling and inhibition of C/EBPα binding to the hepcidin promoter (125), 2 studies suggested that in vivo, the inhibitory effects of anemia or ESAs on hepcidin expression require erythropoietic activity. These studies showed that inhibition of erythropoiesis by chemotherapy, an ESA-blocking antibody, or irradiation prevented hepcidin suppression by either ESAs or anemia (115,126). It has been suggested that proliferating erythrocyte precursors may secrete a substance that circulates to the liver to inhibit hepcidin expression. Recently, 2 modulators of the TGF- β/BMP superfamily signaling pathway, growth and differentiation factor 15 (GDF15) and twisted gastrulation (TWSG1), were reported to be secreted by erythroblast precursors and have a role in hepcidin suppression in thalassemia (127,128). GDF15 is a TGF-β superfamily ligand that activates SMAD2 and SMAD3, and TWSG1 can function as a BMP agonist or antagonist (129-132).

Hepcidin suppression in response to phlebotomy or hemolysis depends on intact erythropoietic activity in mouse models: irradiation and cytotoxic inhibition of erythropoiesis prevent hepcidin suppression (115). GDF15 and TWSG1 are both released by erythroid precursors. High doses of GDF15 are detectable in the serum of patients with ineffective erythropoiesis such as β-thalassemia (127). Such pathological concentrations of GDF15 can suppress hepcidin transcription in cell models, but the underlying molecular mechanism has not yet been characterized. By contrast, lower GDF15 concentrations fail to suppress hepcidin in cellular models and are apparently ineffective in patients with sickle cell anemia, myelodysplastic syndrome, and ACD. TWSG1 expression is increased in thalassemic mice, where it is produced during early erythroblast maturation. In cellular models, the BMP-binding protein TWSG1 inhibits BMP-dependent activation of Smad-mediated signal transduction that leads to hepcidin activation (128). Correlations between TWSG1 expression, serum iron parameters, and hepcidin levels have not yet been studied in human anemias.

Notably, an iron phenotype was not described in mice with GDF15 dysregulation, and sera from patients with sickle cell anemia or myelodysplastic syndrome did not have increased GDF15 levels (133,134), suggesting that the role of these proteins in hepcidin suppression may be limited to anemias such as β-thalassemia with ineffective erythropoiesis.

1.4.6 Hepcidin Assays

Hepcidin excess has been postulated to have a role in the anemia of patients with CKD/ESRD because of decreased renal clearance and induction by inflammatory stimuli, particularly in patients who are ESA resistant. However, it is only recently that hepcidin assays have been developed to start to investigate these hypotheses. The development of immunochemical methods to detect mature hepcidin has been complicated by hepcidin's small size and its conservation in animal species. The first described hepcidin assay was an immunodot assay to measure urinary hepcidin. However, this assay is semiquantitative, laborious, and not suitable for serum hepcidin measurements (75). A commercially available immunoassay was developed to detect serum prohepcidin, but prohepcidin levels do not correlate with biological activity, iron status, or inflammation (135,136). Others have developed mass spectroscopic techniques to measure mature hepcidin in serum and urine. Although this technique has the potential advantage of being able to distinguish among hepcidin-25, hepcidin-22, and hepcidin-20, these assays depend on expensive equipment that is not widely available, and most are semiquantitative, although more recent refinements are improving the quantitative ability (59,62,137-139). In the last year, immunoassays to quantitate mature serum hepcidin have been developed, as well as an assay based on competition against hepcidin-25 labeled with iodine 125 binding to a peptide identical to the ferroportin hepcidin-binding site (88,140,141). A recently published round robin study comparing these various mass spectrometry and immunochemical-based methods to quantify urinary and plasma mature hepcidin has shown that absolute hepcidin concentrations differ widely between methods, but Spearman correlations between individual sample mean hepcidin values obtained using most methods generally were high for 7 of 8 methods tested. Analytical variance generally is low and similar for all methods, indicating the potential suitability of all methods to distinguish hepcidin levels of different samples. It was hypothesized that differences in absolute hepcidin levels between methods may be caused by the use of different calibrators, hepcidin aggregation, hepcidin protein binding, and/or the existence of hepcidin-25, hepcidin-22, and hepcidin-20 isoforms that may be detected to some extent by immunochemical methods, depending on the antibody used. This report called for efforts to further harmonize the various hepcidin assays (142).

1.4.7 Hepcidin Excess and the Anemia of CKD

Using the mentioned immunoassays to measure mature hepcidin, several groups now have confirmed that hepcidin levels are increased in patients with CKD and ESRD and inversely correlate with GFR (23-25) suggesting that decreased renal clearance contributes to the hepcidin increase in this patient population. Hepcidin levels are decreased by dialysis, but return to predialysis levels before the next dialysis session (143). Interestingly, 1 study using a mass spectrometry–based technique to distinguish among hepcidin-25, hepcidin-20, and hepcidin-22 suggested that although hepcidin-25 was increased in patients with ESRD and total hepcidin (encompassing hepcidin-25, hepcidin-20, and hepcidin-22)

correlated inversely with estimated GFR in patients with CKD not requiring dialysis, no significant correlation was found between hepcidin-25 and estimated GFR in patients with CKD not requiring dialysis (63). These results need to be confirmed in larger studies.

Hepcidin levels correlate with markers of iron burden in the CKD population, as in other patient populations, particularly serum ferritin (144). In multivariate analyses, ferritin level is the strongest predictor of serum hepcidin level. Hepcidin levels also are increased by iron administration in the CKD population, as in healthy controls. Interestingly, although hepcidin is stimulated by inflammation and has been increased in patients with inflammation, defined as high C-reactive protein (CRP) levels, initial studies of patients with CKD have not shown a consistent or robust correlation between levels of hepcidin inflammatory markers, such as CRP, erythrocyte sedimentation rate (ESR), or IL-6. One explanation for these findings may be related to the patient populations selected for these studies, some of which excluded patients with active illness or infection.

Hepcidin levels are associated with anemia in dialysis patients, consistent with a role for hepcidin excess in the anemia of CKD; however, there is an inverse correlation between ESA dose and hepcidin level, arguing against a diagnostic role for hepcidin as a predictor of ESA resistance. The likely explanation for this finding is that ESA administration is an inhibitor of hepcidin expression in the CKD population, as in the general population. It has been proposed that the initial decrease in hepcidin levels after starting ESA therapy might be a better indicator of long-term responsiveness to ESAs (145).

Given the limited number and size of studies to date, the unresolved issues surrounding hepcidin assays themselves, and the numerous factors that can modulate hepcidin levels in the CKD/ ESRD population, including iron administration, ESA administration, body iron burden, inflammation, renal clearance, and dialysis (Fig. 6), more studies are needed to determine whether hepcidin will have diagnostic utility as a measure of iron status, inflammatory status, and/or ESA responsiveness or resistance.

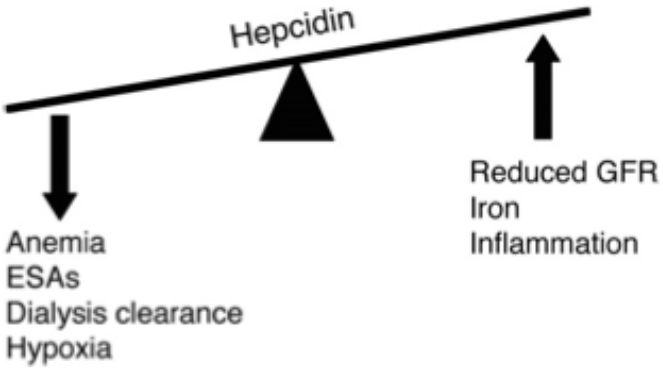


Figure 6. Hepcidin levels in patients with chronic kidney disease and end-stage renal disease. Hepcidin levels are increased in patients with chronic kidney disease and end- stage renal disease and reflect the balance of stimulatory factors: decreased renal clearance (GFR, glomerular filtra- tion rate), inflammation, and iron administration; and inhibitory factors: anemia, erythropoiesis-stimulating agent (ESA) administration, clearance by dialysis, and hypoxia.

1.4.8 Current Management of ACD

Anemia often complicates the underlying chronic diseases and is consistently a predictor of poor prognosis of the disease, longer hospitalization, cognitive impairment, heart failure and increased morbidity.
Although survival benefits have not yet been tested in prospective randomized controlled trials, treatment of anemia has been demonstrated to improve the quality of life and energy levels for hemodialysis, cancer and rheumatoid arthritis patients with concurrent ACD (146-148).
The treatment of choice in ACD is the cure of the underlying chronic disease. However, this is not possible for patients with incurable chronic diseases like cancers and chronic kidney disease. Current therapeutic management of ACD in the clinic involves increasing red blood cells by blood transfusions, erythropoiesis stimulating agents (ESAs) and/or iron administration.
Anemia of chronic kidney disease (CKD) occurs primarily due to a complex interplay of erythropoietin deficiency, shortened red blood cell survival and iron deficiency. Early clinical trials involving recombinant human erythropoietin (rHU EPO) demonstrated increased hematocrit and reticulocytosis in anemia of patients (149). In 1989, the US FDA approved the use of the rHU EPO epoietin alpha for treatment of anemia of CKD. Subsequently, epoietin alpha and other similar ESAs have been used to treat anemia of rheumatoid arthritis, anemia of cancers and other chronic diseases (150-153). Not only did the ESAs improve hemoglobin levels, quality of life scores, energy levels and work capacity were also increased in patients with ACD (154,155).

However, some patients with ACD are poorly responsive to ESAs, leading to a requirement of higher dosing to achieve target hemoglobin levels. Clinical trial results from the Correction of Hemoglobin in Outcomes and Renal Insufficiency (CHOIR) and Trial to Reduce cardiovascular Events with Aranesp Therapy (TREAT) revealed that patients receiving ESA doses to achieve target hemoglobin levels of >13g/dL have a higher incidence of adverse outcomes such as cardiovascular events, strokes, progression of cancers and even death. These findings in CHOIR and TREAT prompted a US Food and Drug Administration black box warning on the labels of ESAs and a cap in criteria of hemoglobin target levels at 11-12g/dL (156-159).

Due to the functional iron deficiency in ACD, iron supplementation is frequently administered either alone or in combination with ESAs. Although oral iron supplement is widely available, cheap, and easy to administer, it is ineffective compared to intravenous (IV) iron therapy in ACD patients owing to hepcidin mediated block in intestinal iron absorption. IV iron administration alone has been used successfully to raise hemoglobin levels in patients with inflammatory bowel disease associated anemia (160). The DRIVE (Dialysis Patient's Response to IV iron and with Elevated Ferritin) trial revealed that anemic hemodialysis patients receiving intravenous ferric glucconate and erythropoietin had a faster and better response in hemoglobin levels compared to those receiving only erythropoietin alone. This suggests that IV iron can relieve the functional iron deficiency in ACD, at least in patients with elevated ferritin and reduced transferrin saturation (161). Subsequent studies confirm the observation that correction of the functional iron deficiency with IV iron supplementation can significantly improve the effects of ESAs (162) and even reducing the required ESA dosage (163).

A recent double-blinded trial, however, finds no additional benefit of providing iron therapy and the ESA darbopoietin alfa in patients with chemotherapy-associated anemia (164). The negative result could be due to higher total body iron load (ferritin levels) or different iron dosing amounts, in this patient population compared to other studies. However, a closer analysis of the data suggests that patients with lower hepcidin levels responded better to parenteral iron with ESAs compared to patients with high hepcidin levels (165).

While IV iron can overcome some degree of iron blockade in ACD, there is also a safety concern for iron therapy in ACD associated with infection or ACDs with coexisting infection. Based on the idea that pathogens need iron for growth, providing iron therapy may be detrimental as it fuels the pathogen's iron needs for survival and propagation. Recommendations for management of these patients tend to err on the safe side while not inhibiting the usage of iron (166). The cause for concern arose from the Pemba trial where universal supplementation of iron and folic acid to children with iron deficiency anemia in malaria endemic regions resulted in a higher incidence of sudden illness and death (167).

Additionally, administering IV iron has been shown to increase hepcidin levels in CKD patients on hemodialysis (24). The rise in hepcidin would later cause iron blockade in the macrophages, rendering the administered iron unavailable for erythropoiesis, thereby potentially worsening the underlying pathophysiology of ACD. Consistent with this, ferritin levels were also increased in these patients (24).

The long term effects of IV iron therapy and concurrent increased iron stores in ACD patients have not been investigated.
Thus, conventional methods using ESAs and iron therapy do not fully address the root cause of the pathophysiology of ACD, that is, excess hepcidin. The use of ESAs is not without risk and may even be fatal. Iron therapy also carries risks of worsening underlying infection and exacerbating anemia by increasing hepcidin. Alternative therapies, which target hepcidin synthesis and its action, are discussed below.

1.4.9 New Therapeutic Strategies for ACD That Target the Hepcidin–Ferroportin Axis

Alternative therapies for ACD that target the hepcidin–ferroportin axis are attractive treatment options. Therapeutics that decrease hepcidin production and increase ferroportin activity would improve iron bioavailability from the diet and would mobilize existing body iron stores for erythropoiesis, without the adverse risks from supraphysiologic iron or ESA therapies. A number of strategies that inhibit hepcidin function (direct hepcidin antagonists), prevent the transcription of hepcidin (hepcidin production inhibitors), or promote resistance of ferroportin to hepcidin action (Ferroportin agonists/stabilizers) are currently under investigation (Fig. 7, red lines.

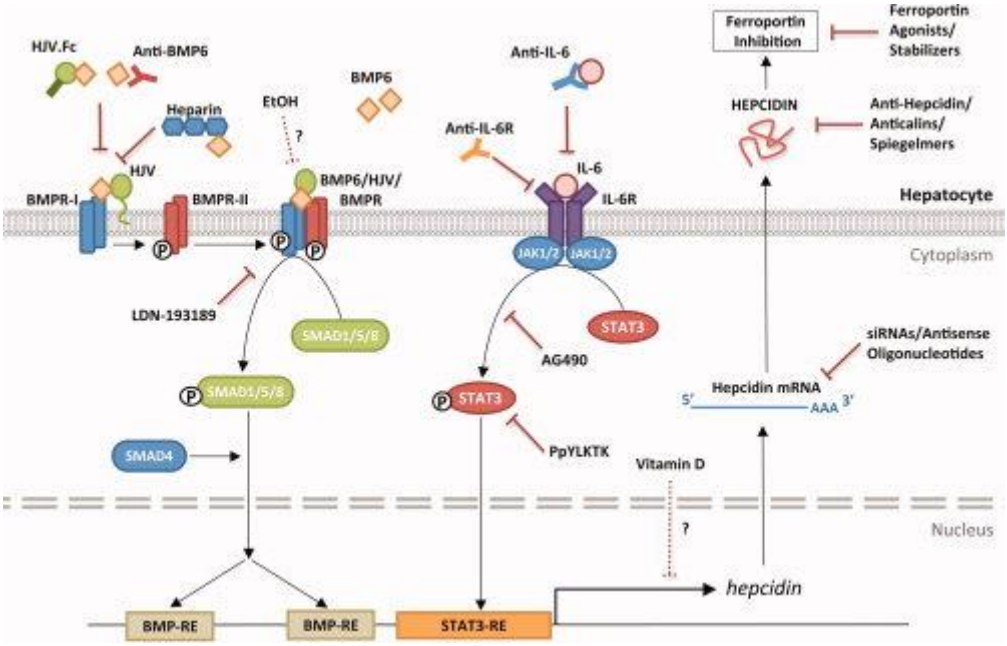


Fig. 7. Inhibitors that target the hepcidin–ferroportin axis are potential therapeutic avenues for treating ACD. As excess hepcidin leads to ACD, blocking the signaling pathways responsible for hepcidin synthesis, neutralizing hepcidin's effect on ferroportin or promoting ferroportin function may ameliorate ACD (red lines). Hepcidin production can be effectively inhibited by the following BMP6–HJV–SMAD inhibitors: soluble HJV.Fc protein (Ferrumax Pharmaceuticals), monoclonal anti-BMP6 antibodies, or the glycosaminoglycan heparin sequester BMP6, preventing its interaction with BMPR and membrane anchored HJV; the dorsomorphin derivative LDN-193189 inhibits BMP Type I receptor activity. Alcohol may also inhibit hepcidin synthesis by dampening the BMP-SMAD pathway. Anti-inflammatory therapeutics suppress IL-6-mediated hepcidin gene expression by: blocking antibody to IL-6 Siltuximab (Janssen Biotech); neutralizing antibody to IL-6 receptor Tocilizumab (Genentech); or using STAT3 pathway inhibitors, which block the phosphorylation of STAT3 (AG490) or its transcription factor binding activity (PpYLKTK). Vitamin D can also downregulate hepcidin transcription, but the mechanism is unknown. Hepcidin protein expression or activity may be inhibited directly by: hepcidin siRNA (Alnylam Pharmaceuticals); hepcidin antisense oligonucleotides (Xenon Pharmaceuticals and ISIS Pharmaceuticals); or direct hepcidin antagonists, including anti-hepcidin antibodies (Amgen, Eli Lilly), hepcidin sequestering anticalins (Pieris AG), or hepcidin binding spiegelmers (NOXXON Pharma). Ferroportin agonists/stabilizers, which modify the ferroportin–hepcidin interaction or increase ferroportin's resistance to hepcidin (Eli-Lilly), may theoretically ameliorate anemia by maintaining ferroportin activity and allowing iron influx.

1.4.9.1 Direct Hepcidin Antagonists

1.4.9.1.1 Antihepcidin antibodies

Therapeutic monoclonal antibodies have revolutionized the management of cancers and autoimmune diseases (168). One strategy under investigation for neutralizing hepcidin activity is with antihepcidin antibodies. Studies performed by Amgen demonstrated that a humanized antihepcidin monoclonal antibody (mAb2.7) can reverse iron restriction and prevent anemia progression in a mouse model of anemia of inflammation when used in combination therapy with ESA (169). However, neither antihepcidin antibodies nor ESA alone could prevent the anemia progression in their model. Recently, the authors performed a pharmacokinetics study on normal cynomolgus monkeys and determined that a dose of 300 mg/kg per week of another monoclonal antihepcidin antibody (Ab12B9m) was required to effectively decrease free hepcidin levels below baseline. Although Ab12B9m has a high affinity (Kd = 1 pM) for hepcidin and a long half life estimated at 16.5 days, the high rate of hepcidin production (7.6 nmol/kg/h) would require large quantities of the antibody to achieve significant blocking effect (170). A US patent (#7820163) was recently assigned to Eli Lilly and Company for the use of antihepcidin monoclonal antibodies as treatment for anemia. These antibodies bind with high affinity (Kd = 80 pM) to human and monkey hepcidin and can block its action on ferroportin. In a cynomolgus monkey IL-6-induced hypoferremia model, antihepcidin monoclonal antibody therapy prevented the hypoferremia for up to 12 hr (171). The antibodies are currently in Phase 1 clinical trials for the treatment of anemia according to the Lilly company website (172).

1.4.9.1.2 Short interference RNA and antisense oligonucleotides against hepcidin

RNA interference (RNAi) and gene silencing antisense oligonucleotides that target transcription or translation of hepcidin represent another approach to developing therapeutics for ACD. Amgen recently described a short hairpin RNA (shRNA) strategy against hepcidin that successfully reduced its expression and corrected anemia in a mouse model of anemia of inflammation . Profound suppression of hepcidin alone was sufficient to redistribute stored body iron into the circulation and to correct the anemia in these mice. Moderate hepcidin suppression alone was unable to correct the anemia and required ESA coadministration to be effective [169]. Alnylam Pharmaceuticals is currently developing hepcidin RNAi (ALN-HPN) to silence hepcidin gene expression and increase serum iron levels. They expect to file an Investigational New Drug application in 2012 for ALN-HPN for the treatment of erythropoietin resistant ACD and iron refractory anemias (173).
Significant technical challenges will need to be overcome to apply RNAi therapeutics for treatment in humans. The challenges include effective design of the RNAi without off-target effects, instability of RNAi in vivo, lack of biocompatibility of the delivery system, and nonspecific targeted delivery to organs/cells (174). Viral delivery systems, while highly efficient in delivering shRNA

or RNAi, can carry risks of random genome integration and induction of unfavorable immunological responses (175). Notably, shRNA against hepcidin delivered in an AAV8 capsid serotype virus system caused some deaths in mice.
Antisense oligonucleotides that inhibit translation of hepcidin or its regulators such as HJV are currently in discovery stages of development. Xenon Pharmaceuticals and ISIS Pharmaceuticals have been collaborating since 2010 to develop this technology to treat anemia of infection (176). Systemic delivery of antisense molecules results in preferential delivery to the liver, making them appealing agents to target hepcidin (177). While the approach is theoretically feasible to dampen hepcidin production, it has not been demonstrated that antisense hepcidin oligonucleotides are effective in increasing serum iron concentration and ameliorating inflammatory anemia in vivo. In addition, antisense oligonucleotides share similar technical challenges with RNAi therapies as described before.

1.4.9.1.3 Hepcidin binding proteins

Lipocalins are secreted proteins whose four-peptide loop cavity forms a binding site with high structural plasticity (178). Their simple structure and natural ability to recognize and bind various small hydrophobic ligands and specific cell-surface receptors make them suitable for engineering a new class of therapeutic proteins for specific blocking purposes (179). These engineered lipocalins are known as anticalins. Scientists at the Technical University Munich and Pieris AG have developed an anticalin PRS-080, which exhibits high affinity binding (Kd = 0.1 nM) to human hepcidin based on enzyme-linked immunosorbent assay (ELISA) studies and surface plasmon resonance. They also demonstrated that pretreatment with 95 mg/kg of PRS-080 can completely neutralize the short-term hypoferremia induced by injection of synthetic human hepcidin in mice (180). According to their company website, Pieris AG recently received support of six million Euro from a EU FP7 Grant to progress PRS-080 from preclinical development through Phase 1b clinical trials (181). Further characterization of this hepcidin blocking anticalin will be needed to determine its safety, tolerability, and efficacy for relieving hepcidin-mediated iron blockade in ACD.

1.4.9.1.4 Hepcidin binding spiegelmers

Aptamers are synthetic single-stranded oligonucleotides that bind ligands with high affinity, representing a novel class of oligonucleotide structures suitable for blocking purposes. Spiegelmers (Spiegel, which means “mirror” in German) are mirror image aptamers trademarked by NOXXON Pharma. Spiegelmers are attractive therapeutic agents because of their high resistance to nuclease activity, good stability in vivo, and low immunogenicity (182,183). NOX-H94 is a polyethylene glycol conjugated (PEGylated) spiegelmer developed by NOXXON Pharma AG that specifically targets and binds to human hepcidin (184). It was demonstrated to be effective in vitro by blocking hepcidin-induced ferroportin degradation in cells. Additionally, it was able to increase serum iron concentration in a IL-6 induced high hepcidin model in cynomolgus monkeys (185). Preclinical

studies suggest spiegelmers are safe, well tolerated, and effective in animals (186-190). The company has initiated a first-in-human clinical trial to evaluate safety, tolerability, and efficacy of NOX-H94. Administration of PEGlyated aptamers was recently shown to lead to an accumulation of the oligonucleotides in macrophages throughout the body, it is currently unknown whether chronic systemic administration of PEGylated aptamers will lead to adverse effects (191).

1.4.9.2 Hepcidin Production Inhibitors

Given the rapid rate of hepcidin production, targeting the positive regulators of hepcidin to reduce its expression may be more effective than direct blockade of hepcidin action. Two signaling pathways have been targeted thus far to inhibit hepcidin synthesis: BMP6–HJV–SMAD pathway inhibitors and IL-6–STAT3 pathway inhibitors.

1.4.9.2.1 BMP6–HJV–SMAD pathway inhibitors

The BMP family of ligands represents the largest subgroup of the TGFβ superfamily. Twenty BMP family members have been identified with diverse functions including embryogenesis, osteogenesis, neurogenesis, and iron metabolism (192,193). Like other members of the family, BMP6 has osteogenic potential. However, BMP6 is crucial in regulating hepcidin and iron metabolism, because the main phenotype of the Bmp6 knockout mice is impaired hepcidin production and severe iron overload (95,96). Blocking hepcidin transcription by targeting the hepatic BMP pathway and more specifically BMP6 may therefore be a useful strategy for treating ACD.

Dorsomorphin is a small molecule inhibitor of BMP receptor Type I kinases that was identified in a zebrafish embryo screen. It was also shown to inhibit BMP-, HJV-, and IL-6-stimulated hepcidin expression in cultured hepatocytes and block iron induced hepcidin mRNA in zebrafish and mice (103). However, dorsomorphin (also known as compound C) is a relatively nonselective kinase inhibitor that also inhibits AMP kinase (194). A derivative of dorsomorphin, LDN-193189 was shown to have improved potency and selectivity as a BMP inhibitor (195) and was able to inhibit excessive BMP signaling in vivo (196). We have recently succeeded in using LDN-193189 to reverse anemia associated with streptococcal peptidoglycan-polysaccharide (PG-APS)-induced chronic arthritis in rats (197). Compared to mock treatment, LDN-193189 treatment over a period of 4 weeks reduced hepatic hepcidin mRNA levels, increased serum iron concentration, increased ferroportin expression in splenic macrophages, and importantly, improved hemoglobin levels and hematocrit in anemic rats. Consistent with our data, LDN-193189 treatment prevented an acute inflammatory anemia induced by turpentine injections in mice (198). However, profiling of LDN-193189 against a panel of 123 human kinases revealed that it not only blocks the BMP pathway but also potently inhibits VEG-F and components of the mitogen-activated protein kinase (MAPK)–extracellular signal-regulated protein kinase (ERK) pathway (199). Thus, LDN-193189 is not as

specific a BMP inhibitor as initially thought, and caution must be used when interpreting results from studies using LDN-193189.

We have shown that a soluble form of the human hemojuvelin protein linked to the constant region of IgG1 (HJV.Fc) can decrease BMP-mediated hepcidin expression in vitro. Additionally, HJV.Fc administration into healthy rodents blocked SMAD activation, decreased hepcidin expression, mobilized splenic iron stores, and increased serum iron levels (200). In collaboration with Ferrumax Pharmaceuticals, our recent study in rats with PG-APS-induced ACD indicated that pharmacologic inhibition of hepatic BMP signaling with HJV.Fc reduced hepcidin mRNA within 24 hr. Longer-term treatment for 4 weeks showed that HJV.Fc inhibited phosphorylation of hepatic SMAD 1/5/8, increased splenic ferroportin levels and serum iron levels, and rescued the anemia in ACD rats (197). The safety and efficacy of HJV.Fc in human patients has not yet been determined.

Anti-BMP6 monoclonal antibody therapy is another option to specifically block BMP6-mediated hepcidin regulation. Administration of anti-BMP mAb in healthy mice decreased hepatic hepcidin expression and increased serum iron levels (95). In the Hfe transgenic mouse model of excess hepcidin and IDA, anti-BMP6 administration improved the anemia by lowering hepcidin levels (201). Several caveats surround the targeting of BMP6 as a therapeutic strategy. Whether BMP6 is the most important BMP in the physiologic regulation of hepcidin in humans has not yet been shown. Due to high amino acid sequence similarity among the BMP family of ligands (202), one technical challenge is to generate antibodies that can specifically recognize BMP6 without significant cross-reactivity to other BMPs. Finally, the safety of lowering BMP6 in humans is unknown.

Recently, heparin was implicated in sequestering BMP activity and lowering hepcidin expression. Heparin is a highly sulfated glycosaminoglycan that is widely used pharmacologically as an anticoagulant. Pretreatment of cultured hepatoma cell lines with heparin decreased basal levels of BMP-SMAD signaling and inhibited BMP6-mediated hepcidin transcription. Additionally, injection of heparin (50 mg/kg) into healthy mice decreased hepatic SMAD phosphorylation and reduced hepcidin expression, leading to mobilizing of spleen iron stores and increased circulating iron. Moreover, deep vein thrombosis patients treated with heparin have decreased serum hepcidin levels and increased serum iron levels (203). Whether heparin will become a useful strategy to treat ACD remains to be determined, as heparin is known to have significant adverse side effects including life-threatening bleeding, heparin-induced thrombocytopenia, hyperkalemia, alopecia, and osteoporosis (204).

Alcohol loading in mice has been shown to decrease hepcidin mRNA and increase iron absorption through the intestines (205). The mechanism was recently elucidated to involve inhibition of the BMP-SMAD pathway (206). Chronic alcohol consumption in humans is associated with excess iron accumulation in the liver, and hepcidin may be a contributing factor to the progression of alcoholic liver disease (207). The therapeutic potential of using alcohol for the treatment of ACD is an interesting strategy that must be tempered by the well-known deleterious side effects of excess alcohol use.

1.4.9.2.2 IL-6 pathway inhibitors

Inhibitors of the IL-6 pathway have been shown to downregulate hepcidin expression and improve anemia of inflammation in Multicentric Castleman's Disease, a rare lymphoproliferative disorder marked by excessive production of IL-6 in the lymph nodes and associated with hypochromic and microcytic anemia. Serum hepcidin levels were also elevated in these patients, most likely due to IL-6-mediated hepcidin synthesis. Most MCD patients (5 out of 6) when treated with the anti-IL-6 receptor antibody (anti-IL-6R) Tocilizumab for 6–12 months had lower serum hepcidin levels, normalization of hemoglobin levels, and experienced beneficial effects on disease symptoms such as reduced fatigue, increased weight, and alleviation of fever (208). Another study demonstrated that IV administration of anti-IL-6R antibodies ameliorated anemia in a monkey model of collagen-induced arthritis. In this model, anti-IL-6R antibody therapy effectively lowered hepcidin levels, decreased C-reactive protein (CRP) levels within 1 week, and improved hematological parameters over a treatment period of 4 weeks (209).
Blocking antibodies to the IL-6 ligand also resulted in similar hepcidin lowering effects. The anti-IL-6 chimeric monoclonal antibody, Siltuximab, was recently demonstrated to increase hemoglobin levels by 2.1 g/dL in an open label, dose finding, Phase 1 study in MCD patients (210). The major complication of blockade of IL-6 activity appears to be increased risk of infections (211,212).
In addition to IL-6 ligand/receptor blockade, inhibition of the JAK1/2–STAT3 signaling cascade can also lower hepcidin expression. The JAK2 inhibitor AG490 and synthetic peptide inhibitor of STAT3 (PpYLKTK) have been actively studied to target cancers with elevated JAK/STAT activity (213-215). AG490 inhibits the phosphorylation of STAT3 by JAK2, while the PpYLKTK peptide disrupts pSTAT3 dimerization, which is required for binding target genes. Faith et al. demonstrated that both compounds were able to decrease phosphorylation of STAT3 and downregulate IL-6-mediated hepcidin expression in a mouse liver coculture system (216). The use of STAT3 inhibitors to modulate hepcidin production in vivo has not yet been reported.

1.4.9.2.3 Vitamin D

Vitamin D is a hormone synthesized by the skin on exposure to UV light and activated in the kidney. Primarily known for regulating calcium and promoting bone health, vitamin D has also been implicated in a wide range of cellular activities including differentiation of hematopoietic cells and down regulation of proinflammatory cytokines transcripts (217,218). Vitamin D deficiency is associated with a higher prevalence of anemia of inflammation in elderly people (219). Vitamin D deficiency is also a common occurrence in CKD and hemodialysis patients (220). In a recent pilot study, vitamin D supplementation had an erythropoietin (EPO) sparing effect in vitamin D deficient hemodialysis patients (221). The results were consistent with observations from a retrospective study where vitamin D repletion in anemic CKD patients not on hemodialysis correlated with a lowering of EPO dose requirements (222).

A mechanism for the EPO sparing effects of vitamin D is suggested by recent data demonstrating a hepcidin lowering effect of vitamin D. In vitro treatment with vitamin D of monocytes isolated from hemodialysis patients downregulated hepcidin transcription. Furthermore, oral administration of vitamin D in healthy volunteers lowered serum levels of hepcidin by 50% compared to baseline levels within 24 hr and persisted for 72 hr (223). Supplementation with vitamin D has also been reported to have beneficial effects on increasing erythropoiesis (224) and decreasing inflammation (225). These initial results are promising, and a randomized controlled study is warranted to determine whether correction of vitamin D deficiency can ameliorate ACD.

1.4.9.2.4 Ferroportin Agonists/Stabilizers

Hepcidin excess prevents iron absorption from the diet and blocks iron release from body stores by binding to and inducing the degradation of the iron export protein ferroportin. In addition to reducing hepcidin production and blocking its activity, agents that stabilize ferroportin on the cell surface may be useful for correcting the functional iron deficiency in ACD. In a study characterizing the molecular mechanism of ferroportin disease with parenchymal iron overload and resistance to hepcidin, the thiol form of Cys326 in ferroportin was found to be essential in hepcidin–ferroportin interaction (226). In a high-throughput screening approach using human embryonic kidney (HEK) cells expressing ferroportin–green fluorescent protein (GFP) fusion protein, they discovered thiol modifier compounds that prevented ferroportin–hepcidin binding and blocked internalization of ferroportin in the presence of excess hepcidin. This screening approach also identified molecules, such as cardiac glycosides, which do not interfere with hepcidin binding but appear to prevent ferroportin internalization (227). An antiferroportin mAb developed by Eli Lilly (Patent Application #20110129480), which binds to the extracellular loop of ferroportin, can block the hepcidin–ferroportin interaction, while maintaining ferroportin function (228). These emerging strategies would promote ferroportin activity and allow continuous iron influx, potentially preventing iron-restrictive erythropoiesis due to hepcidin excess. The safety and efficacy of these approaches in humans have not yet been determined.

1.4.9.3 Conclusions

Recent advances in the iron metabolism field provided valuable insights into the molecular pathophysiology of ACD as well as new potential targets for therapy. Targeting the hepcidin–ferroportin axis with novel therapeutics that inhibit the BMP6–HJV–SMAD and the IL-6–STAT3 pathways for hepcidin production, antagonize hepcidin activity, or promote ferroportin function may lead to better management of the iron maldistribution and its contribution to the consequent anemia of this common but clinically important disorder.

2. AIM

In patients with CKD the behaviour of hepcidin and other molecules related to iron absorption and transport remains unexplored.
To investigate these effects and the responses of these components, an animal model that represents renal anaemia as seen in patients with CKD is necessary. Therefore, we focused on rats with adenine-induced renal failure.
Adenine treatment in rats has been proposed as an animal model of anemia of CKD with high hepcidin levels that mimics the condition in patients (229).
This model is generated by giving rats a diet supplemented with 0.75% adenine for 8 weeks, inducing irreversible kidney disease and severe anemia within 4 weeks that persists for another 4 weeks. However, this model has a high mortality rate, and the hematologic and iron phenotypes have not been fully characterized.
In this study, we generated a modified adenine diet model that improves the survival rate while maintaining irreversible renal failure and anemia. Rats were given 0.75% adenine diet for 3 weeks followed by a normal diet for 3 weeks, and then characterized for hematological profile, renal parameters, iron status, and hepcidin expression. We then investigated whether the small molecule BMP inhibitor LDN-193189 was able to inhibit hepatic hepcidin mRNA expression and mobilize stored iron into plasma in adenine-treated rats to correct the reticuloendothelial cell iron blockade and anemia.

3. MATERIALS AND METHODS

3.1 Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at the Massachusetts General Hospital.
Eight-week-old Wistar male rats (Charles River Labs) were housed in the Massachusetts General Hospital rodent facility. Rats were randomized into control diet and adenine diet groups each receiving equal amounts of either standard/ control (Prolab 5P75 Isopro RMH 3000 diet containing 380 p.p.m. iron) or 0.75% adenine supplemented rodent chow (Harlan-Teklad) with similar iron composition.
To establish the original adenine model, rats in the adenine diet group (n = 12) and control diet group (n = 8) were given 0.75% adenine diet and standard rodent chow respectively for 6 weeks. Tail vein blood draws were performed weekly and rats were sacrificed at week 4 and 6.
To establish the modified adenine model, rats in the adenine diet group (n = 57) were fed on a 0.75% adenine diet for 3 weeks and then fed on control diet over a subsequent 5 weeks period. Rats in the control diet group (n = 39) were given a control diet through all 8 weeks. Tail vein blood draws were performed every 2 weeks and rats were sacrificed at time points 1, 2, 4, 6, and 8 weeks.
For LDN-193189 treatment experiments, 1 week after adenine diet administration, 8-week-old Wistar male rats in the modified adenine group received either intraperitoneal injections of LDN (n = 8) (kindly provided by Charles Hong, Vanderbilt University) at 8 mg/ kg daily for 5 wks or the vehicle 2% 2-hydroxypropyl-B-cyclodextrin (Sigma-Aldrich) in PBS (n = 8) using the same dosing regimen. Control diet groups (n = 6) were fed on control diet all six weeks. Schematic of experimental design is depicted in Figure 1. Six hours after last injection, rats were sacrificed, blood was collected by cardiac puncture and liver tissues were harvested for analysis.
LDN-193189 is a cell permeable small molecule inhibitor of bone morphogenetic protein (BMP) type I receptors ALK2 and ALK3 (IC_{50} = 5 nM and 30 nM respectively) (196). LDN-193189 was derived from structure-activity relationship studies of Dorsomorphin and functions primarily through prevention of Smad1, Smad5, and Smad8 phosphorylation (103,195). LDN-193189 only weakly inhibits ALK4, ALK5, and ALK7.

3.2 RNA extraction and quantitative real-time PCR

3.2.1 Preparing RNA

Total RNA was isolated from rat liver tissue using the RNeasy Mini Kit (Qiagen), with deoxyribonuclease digestion using the RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions.
The total RNA had an A260/280 greater than 1.9

3.2.2 Converting Total RNA to cDNA

cDNA synthesis was performed using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions.
iScript cDNA synthesis kit provides a sensitive and easy-to-use solution for two-step RT-PCR. The iScript reverse transcriptase is RNase H+, which provides greater sensitivity than RNase H-enzymes in quantitative PCR. iScript is a modified MMLV-derived reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA. The enzyme is provided preblended with RNase inhibitor. The unique blend of oligo(dT) and random hexamer primers in the iScript reaction mix works exceptionally well with a wide variety of targets. This blend is optimized for the production of targets <1kb in length.

3.2.3 Quantitative real-time PCR

Real-time PCR is the ability to monitor the progress of the PCR as it occurs. Data is collected throughout the PCR process rather than at the end of the PCR process.
In real-time PCR, reactions are characterized by the point in time during cycling when amplification of a target is first detected rather than the amount of target accumulated after a fixed number of cycles
Real-time quantification of mRNA transcripts was performed using 2-step reverse-transcribed polymerase chain reaction (RT-PCR) using the ABI Prism 7900HT Sequence Detection System.
The two-step method, reverse transcription (RT) and PCR are performed in separate reactions, is useful for detecting multiple transcripts from a single cDNA template or for storing cDNA aliquots for later use.
It was performed absolute quantitative real time PCR with a set of standards that is generated by making serial dilutions from a sample of known quantity.
Absolute quantitation (AQ) using standard curve is the process that determines the absolute quantity of a single nucleic acid target sequence within an unknown sample. AQ assays use a standard curve to calculate the quantity of an unknown target sequence. The results of AQ experiments are reported in the same units of measure as the standard curve
Rat Hamp and Hprt transcripts were amplified with specific primers summarized in Table 1 and detected using the Power SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer's instructions.
SYBR Green reagents use SYBR® Green I dye, a double-stranded DNA binding dye, to detect PCR products as they accumulate during PCR cycles.
Samples were analyzed in duplicate or triplicate and expression levels were normalized to the housekeeping gene Hprt.

Table 1. Primers

Gene	Forward (5'-3')	Reverse (5'-3')
Hamp	ACAGAAGGCAAGATGGCACT	GAAGTTGGTGTCTCGCTTCC
Crp	GCTTTTGGTCATGAAGACATG	TCACATCAGCGTGGGCATAG
Hprt	CTCATGGACTGATTATGGACAGGAC	GCAGGTCAGCAAAGAACTTATAGCC

3.3 Blood collection

Blood was collected via at tail vein puncture under isofluorane anaesthesia at intervals of 1 or 2 weeks during the course of the study. Whole blood was collected in BD Microtainer tubes with EDTA (BD Medical No. 365973) for complete blood count analysis and reticulocyte hemoglobin analysis at the hematology core facility at Children's Hospital. Blood serum samples were obtained using BD Microtainer serum separator tubes (BD Medical No. 365956), according to the manufacturer's instructions and stored at -80 ^{o}C until time of assessment.

3.4 Hematological analysis

Hemoglobin concentration (Hb), hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin (MCH), red blood cell count (RBC), reticulocyte count (Retic), content of hemoglobin in reticulocytes (CHr), mean cell volume of reticulocytes (MCVr), platelets (PLT), and white blood cell (WBC) counts were assessed in whole blood EDTA using the ADVIA 2120i and analyzed with multispecies software using rat settings (ADVIA 2120/2120i).
The hematocrit (Ht or HCT) or packed cell volume (PCV) or erythrocyte volume fraction (EVF) is the volume percentage (%) of red blood cells in blood.
The mean corpuscular volume, or "mean cell volume" (MCV), is a measure of the average red blood cell size. The MCV is calculated by dividing the total volume of packed red blood cells (hematocrit) by the total number of red blood cells. The resulting number is then multiplied by 10.
In patients with anemia, it is the MCV measurement that allows classification as either a microcytic anemia (MCV below normal range), normocytic anemia (MCV within normal range) or macrocytic anemia (MCV above normal range).
The mean corpuscular hemoglobin, or "mean cell hemoglobin" (MCH), is the average mass of hemoglobin per red blood cell in a sample of blood.
A red blood cell (RBC) *count* is the number of red blood cells per volume of blood, and is reported in either millions in a microliter or millions in a liter of blood.

A reticulocyte count is a blood test performed to assess the body's production of immature red blood cells (reticulocytes).
Content of hemoglobin in reticulocytes (CHr) represents the hemoglobin content in reticulocytes, the youngest red blood cells (RBC) which transition to mature red blood cell in 1 to 2 days.

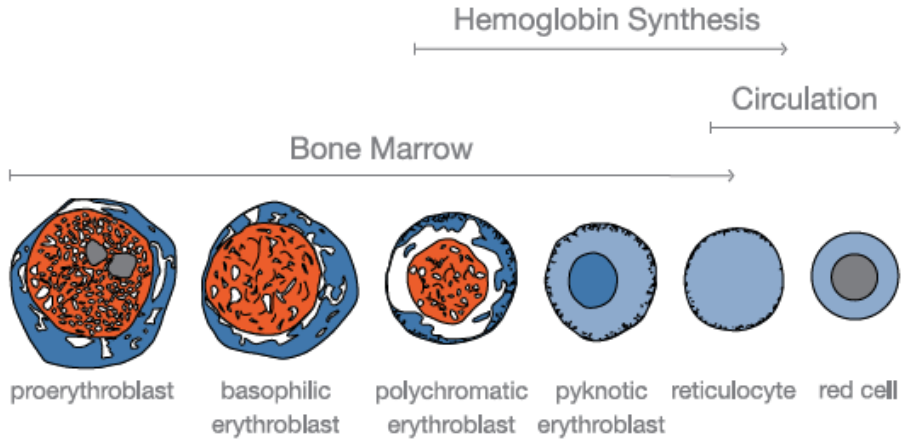


Figure 8. The stages of red blood cell maturation.

The PLT blood test is also known as the *platelet blood test* or a platelet count. PLT in blood test stands for 'platelet'. PLT testing is done to count how many blood platelets there are in our blood.
White blood cells, or leukocytes play a role in reducing inflammation in the body. A high white blood cell count could mean that there is inflammation

3.5 Renal and liver function and electrolyte analyses

Serum samples were analyzed using a DRI-CHEM veterinary chemistry analyzer (Heska Corportation, Loveland, CO) at the MGH Clinical Pathology Core for the following biochemical parameters: Serum creatinine (Cre), blood urea nitrogen (BUN), total protein, albumin (Alb), globulin, glucose (Glu), cholesterol, Alanine transaminase (ALT), Alkaline phosphatase (ALP), Gamma glutamyl transpeptidase (GGT), total bilirubin (TBIL), sodium (Na), potassium (K), calcium (Ca), inorganic phoshorus (P), Choride, and Na/K ratio.
Creatinine is a non-protein waste product of creatine phosphate metabolism by skeletal muscle tissue. Creatinine production is continuous and is proportional to muscle mass. Measuring serum creatinine is a useful and inexpensive method of evaluating renal dysfunction.
Creatinine is freely filtered and therefore the serum creatinine level depends on the Glomerular Filtration Rate (GFR). Renal dysfunction diminishes the ability to filter creatinine and the serum creatinine rises. If the serum creatinine level doubles, the

GFR is considered to have been halved. A threefold increase is considered to reflect a 75% loss of kidney function.
The blood urea nitrogen test is a measure of the amount of nitrogen in the blood in the form of urea, and a measurement of renal function. Urea is a by-product from metabolism of proteins by the liver and is removed from the blood by the kidneys.
The liver produces urea in the urea cycle as a waste product of the digestion of protein.
The total protein test measures the total amount of two classes of proteins found in the fluid portion of your blood: albumin and globulin.
This test is often done to diagnose nutritional problems, kidney disease or liver disease.
Serum glucose (BG, Glu).
The liver's ability to produce glucose (gluconeogenesis) is usually the last function to be lost in the setting of fulminant liver failure.
A complete cholesterol test — also called a lipid panel or lipid profile — is a blood test that can measure the amount of cholesterol and triglycerides in the blood. A cholesterol test can help determine risk of atherosclerosis, the buildup of plaques in arteries that can lead to narrowed or blocked arteries.
Alanine transaminase or ALT is a transaminase enzyme.
ALT is found in serum and in various bodily tissues, but is most commonly associated with the liver. It catalyzes the two parts of the alanine cycle.
It is measured clinically as a part of a diagnostic evaluation of hepatocellular injury, to determine liver health.
The alkaline phosphatase (ALP) test measures the amount of the enzyme ALP in the blood. The test is used to help detect liver disease or bone disorders.
Gamma-glutamyl transpeptidase (GGT) is a test to measure the amount of the enzyme GGT in the blood.
This test is used to detect diseases of the liver or bile duct disorders and bone disease.
Bilirubin is a breakdown product of heme (a part of hemoglobin in red blood cells). The liver is responsible for clearing the blood of bilirubin. Bilirubin is measured to diagnose and/or monitor liver diseases.
A sodium test checks how much sodium (an electrolyte and a mineral) is in the blood. Sodium is both an electrolyte and mineral. It helps keep the water (the amount of fluid inside and outside the body's cells) and electrolyte balance of the body. Sodium is also important in how nerves and muscles work.
Electrolytes test detects a problem with the body's fluid and electrolyte balance.

3.6 Serum iron measurements

Serum was collected and analyzed for iron concentration and unsaturated iron-binding capacity. Serum iron and unsaturated iron-binding capacity (UIBC) were measured by colorimetric assay using the Iron/UIBC kit (Thermo Electron Corp.). Total iron-binding capacity (TIBC) was calculated as the sum of serum iron and UIBC measurements, and transferring saturation percentage was calculated as serum iron / TIBC × 100.

3.7 Tissue iron measurement

Immediately after harvest, livers and spleen were sectioned into 2mm cubes and weighed. Quantitative measurement of nonheme iron was performed according to the method of Torrence and Bothwell [41]. Results are reported as micrograms iron/gram wet weight tissue.

3.8 EPO ELISA

Serum erythropoietin concentrations were measured using a mouse/rat EPO serum/plasma singleplex immunoassay kit (Meso Scale Discovery, Gaithersburg, MD). Analysis was performed per manufacturer's protocol

3.9 Statistics

Results were expressed as mean ± SEM values. A two-tailed Student's *t*-test with $P < 0.05$ was used to determine statistical significance.

4. RESULTS

4.1 Characterization of a modified adenine model of anemia of CKD

4.1.1 A modified adenine model improves survival and maintains irreversible renal failure

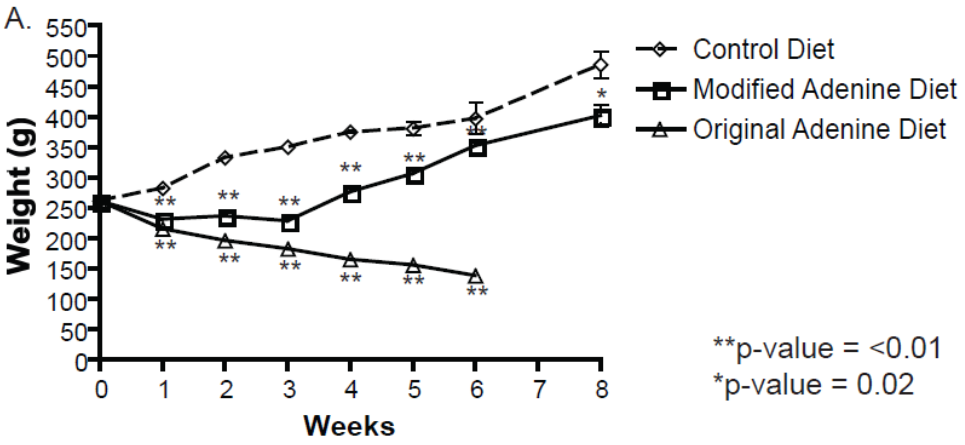
Treatment of rats with a diet supplemented with 0.75% adenine for 8 weeks has previously been proposed as an animal model of anemia of CKD with high hepcidin levels that mimics the condition in patients (229,230). We therefore tested this model in our laboratory to perform a more detailed characterization of the anemia phenotype, with the ultimate aim to test hepcidin lowering agents as a new strategy to treat disordered iron homeostasis and anemia of CKD. We found that treatment with a 0.75% adenine diet continuously lead to a very high morality rate of 66.7% (4/6) by 6 weeks. Although mortality was not mentioned in the paper by Yokozawa et al. (229) a similarly high mortality rate has been seen by other groups (231).

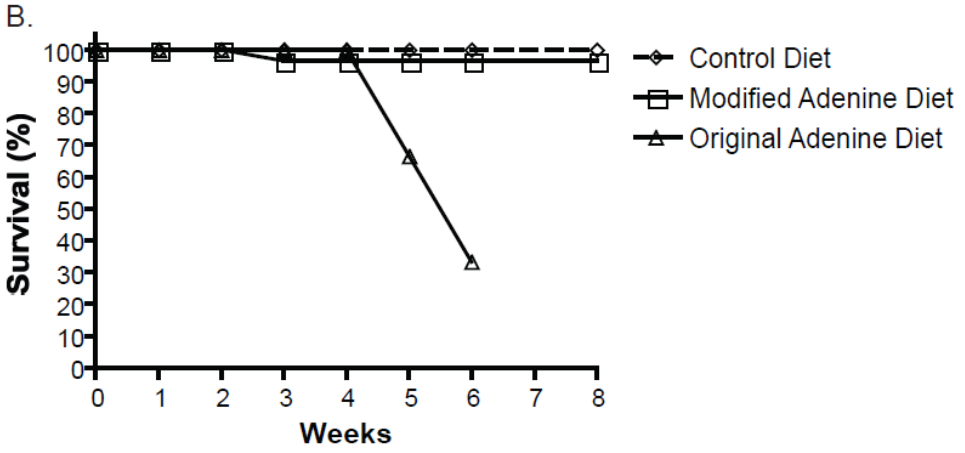
We therefore developed a modified adenine treatment model with the aim to maintain irreversible renal failure and anemia, but to improve survival. It has previously been reported that treatment with 0.75% adenine diet for 4 weeks followed by a normal diet for 4 weeks maintained irreversible renal failure and anemia with improved survival to 81. Administration of 0.75% adenine diet for 2 weeks followed by a normal diet for 4 weeks improved survival to 100%, but resulted in reversible renal failure and only a mild anemia (231) To try to improve survival further, but maintain irreversible renal failure and anemia, we tested the effects of 0.75% adenine diet treatment for 3 weeks followed by a control diet for 5 weeks -“Modified Adenine Diet”. Survival, weights and renal function parameters were compared to the continuous adenine treatment for 6 weeks –“Original Adenine Diet” and rats maintained on a control diet for 8 weeks -“Control Diet” (Figure 9).

Control diet rats had 100% survival and gained an average of 9% in weight every week up to 86% increase above the initial weight. Modified adenine diet rats at the end of the 3 week period had an 13% loss in weight compared to the pretreatment value. However, a change to control diet for the following 3 weeks allowed a steady weight gain to 53% above the initial weight by the end of the experiment (Figure 9A). In contrast, rats receiving continuous adenine diet for 6 weeks continued to lose weight, down to 51% of initial weight by the end of the experiment (Figure 9A). Rats receiving the modified adenine treatment greatly improved their survival rate to 96.4% (54/56 survived) (Figure 9B), compared to rats receiving continuous adenine diet for 6 weeks which had a 33.3% survival rate (2/6 survived) (Figure 9B).

Modified adenine diet rats had a significant increase in creatinine (Cre) and blood urea nitrogen (BUN) by week 1 that remained increased at week 6 despite a change to normal diet after week 3, indicating irreversible deterioration of kidney function with just 3 weeks of adenine-diet treatment (Figure 9C-D). Indeed, kidneys from these rats appeared pale and enlarged at the time of sacrifice at 6 weeks,

similar to the kidneys from rats receiving continuous adenine diet for 6 weeks. This increase in Cre and BUN persisted even to 8 weeks (Figure 9C-D). We observed a small increase in serum potassium in adenine treated rats, but did not observe any other significant electrolyte imbalance in this model compared to the control diet group (Table 2). We also did not observe any changes in albumin, bilirubin, ALT and ALP, indicating that the adenine treatment did not contribute to hepatic injury or toxicity (Table 2).





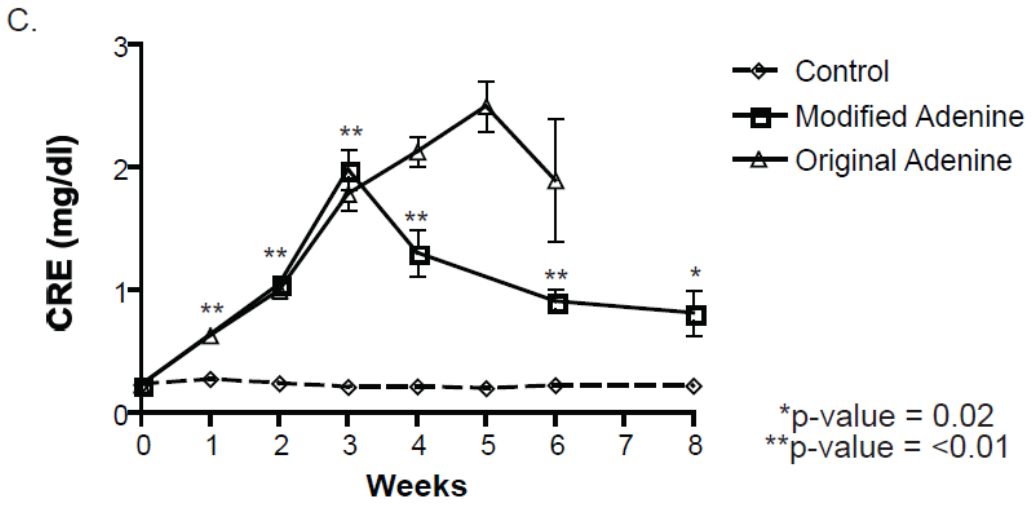


Figure 9. Increased survival and maintenance of irreversible renal failure in Modified Adenine Model compared to the Original Adenine Model

*A) Body weights (grams) for rats on control diet, adenine diet for 3 weeks followed by 5 weeks of control diet -modified adenine diet, and continuous adenine diet - original adenine diet were monitored weekly for a duration of 8 weeks. Rats on control diet (open diamonds) increased in weight each week. In contrast, rats on original adenine diet (open triangles) steadily lost weight throughout the experimental period. Rats on the modified adenine diet (open squares) lost weight in the first 3 weeks while on the adenine diet, however when control diet was introduced, their weights steadily increased almost to the weights of their control diet only counterparts by the end of 8 weeks. For each group, significant changes are shown as (** P<0.01, * P=0.02) in comparison to the control diet.*
B) Survival rates for control diet, modified adenine diet and original adenine diet were plotted as percent survival. All rats on the control diet (open diamonds) survived the 8 week experimental period, rats on the original adenine diet (open triangles) had a 30% survival rate at week 6 while rats on the modified adenine diet (open squares) had a 96.4% survival rate at 8 weeks. The surviving rats on the original adenine diet were in such poor health condition that they were sacrificed at week 6.
C) Serum creatinine (CRE) and D) serum blood urea nitrogen (BUN) were collected from all groups to measure renal function. Rats receiving control diet had low CRE and BUN levels indicating normal functioning kidneys, while rats on the original adenine diet increased CRE and BUN levels continuously indicating severe renal failure. Rats on the modified adenine diet had a steady increase in CRE and BUN until 3 weeks and maintained the increase throughout the experimental period, indicating irreversible renal failure.

Table 2. Renal and Liver biochemical parameters in Adenine rats at 6 weeks

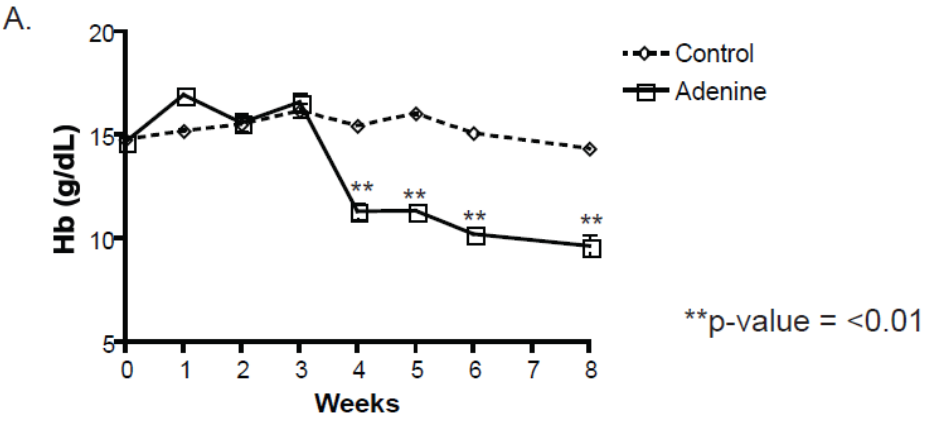
	Units	*Control Diet (N=4)*	*Adenine Diet (N=8)*
BUN	mg/dl	21.70 ± 1.8	66.36 ± 19.45*
Creatinine	mg/dl	0.25 ± 0.10	0.94 ± 0.29*
Phosphorus	mg/dl	7.28 ± 0.57	7.53 ± 1.10
Calcium	mg/dl	9.83 ± 0.92	11.13 ± 0.18
Total Protein	g/dl	5.10 ± 0.47	4.98 ± 0.21
Albumin	g/dl	3.40 ± 0.24	3.48 ± 0.17
Globulin	g/dl	1.70 ± 0.27	1.50 ± 0.11
Glucose	mg/dl	189.00 ± 25.73	166.00 ± 10.10
Cholesterol	mg/dl	63.25 ± 9.64	94.13 ± 12.96*
ALT (GPT)	U/l	28.50 ± 1.29	33.38 ± 7.01
ALP	U/l	196.00 ± 32.16	236.00 ± 28.08
GGT	U/l	11.67 ± 1.53	10.25 ± 0.50
Total Bilirubin	mg/dl	0.28 ± 0.10	0.38 ± 0.16
Sodium	mEq/l	134.50 ± 6.45	141.50 ± 2.39
Potassium	mEq/l	4.38 ± 0.25	5.08 ± 0.65*
Chloride	mEq/l	95.25 ± 4.92	101.88 ± 3.09
Na/K Ratio		30.75 ± 1.50	28.38 ± 4.07

**p-values <0.01*

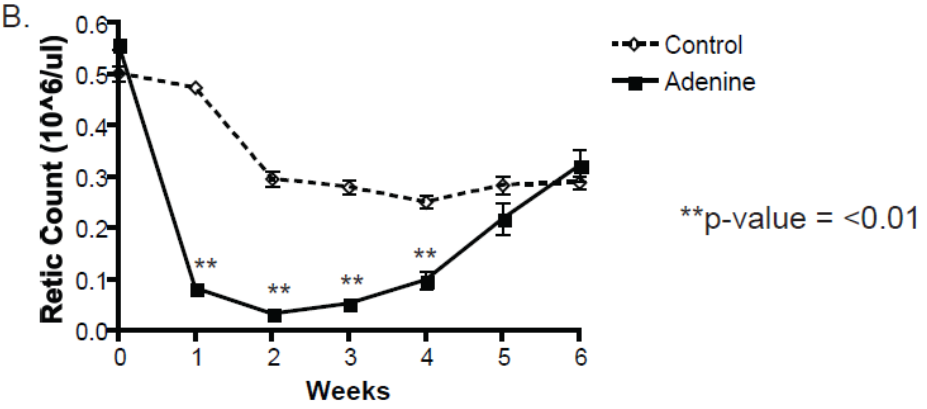
4.1.2 A modified adenine model exhibits irreversible anemia, hypoferremia, reticuloendothelial cell iron blockade and hepcidin excess.

The modified adenine diet rats developed anemia associated with renal failure at week 4 with hemoglobin (Hb) and hematocrit (HCT) levels at levels at 11g/dL and 35% compared to control diet rats with normal hemoglobin (Hb) and HCT levels at 15g/dL and 50% (Fig 10A and 11A). The anemia was long lasting, with Hb and HCT levels remaining at 10g/dL and 30% at week 6 and 9g/dL and 25% respectively at week 8 (Fig 10A and 11A). The decrease in HCT was due largely to a drastic reduction in reticulocytes (retic) after week 1 on the adenine diet, which remained decreased until week 5, at which time it approached the levels in control animals, but remained inappropriately low relative to the degree of anemia (Figure 10B). Similarly, the mean corpuscular volume of reticulocytes (MCVr) was also decreased (Figure 10C). There was also a marked decrease in iron incorporation into reticulocytes as measured by content of hemoglobin in reticulocytes (CHr) from weeks 1-3 in the adenine-treated rats compared to controls (Figure 10D). Effects on other complete blood count parameters are shown in Figure 11. These blood parameters indicate that the adenine-treated rats developed anemia within 4 weeks, characterized by a decreased production of red blood cells, and a clear lack of iron incorporation into the reticulocytes up to week 3.

A.


Hb (g/dL)
20
15
10
5
0 1 2 3 4 5 6 7 8
Weeks
Control
Adenine
**
**
**
**
**p-value = <0.01

B.


Retic Count (10^6/ul)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 1 2 3 4 5 6
Weeks
Control
Adenine
**
**
**
**
**p-value = <0.01

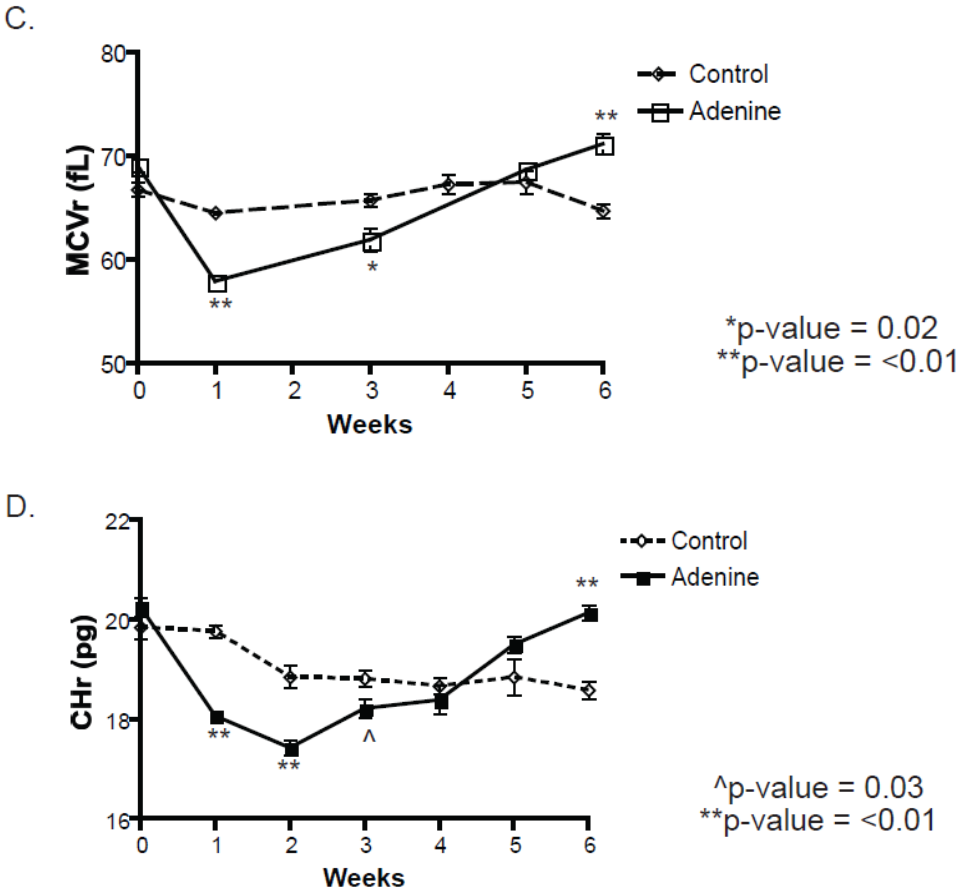
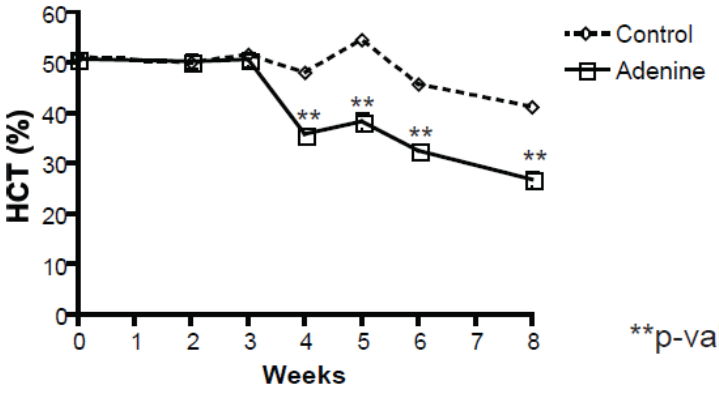


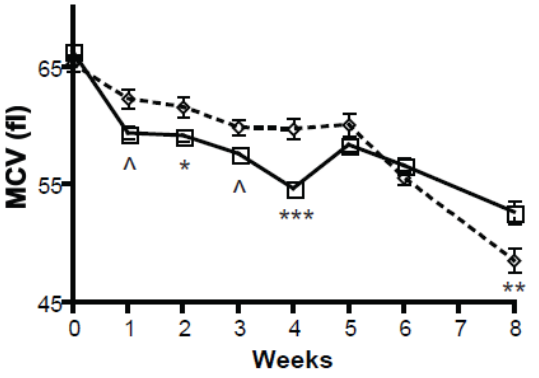
Figure 10. Characterization of Anemia in Modified Adenine Model
*A) Rats on the modified adenine diet (open squares) are anemic at week 4, with hemoglobin levels at 11 g/dL compared to 15 g/dL in control diet rats (open diamonds). The anemia associated with renal failure continues to week 8. B) Reticulocyte analysis in modified adenine diet rats show a significant reduction in reticulocyte count after 1 week on the adenine diet and remained significantly decreased until week 5, contributing to low hematocrit (HCT, Supplemental Figure 1A). C) Similarly, due to the decrease in reticulocyte number, the mean corpuscular volume of reticulocytes (MCVr) is decreased in the first 1-3 weeks on adenine diet. D) Modified adenine diet rats have decreased iron incorporation into the reticulocytes within this first 1-3 weeks as evidenced by lowered content of hemoglobin in reticulocytes (CHr) compared to control, indicating iron deficiency. Differences between groups were tested by unpaired t tests. ** P<0.01, *P=0.01, ^P=0.03 compared to control rats, comparisons were made for each time point.*

A.



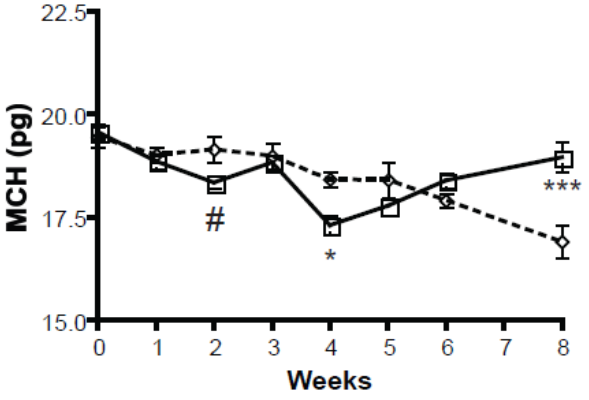
**p-value = <0.01

B.



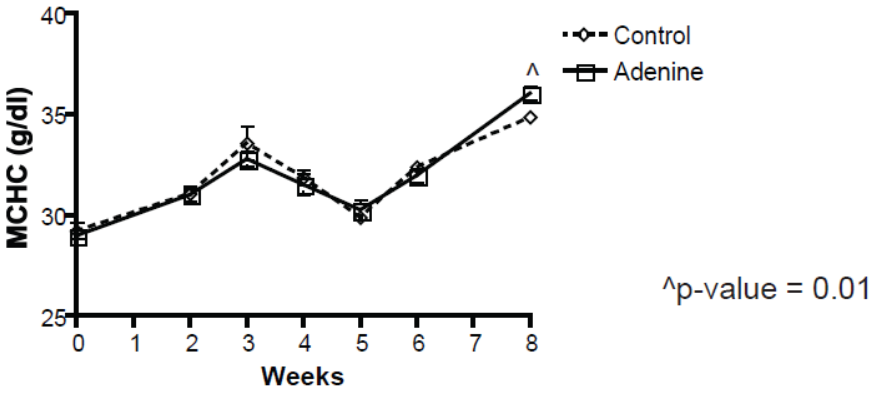
^p-value= 0.01
*p-value = 0.03
**p-value = 0.02
***p-value = <0.01

C.

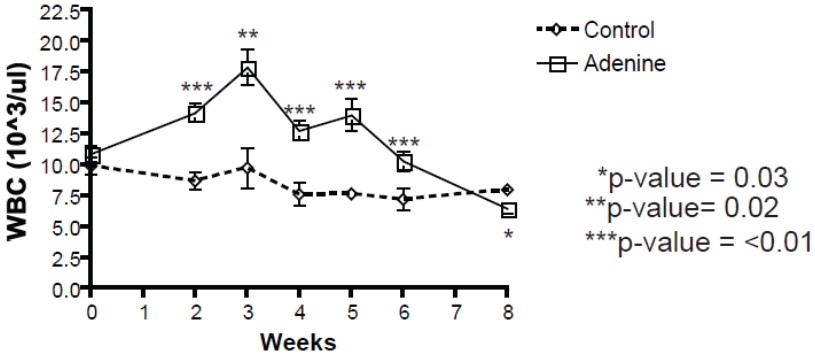


#p-value = 0.05
*p-value = 0.02
***p-value = <0.01

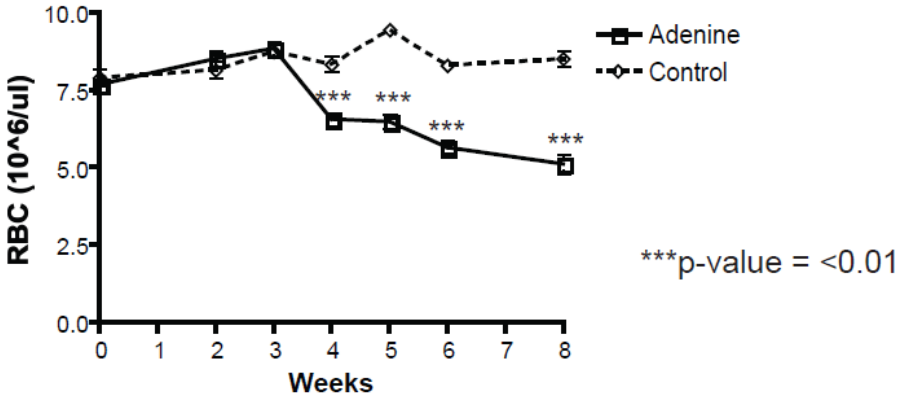
D.


MCHC (g/dl)
40
35
30
25
0 1 2 3 4 5 6 7 8
Weeks
Control
Adenine
^
^p-value = 0.01

E.


WBC (10^3/ul)
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
0 1 2 3 4 5 6 7 8
Weeks
Control
Adenine
**
***
***
***
***
*
*p-value = 0.03
**p-value= 0.02
***p-value = <0.01

F.


RBC (10^6/ul)
10.0
7.5
5.0
2.5
0.0
0 1 2 3 4 5 6 7 8
Weeks
Adenine
Control
***
***
***
***
***p-value = <0.01

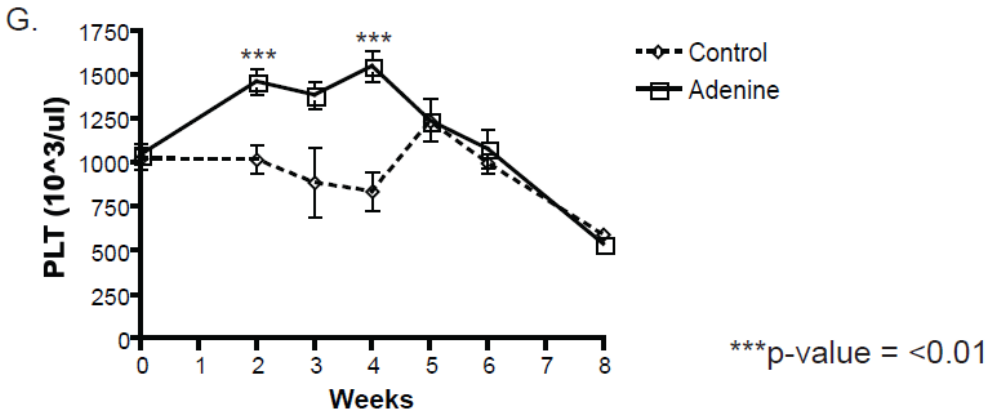
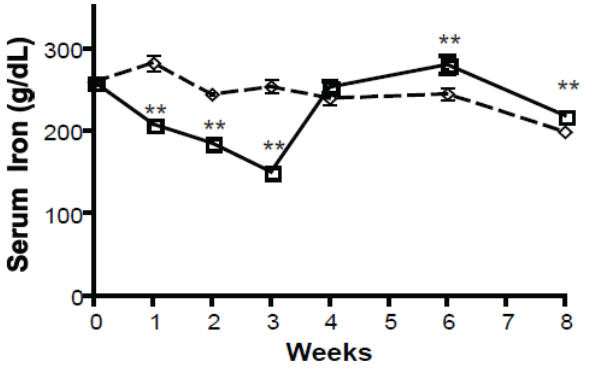


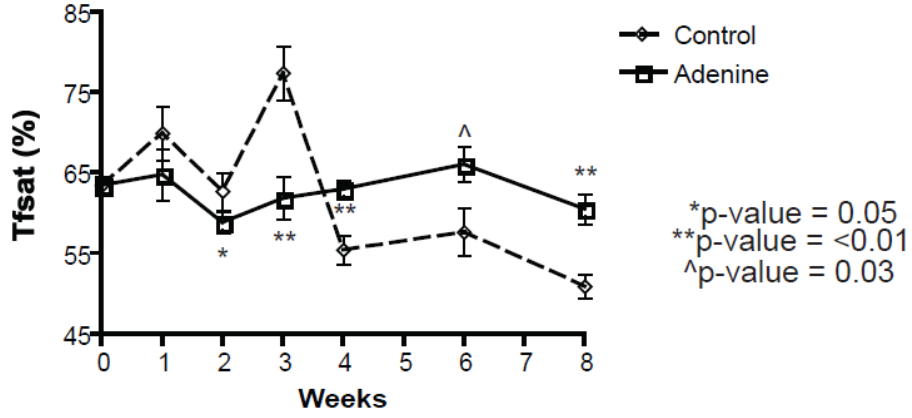
Figure 11. Complete blood count parameters for modified adenine diet rats
*A) Rats on the modified adenine diet (open squares) are anemic and display low hematocrit (HCT) compared to control diet rats (open diamonds) at week 4. The anemia associated with renal failure continues to week 8. B) Adenine diet rats also have low mean corpuscular volume (MCV) and C) mean corpuscular hemoglobin (MCH) from week 1-4, with no changes in D) mean corpuscular hemoglobin content (MCHC) until week 8. E) White blood cell counts are significantly increased in adenine diet rats from week 1-6 while F) red blood cell counts are decreased in adenine diet rats from week 4-8 compared to controls. G) Platelet counts are significantly increased in adenine diet rats from week 2-4 and returned to control levels from week 5-8. Differences between groups were tested by unpaired t tests. ^P=0.01, *** P<0.01, **P=0.02, *P=0.03, compared to control rats, comparisons were made for each time point.*

Consistent with the decreased iron content in reticulocytes, the modified adenine diet rats exhibited reduced serum iron and transferrin saturation from weeks 1-3 compared with control rats (Figure 12A-B). Adenine treated rats also exhibited iron retention in the spleen throughout the entire experimental period (Figure 12C). These data are consistent with a reticuloendothelial cell iron blockade that is characteristic of anemia of chronic kidney disease. Interestingly, by weeks 5-6, the serum iron levels and transferrin saturation, MCVr, and CHr increased to surpass the level in control animals, suggesting that there was some improvement in iron availability after week 3 in this model.

A.


Serum Iron (g/dL)
300
200
100
0
0 1 2 3 4 5 6 7 8
Weeks
Control
Adenine
**p-value = <0.01

B.


Tfsat (%)
85
75
65
55
45
0 1 2 3 4 5 6 7 8
Weeks
Control
Adenine
*p-value = 0.05
**p-value = <0.01
^p-value = 0.03

C.

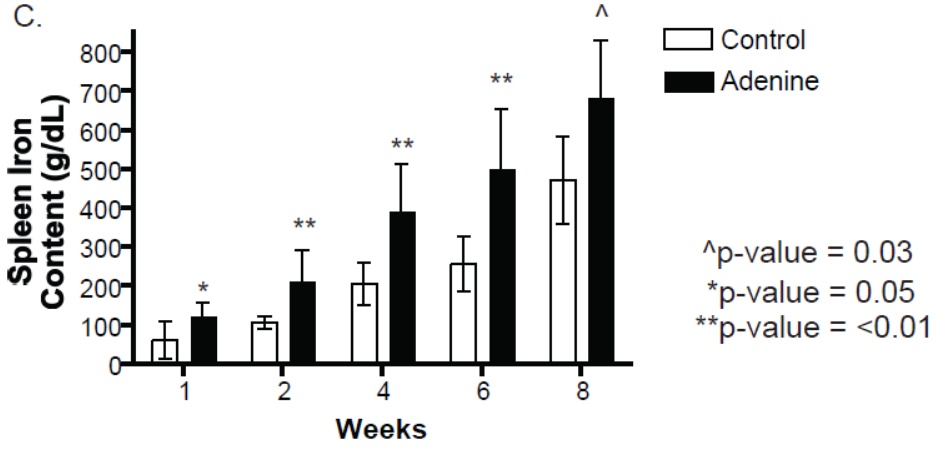

Spleen Iron Content (g/dL)
800
700
600
500
400
300
200
100
0
1 2 4 6 8
Weeks
Control
Adenine
^p-value = 0.03
*p-value = 0.05
**p-value = <0.01

Figure 12. Decreased serum iron concentration and increasing spleen iron content within 3 weeks on Adenine diet
*A) Rats receiving adenine diet (open squares) had decreased levels of serum iron and B) lowered transferrin saturation (Tfsat) during the first 3 weeks compared to those on control diet (open diamonds). However, when adenine rats received control diet after week 3, the serum iron levels and Tfsat returned to control levels. C) The reduction of available circulating iron is due to iron accumulation in the splenic reticuloendothelial macrophages as adenine diet rats have significantly increased values of spleen iron content compared to control rats throughout the 8 week experimental period. Differences between groups were tested by unpaired t tests. ** P<0.01, *P=0.05, ^P=0.03, compared to control rats, comparisons were made for each time point.*

Hepcidin excess has been implicated in causing the functional iron deficiency and reticuloendothelial cell iron blockade often seen in patients with anemia of CKD. We therefore measured the levels of hepatic hepcidin mRNA in the adenine-treated rats compared to control diet rats. Hepcidin expression in the liver was increased by 3-fold within 1 week of adenine treatment (Figure 13A). Hepcidin expression remained high at 2 and 4 weeks in the adenine-treated rats, but then decreased compared to control animals at week 6 to 8. Presumably, the decrease in hepcidin at the later time points is a response to the anemia, which is a potent suppressor of hepcidin expression. In fact, the hepcidin levels in weeks 6 to 8 may still be inappropriately high relative to the degree of anemia, since mice with a similar degree of anemia have about 10-fold lower hepcidin levels compared with nonanemic mice (ref). The decrease in hepcidin mRNA levels at later time points corresponds to increased iron availability for erythropoiesis, manifest by increased serum iron, transferrin saturation, and CHr (Figure 12A, B, Figure 10D). These data suggest that hepcidin excess may be at least in part responsible for the reticuloendothelial cell iron blockade and anemia of this modified adenine model.
Hepcidin excess in CKD has been attributed to reduced renal clearance and/or stimulation by inflammation. As an indicator of an inflammatory response in the liver, we assessed if C-reactive protein (CRP) mRNA levels in liver were increased in adenine-treated rats. CRP mRNA levels in the livers of adenine rats were slightly but significantly increased at 2 weeks of adenine treatment compared to control (Figure 13B).

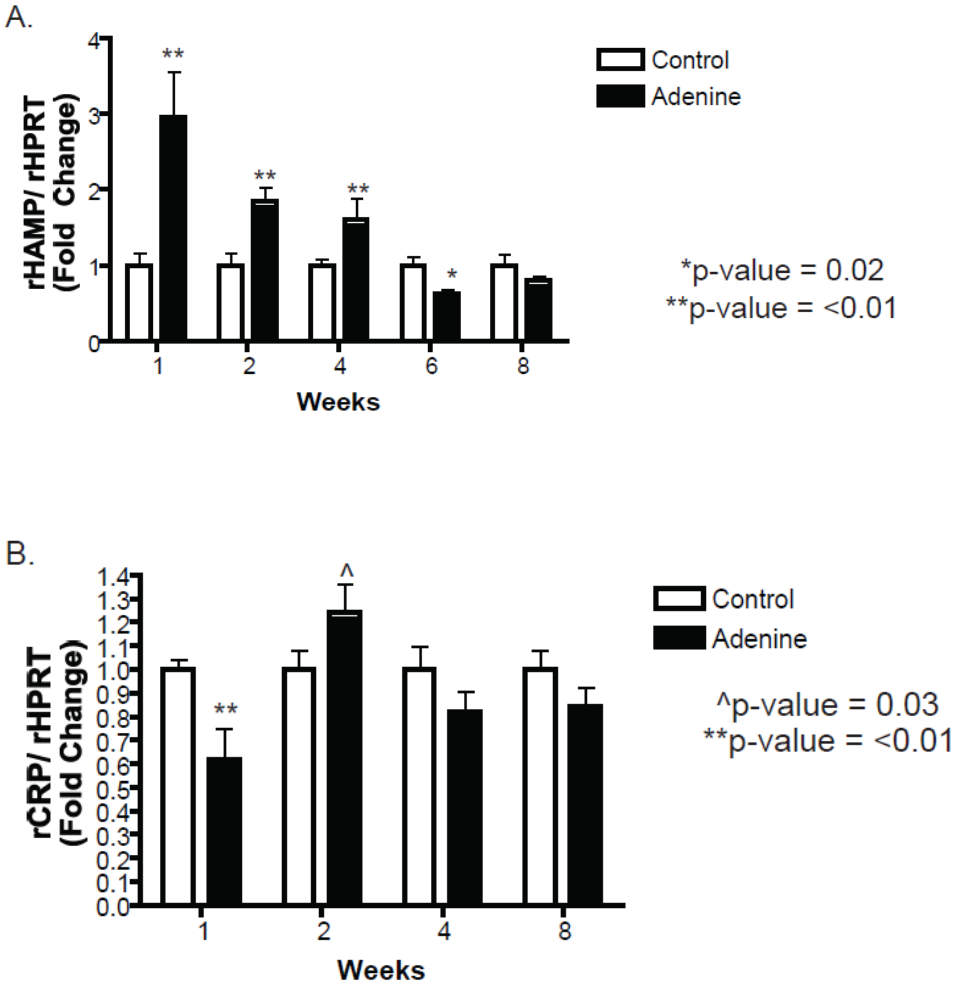


Figure 13. Hepatic hepcidin mRNA expression is increased and is correlated with increased hepatic CRP mRNA expression in Modified Adenine Model
*A) Rats on modified adenine diet (open squares) increased hepcidin mRNA expression by 3 fold at week 1 and remained significantly increased until week 4 compared to control diet rats (open diamonds). B) C-reactive protein, CRP, a marker of inflammation was increased at week 2. Results are shown as fold change of HAMP/HPRT ratio or CRP/HPRT where expression of hypoxanthine phosphoribosyltransferase, HPRT, served as an internal control. Differences between groups were tested by unpaired t tests. ** P<0.01, *P=0.02, ^P=0.03, compared to control rats, comparisons were made for each time point*

4.2 Treatment with LDN-193189 lowers hepcidin, mobilizes spleen iron stores, increases serum iron and increases iron incorporation into reticulocytes, but does not improve anemia in a modified adenine model.

Since the modified adenine-induced renal failure model is a high hepcidin model reflective of the reticuloendothelial cell iron blockade and anemia associated with CKD patients, we investigated whether hepcidin inhibition, by pharmacologic treatment with a small molecule BMP signaling inhibitor LDN-193189 (LDN), would mobilize stored iron from the reticuloendothelial macrophages, increase iron availability for erythropoiesis, and correct the anemia. We treated rats at 1 week post adenine diet administration (Figure 14) since hepcidin was maximally increased. Furthermore, we observed reduced serum iron, spleen iron sequestration, and reduced iron incorporation into reticulocytes suggestive of functional iron deficiency at this time point.

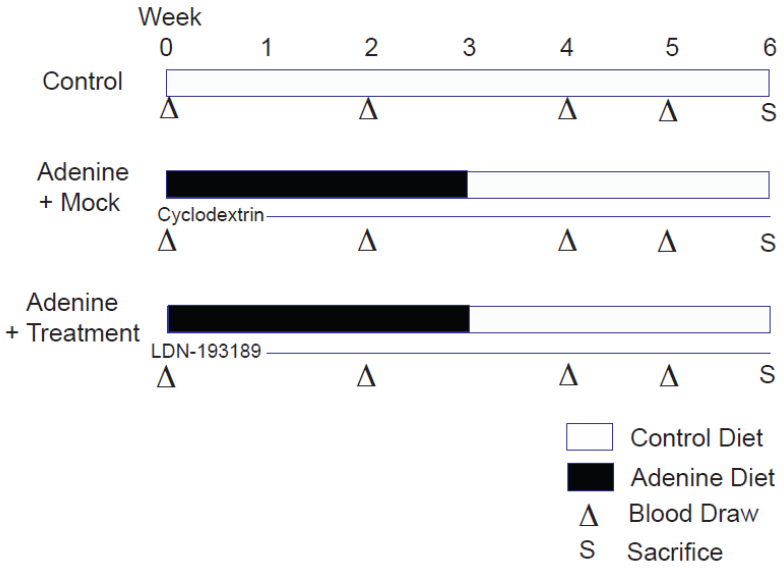


Figure 14. Schematic of LDN-193189 treatment strategy in modified adenine-induced renal failure model. 8-week old Wistar male rats were given either control diet (open bars) for duration of 6 weeks or 0.75% adenine supplemented diet (black bars) for 3 weeks followed by control diet (open bars) for another 3 weeks. The adenine diet group was further divided into a mock treatment arm or treatment arm receiving daily injections of either cyclodextrin or LDN-193189 at a dose of 8mg/kg, respectively, starting at week 1. Tail-vein blood draws (indicated by triangles) were performed at week 0, 2, 4, and 5 for all groups. Animals were sacrificed (S) at week 6 with blood collection by cardiac puncture, followed by harvesting of the liver for analysis of hepatic hepcidin mRNA expression.

After 5 weeks of treatment with LDN-193189, hepatic hepcidin expression was dramatically downregulated compared to rats treated with vehicle only and rats on a control diet (Figure 15).

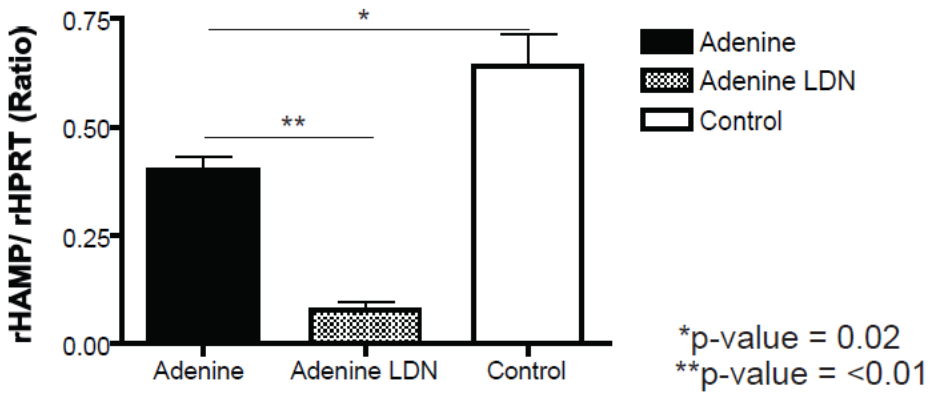
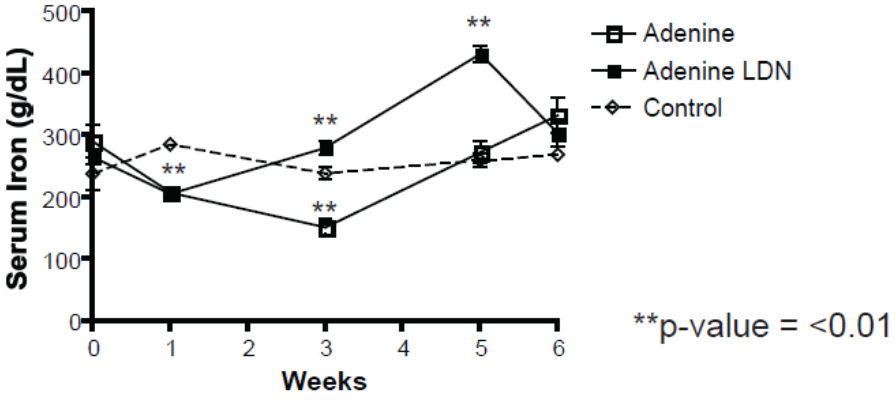


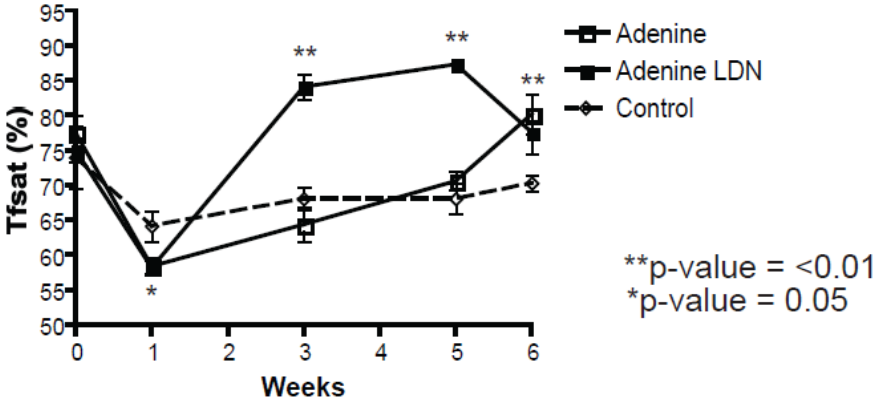
*Figure 15. Effective inhibition of hepcidin expression in LDN treated Adenine rats at 6 weeks. Hepcidin mRNA expression relative to the housekeeping gene HPRT was determined in livers of control and adenine diet rats which were daily treated with either a single injection of vehicle or LDN-193189 (8mg/kg) from week 1 to week 6 and sacrificed 6 hours after last injection. LDN treatment significantly reduced liver hepcidin mRNA expression in the adenine diet rats (dotted bar) compared to adenine rats receiving cyclodextrin vehicle treatment (black bar). Hepcidin mRNA levels in adenine rats (black bar) are low compared to control diet rats (white bar) at week 6. Differences between groups were tested by unpaired t tests. ** P<0.01, compared to adenine diet rats receiving vehicle control and *P=0.02, compared to control rats.*

The effect of hepcidin inhibition was evident by week 3 and week 5, where serum iron concentrations and transferrin saturation in LDN treated adenine rats were significantly increased compared to vehicle-treated adenine rats (Fig. 16A and 16B) This surge in circulating levels of serum iron was at least in part due to release of stored iron from the reticuloendothelial macrophages in the spleen since LDN treated rats had significantly less iron content in the spleen compared to their vehicle treated counterparts, with iron content levels similar to the control diet group (Figure 16C). Importantly, this surge of iron availability correlated with an increase in hemoglobin content of reticulocytes (Figure 17A) and mean corpuscular volume of reticulocytes (Figure 17B) at week 3 and week 5, suggesting that the iron was efficiently being incorporated into reticulocytes. The overall mean cell volume (MCV) and mean cell hemoglobin (MCH) were also significantly increased by week 6 (Fig. 17E-F) However, LDN treatment alone caused only a trend towards increased reticulocyte count, and did not correct Hb levels in the adenine rats (Figure 17C-D).

A.


Serum Iron (g/dL)
500
400
300
200
100
0
0
1
2
3
4
5
6
Weeks
**
**
**
**
Adenine
Adenine LDN
Control
**p-value = <0.01

B.


Tfsat (%)
95
90
85
80
75
70
65
60
55
50
0
1
2
3
4
5
6
Weeks
**
**
**
*
Adenine
Adenine LDN
Control
**p-value = <0.01
*p-value = 0.05

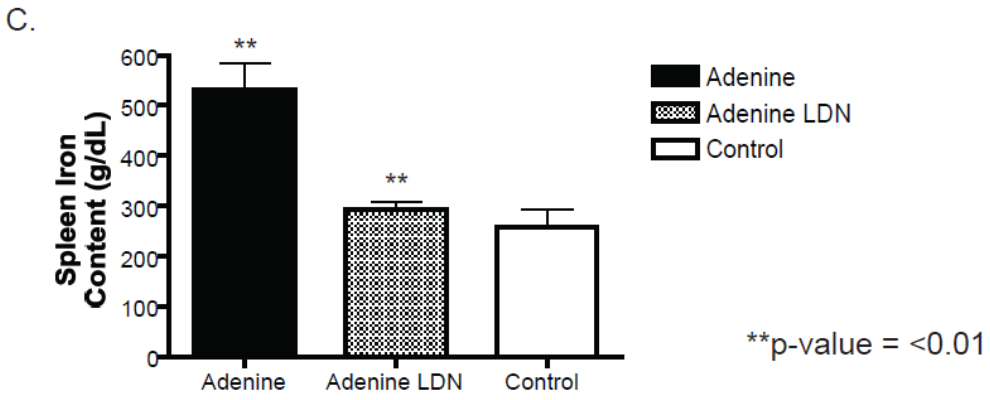
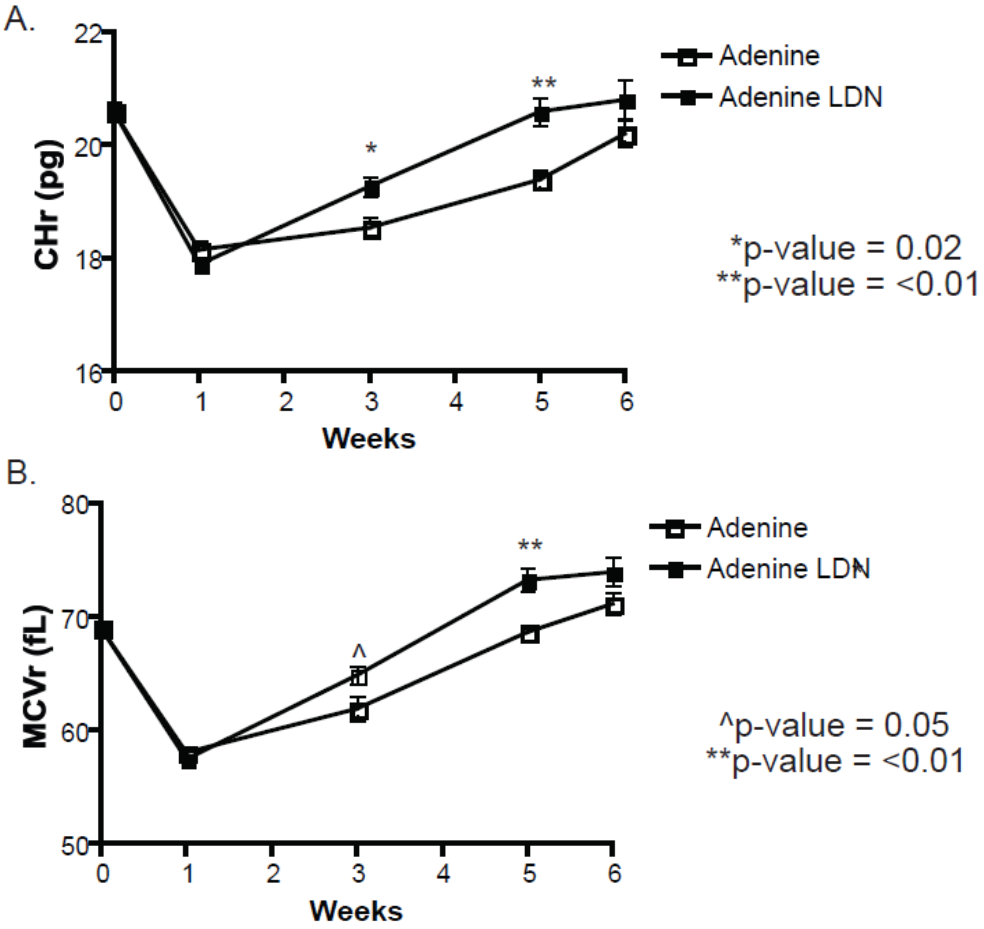
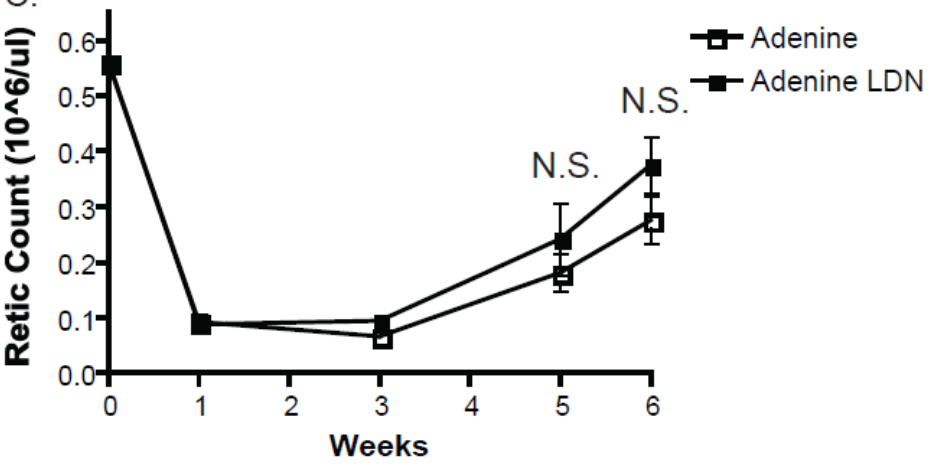


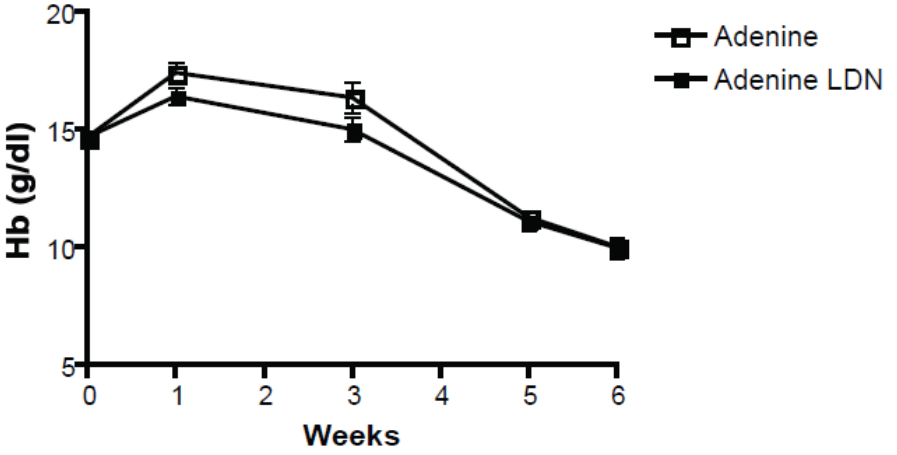
*Figure 16. LDN treated Adenine rats show mobilization of stored iron into plasma A) LDN treatment of adenine rats (closed squares) resulted in a significant increase of serum iron levels and B) transferrin saturation (Tfsat) by week 3 and 5 compared with vehicle treated controls (open squares). C) The increase in available serum iron is due to iron mobilization from spleen with significantly decreased values of spleen iron content in adenine rats treated with LDN (dotted bar) compared to vechicle treated control (black bar). Differences between groups were tested by unpaired t tests. ** P<0.01, *P=0.05, compared to adenine diet vehicle control rats, comparisons were made for each time point.*


A.
CHr (pg)
22
20
18
16
0
1
2
3
4
5
6
Weeks
*
**
Adenine
Adenine LDN
*p-value = 0.02
**p-value = <0.01
B.
MCVr (fL)
80
70
60
50
0
1
2
3
4
5
6
Weeks
^
**
Adenine
Adenine LDN
^p-value = 0.05
**p-value = <0.01

C.


Retic Count (10^6/ul)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
6
Weeks
N.S.
N.S.
Adenine
Adenine LDN

D.


Hb (g/dl)
20
15
10
5
0
1
2
3
4
5
6
Weeks
Adenine
Adenine LDN

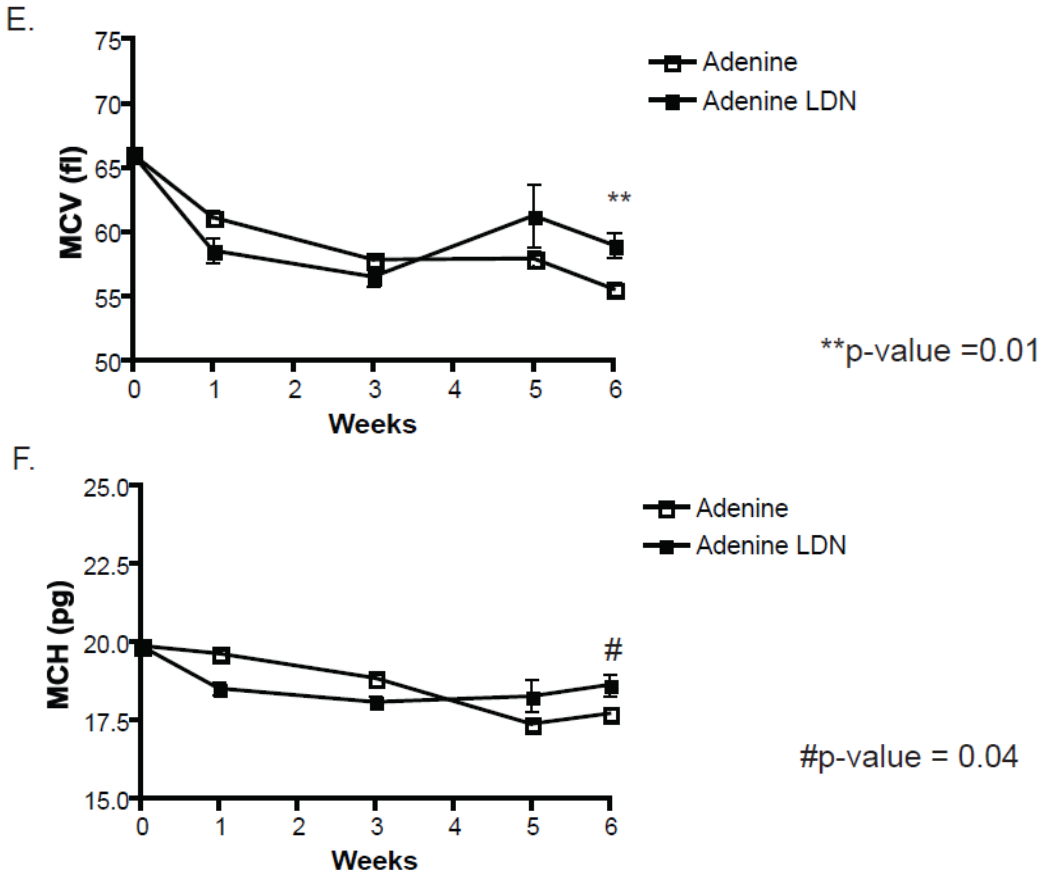


Figure 17. LDN treated Adenine rats show significant increase of hemoglobin incorporation into reticulocytes and increased mean cell volume of reticulocytes which ultimately increased mean corpuscular volume and mean cell hemoglobin at week 6, but LDN treatment did not increase overall Hb levels.
*A) The reticulocyte analysis shows a significant increase of hemoglobin incorporation into reticulocytes (CHr) and B) increased mean cell volume of reticulocytes (MCVr) in LDN treated rats (closed squares) at week 3 and week 5. C) Reticulocyte counts recovered spontaneously due to switching of control diet at week 3 and LDN treatment had no additional effect of increasing retic count. D) LDN treatment did not increase overall hemoglobin levels (Hb) but did increase mean corpuscular volume (MCV) and mean cell hemoglobin (MCH) by the end of the experimental period at week 6. Differences between groups were tested by unpaired t tests. ** P<0.01, *P=0.02, ^P= 0.05, #P=0.04 compared to adenine diet vehicle control rats, comparisons were made for each time point.*

4.3 Adenine induces an acute decrease in circulating erythropoietin to undetectable levels.

In an effort to understand the contribution of erythropoietin (232) deficiency to the anemia of the adenine-induced renal failure model, we examined the serum EPO levels in the adenine-treated rats at week 1 and at week 6 in the absence and presence of LDN treatment. Serum EPO levels were well below the limit of detection after 1 week of adenine-treatment compared to controls (Figure 18). This drop in EPO levels is directly correlated to the precipitous drop in reticulocyte count at week 1 of adenine-treatment (Figure 10B). However, at week 6, the EPO levels in adenine-treated rats returned to a similar level as rats on a control diet, comparable to what has been reported in human patients with CKD. Such "normal" EPO levels in CKD patients with anemia are suggested to be inappropriately low relative to the degree of anemia, since human patients with anemia in the absence of kidney disease typically have EPO levels about 10-100-fold higher than baseline (ref). Interestingly, there was a trend towards an increase in EPO levels in LDN treated adenine rats, albeit not significant.

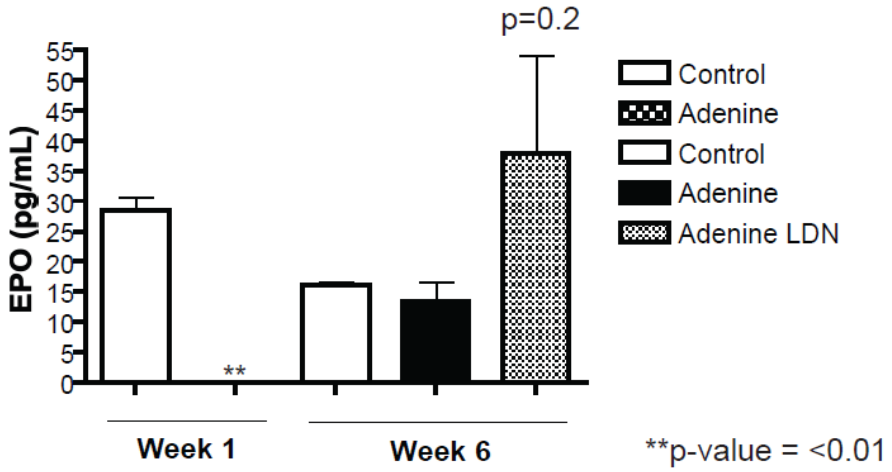


*Figure 18. Serum EPO levels in Modified Adenine Model at week 1 and effect of LDN treatment on EPO levels at week 6. A) Serum EPO levels are below the limit of detection in adenine diet rats at week 1, these values correlate with the significant reduction of reticulocyte number (Figure 3B). At week 6, adenine diet rats display relative EPO deficiency due to anemia as evidenced by similar levels of EPO in both adenine diet rats and control rats. LDN treatment of adenine rats induced an increase in EPO levels but is not significant. Differences between groups were tested by unpaired t tests. ** P<0.01, compared to control rats.*

5. DISCUSSION

We developed an animal model of anemia of CKD with hepcidin excess and demonstrated for the first time that pharmacologic intervention to lower hepcidin expression can correct the functional iron deficiency in anemia of CKD.

The adenine-induced kidney disease model in rats was first published in 1986 by Yokozawa et al. (229,233). Dietary supplementation with adenine is thought to induce kidney disaese in rats primarily due to obstruction of renal tubules by 2,8-dihydroxyadenine crystals (231). The adenine model is often used to study anemia associated with CKD because it reflects the severe anemia seen in patients better compared to the other widely used 5/6 nephrectomy CKD model that is associated with a more mild anemia without alterations in serum iron parameters (234-236). The adenine model was also recently proposed to exhibit elevated hepcidin levels (237), similar to what has been demonstrated in human patients with CKD. However, a full characterization of the hematologic and,iron parameters in the adenine model has been limited (230,231,238) and the mortality rate in the Yokozawa model is prohibitively high (67%) both in ours and others hands. We therefore developed a modified adenine model by giving a diet supplemented with 0.75% adenine for 3 weeks followed by adenine free normal diet for another 3 weeks, which we demonstrated had a higher survival rate than the Yokozawa model and other previously published models, while maintaining irreversible renal failure and anemia. Moreover, we demonstrated that adenine-treated rats have increased hepatic hepcidin levels, decreased serum iron concentration, increased spleen iron content, low hemoglobin levels and inappropriately low EPO levels relative to the degree of anemia, reflective to some extent of the clinical condition in patients with anemia of CKD.

Current management of anemia of CKD are co-administration of ESAs and supplemental iron (239-241). However, ESA therapies have been associated with increased morbidity and mortality in several recent clinical trials. The Correction of Hemoglobin in Outcomes and Renal Insufficiency (CHOIR) and Trial to Reduce cardiovascular Events with Aranesp Therapy (TREAT) studies revealed that CKD patients who received ESA doses to achieve target hemoglobin levels >13g/dL had a higher incidence of cardiovascular events, stroke, progression of cancer and death (242,243). In addition, not all patients respond to ESAs or iron therapy adequately, and these therapies do not fully address the underlying pathophysiologic mechanisms of this disease. For example, CKD patients, particularly patients on hemodialysis, have excess levels of the main iron regulatory hormone hepcidin, and hepcidin excess has been proposed to contribute to the anemia of CKD by limiting iron availability for erythropoiesis. Providing supraphysiologic iron to these patients would worsen the iron restriction and functional iron deficiency in these patients since intravenous iron can potently stimulate hepcidin production by threefold in CKD patients (24).

Therefore, we tested whether pharmacologic intervention to lower hepcidin expression can correct the functional iron deficiency that contributes to the anemia in our model of anemia of CKD. We showed here that the small molecule inhibitor LDN-193189 lowered endogenous hepatic hepcidin production, mobilized stored

iron into plasma and increased circulating serum iron levels within 2 and 4 weeks of LDN treatment. The available iron was efficiently incorporated into hemoglobin in reticulocytes, the precursors to mature red blood cells. Previous studies in patients with CKD have suggested that absolute iron deficiency can contribute to anemia and ESA hyporesponsiveness, and that iron therapy can raise hemoglobin levels and reduce ESA requirements (161,244). Our data provide the first proof of concept evidence that pharmacologic hepcidin lowering strategies can improve iron availability for erythropoiesis in an animal model of anemia of CKD. By improving iron absorption from the diet and iron mobilization from the patient's own body stores, hepcidin lowering strategies could provide an alternative and/or adjunctive therapy to IV iron and ESAs that can minimize the potential adverse consequences of high doses of ESAs and repeated IV iron administration. This would be a particularly attractive option for patients with low serum transferrin saturation and high serum ferritin, for whom current guidelines are unclear how to manage.

Despite an increase in serum iron and improvement in iron incorporation into red blood cells, treatment with LDN-193189 alone did not prevent the anemia progression in our model. This contrasts with studies in two other models of anemia of chronic disease in the absence of kidney disease, a group A streptococcal peptidoglycan-polysaccharide (PG-APS) model of relapsing arthritis or chronic turpentine treatment, where that treatment with LDN193189 alone was able to ameliorate anemia. In another anemia of chronic disease model induced by injection of heat-killed Brucella abortus, treatment with a blocking hepcidin antibody alone was not able to prevent anemia development, but did improve anemia when co-administered with ESAs compared to ESA administration alone. One explanation for the difference in our model compared to other animal models is that erythropoietin deficiency may play a more prominent role in anemia in CKD compared with other chronic diseases. Our data suggests that hepcidin excess and restricted iron availability are not limiting factors for the development of anemia in the modified adenine model. Future studies will be needed to test whether co-adminstration of hepcidin lowering agents such as LDN-193189 will be useful as adjunctive therapy with ESAs to improve hemoglobin levels while reducing ESA dose in models of anemia of chronic kidney disease.

One limitation of the modified adenine model is that EPO levels dramatically fell and were undetectable at 1 week, corresponding to the precipitous drop in reticulocyte counts. Endogenous serum EPO levels during the early weeks of the adenine-induced renal failure model have not been reported. This finding was surprising as published reports in CKD patients demonstrate relative EPO deficiency in renal anemia compared to healthy controls rather than an absolute absence of EPO. Therefore, this result is not reflective of the EPO levels measured in anemic CKD patients, which are generally in the normal range or slightly increased, albeit inappropriately low relative to the degree of anemia (245). It seems likely that the severe EPO deficiency characteristic of the early phase of this model was a limiting factor in the correction of anemia with LDN-193189 alone. Another limitation of this model is that hepcidin levels fell and serum iron levels rose on their own after 4 weeks, thereby limiting the therapeutic window for hepcidin lowering agents in this model. The mechanism of hepcidin excess in

patients with CKD is thought to be due to reduced renal clearance and stimulation by inflammatory cytokines (237). We were unable to measure circulating levels of bioactive hepcidin in this model because there is no currently available assay for this in rats. If reduced renal clearance were present in this model, circulating bioactive hepcidin levels could be even higher than reflected by the liver mRNA levels. However, it seems unlikely that hepcidin excess was present after 4 weeks, given the rise in serum iron levels above baseline. Future studies will be needed to develop animal models of anemia of chronic kidney disease that more accurately reflect the characteristics of human patients with this disease to help further test alternative treatment strategies for this disease.

In summary, we have provided the first experimental evidence that lowering hepcidin can increase iron availability for incorporation into red blood cells in an animal model of anemia of CKD. By correcting hepcidin excess to allow normal dietary iron absorption and mobilization of iron from body stores, such strategies could be anticipated to reduce the requirement for intravenous iron therapy and high dose ESAs to improve safety and efficacy of anemia management in CKD patients.

6. BIBLIOGRAPHY

1. National Kidney Foundation. Chronic kidney disease. <www.kidney.org/ kidneydisease/ckd/index.cfm>; 2009 [accessed 7.07.09].
2. Weiner DE, Tighiouart H, Vlagopoulos PT, et al. Effects of anemia and left ventricular hypertrophy on cardiovascular disease in patients with chronic kidney disease. J Am Soc Nephrol 2005;16:1803–10.
3. World Health Organization. Database of anemia. Worldwide prevalence of anaemia 1993–2005. <www.who.int/vmnis>; 2009 [accessed 7.07.09].
4. Hsu CY, McCulloch CE, Curhan GC. Epidemiology of anemia associated with chronic renal insufficiency among adults in the United States: results from the third national health and nutrition examination survey. J Am Soc Nephrol 2002;13:504–10.
5. Astor BC, Muntner P, Levin A, Eustace JA, Coresh J. Association of kidney function with anemia: the third national health and nutrition examination survey (1988–1994). Arch Intern Med 2002;162:1401–8.
6. Fehally J, Floege J, Johnson R. Comprehensive clinical nephrology. Philadelphia: Mosby Elsevier; 2003, p. 853–60.
7. Eckardt KU. Erythropoietin: oxygen-dependent control of erythropoiesis and its failure in renal disease. Nephron 1994;67:7–23.
8. Eschbach JW, Adamson JW, Cook JD. Disorders of red cell production in uremia. Arch Intern Med 1970;126:812–5.
9. Koch KM, Patyna D, Shaldon S, Werner E. Anemia of the regular hemodialysis patient and its treatment. Nephron 1974;12:405–19.
10. Geerlings W, Morris RW, Brunner FP, et al. Factors influencing anaemia in dialysis patients. A special survey by the EDTA-ERA registry. Nephrol Dial Transplant 1993;8:585–9.
11. Katzarski KS, Charra B, Luik AJ, et al. Fluid state and blood pressure control in patients treated with long and short dialysis. Nephrol Dial Transplant 1999;14:369–75.
12. Locatelli F, Del Vecchio L. Dialysis adequacy and response to erythropoietic agents: what is the evidence base? Nephrol Dial Transplant 2003;18(Suppl. 8): viii29–35.
13. National Kidney Foundation. KDOQI Clinical Practice Guidelines and Clinical Practice Recommendations for Anemia in Chronic Kidney Disease. Am J Kidney Dis. 2006;47(5 suppl 3):S1-145.
14. Besarab A, Bolton WK, Browne JK, et al. The effects of normal as compared with low hematocrit values in pa-tients with cardiac disease who are receiving hemodialysis and epoetin. N Engl J Med. 1998;339(9):584-590.
15. Singh AK, Szczech L, Tang KL, et al. Correction of anemia with epoetin alfa in chronic kidney disease. N Engl J Med. 2006;355(20):2085-2098.
16. Szczech LA, Barnhart HX, Inrig JK, et al. Secondary analysis of the CHOIR trial epoetin-alpha dose and achieved hemoglobin outcomes. Kidney Int. 2008;74(6):791-798.

17. Weiss G, Goodnough LT. Anemia of chronic disease. N Engl J Med. 2005;352(10):1011-1023.
18. Malyszko J, Mysliwiec M. Hepcidin in anemia and inflammation in chronic kidney disease. Kidney Blood Press Res. 2007;30(1):15-30.
19. Macdougall IC, Tucker B, Thompson J, Tomson CR, Baker LR, Raine AE. A randomized controlled study of iron supplementation in patients treated with erythropoietin. Kid- ney Int. 1996;50(5):1694-1699.
20. Fudin R, Jaichenko J, Shostak A, Bennett M, Gotloib L. Correction of uremic iron deficiency anemia in hemodia- lyzed patients: a prospective study. Nephron. 1998;79(3):299- 305.
21. Markowitz GS, Kahn GA, Feingold RE, Coco M, Lynn RI. An evaluation of the effectiveness of oral iron therapy in hemodialysis patients receiving recombinant hu- man erythropoietin. Clin Nephrol. 1997;48(1):34-40.
22. Eleftheriadis T, Antoniadi G, Liakopoulos V, Kartsios C, Stefanidis I. Disturbances of acquired immunity in hemodialysis patients. Semin Dial. 2007;20(5):440-451.
23. Ganz T, Olbina G, Girelli D, Nemeth E, Westerman M. Immunoassay for human serum hepcidin. Blood. 2008; 112:4292-4297.
24. Ashby DR, Gale DP, Busbridge M, et al. Plasma hepcidin levels are elevated but responsive to erythropoietin therapy in renal disease. Kidney Int. 2009;75(9):976-981.
25. Zaritsky J, Young B, Wang HJ, et al. Hepcidin—a potential novel biomarker for iron status in chronic kidney disease. Clin J Am Soc Nephrol. 2009;4:1051-1056.
26. Finch C. 1994. Regulators of iron balance in humans. Blood 84: 1697–1702.
27. Poss KD, Tonegawa S. 1997. Heme oxygenase 1 is required for mammalian iron reutilization. Proc Natl Acad Sci 94: 10919–10924.
28. Beguin Y, Stray SM, Cazzola M, Huebers HA, Finch CA. 1988. Ferrokinetic measurement of erythropoiesis. Acta Haematol 79: 121–126.
29. Breuer W, Ronson A, Slotki IN, Abramov A, Hershko C, Cabantchik ZI. 2000. The assessment of serum nontrans- ferrin-bound iron in chelation therapy and iron supple- mentation. Blood 95: 2975–2982.
30. Ohgami RS, Campagna DR, McDonald A, Fleming MD. 2006. The Steap proteins are metalloreductases. Blood 108: 1388–1394.
31. Soe-Lin S, Apte SS, Andriopoulos B Jr, Andrews MC, Schranzhofer M, Kahawita T, Garcia-Santos D, Ponka P. 2009. Nramp1 promotes efficient macrophage recycling of iron following erythrophagocytosis in vivo. Proc Natl Acad Sci 106: 5960–5965.
32. Shi H, Bencze KZ, Stemmler TL, Philpott CC. 2008. A cytosolic iron chaperone that delivers iron to ferritin. Sci- ence 320: 1207–1210.
33. Nandal A, Ruiz JC, Subramanian P, Ghimire-Rijal S, Sinna- mon RA, Stemmler TL, Bruick RK, Philpott CC. 2011. Activation of the HIF prolyl hydroxylase by the iron chaperones PCBP1 and PCBP2. Cell Metab 14: 647 – 657.

34. Sheftel AD, Zhang AS, Brown C, Shirihai OS, Ponka P. 2007. Direct interorganellar transfer of iron from endosome to mitochondrion. Blood 110: 125–132.
35. Paradkar PN, Zumbrennen KB, Paw BH, Ward DM, Kaplan J. 2009. Regulation of mitochondrial iron import through differential turnover of mitoferrin 1 and mitoferrin 2. Mol Cell Biol 29: 1007–1016.
36. Chen W, Dailey HA, Paw BH. 2010. Ferrochelatase forms an oligomeric complex with mitoferrin-1 and Abcb10 for erythroid heme biosynthesis. Blood 116: 628–630.
37. Casey JL, Hentze MW, Koeller DM, Caughman SW, Rouault TA, Klausner RD, Harford JB. 1988. Iron-responsive ele- ments: Regulatory RNA sequences that control mRNA levels and translation. Science 240: 924–928.
38. Salahudeen AA, Thompson JW, Ruiz JC, Ma HW, Kinch LN, LiQ,GrishinNV,BruickNK.2009.An E3 ligase possessing an iron-responsive hemerythrin domain is a regulator of iron homeostasis. Science 326: 722–726.
39. Vashisht AA, Zumbrennen KB, Huang X, Powers DN, Durazo A, Sun D, Bhaskaran N, Persson A, Uhlen M, Sang- felt O, et al. 2009. Control of iron homeostasis by an iron- regulated ubiquitin ligase. Science 326: 718–721.
40. Bullock GC, Delehanty LL, Talbot AL, Gonias SL, Tong WH, Rouault TA, Dewar B, Macdonald JM, Chruma JJ, Goldfarb AN. 2010. Iron control of erythroid development by a novel aconitase-associated regulatory pathway. Blood 116: 97–108.
41. Talbot AL, Bullock GC, Delehanty LL, Sattler M, Zhao ZJ, Goldfarb AN. 2011. Aconitase regulation of erythropoi- esis correlates with a novel licensing function in erythro- poietin-induced ERK signaling. PLoS ONE 6: e23850.
42.
43. Sanchez M, Galy B, Schwanhaeusser B, Blake J, Bahr-Iva- cevic T, Benes V, Selbach M, Muckenthaler MU, Hentze MW. 2011. Iron regulatory protein-1 and -2: Transcrip- tome-wide definition of binding mRNAs and shaping of the cellular proteome by iron regulatory proteins. Blood 118: e168–e179.
44. Zhang DL, Hughes RM, Ollivierre-Wilson H, Ghosh MC, Rouault TA. 2009. A ferroportin transcript that lacks an iron-responsive element enables duodenal and erythroid precursor cells to evade translational repression. Cell Metab 9: 461 – 473.
45. Chen JJ. 2007. Regulation of protein synthesis by the heme- regulated eIF2a kinase: Relevance to anemias. Blood 109: 2693 – 2699.
46. LiuS, BhattacharyaS, HanA, SuraganiRN, ZhaoW, FryRC, Chen JJ. 2008. Haem-regulated eIF2a kinase is necessary for adaptive gene expression in erythroid precursors un- der the stress of iron deficiency. Br J Haematol 143: 129 – 137.

47. Mastrogiannaki M, Matak P, Keith B, Simon MC, Vaulont S, Peyssonnaux C. 2009. HIF-2a, but not HIF-1a, promotes iron absorption in mice. J Clin Invest 119: 1159–1166.
48. Krause A, Neitz S, Magert HJ, Schulz A, Forss- mann WG, Schulz-Knappe P, Adermann K. LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Lett 2000;480:147–50.
49. Pigeon C, Ilyin G, Courselaud B, Leroyer P, Turlin B, Brissot P, Loreal O. A new mouse liver- specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is over- expressed during iron overload. J Biol Chem 2001;276:7811–9.
50. Jordan JB, Poppe L, Haniu M, Arvedson T, Syed R, Li V, et al. Hepcidin revisited, disulfide connectivity, dynamics, and structure. J Biol Chem 2009;284:24155– 67.
51. Farnaud S, Patel A, Evans RW. Modelling of a metal-containing hepcidin. Biometals 2006;19: 527–33.
52. Farnaud S, Rapisarda C, Bui T, Drake A, Cammack R, Evans RW. Identification of an iron-hepcidin complex. Biochem J 2008;413:553–7.
53. Gerardi G, Biasiotto G, Santambrogio P, Zanella I, Ingrassia R, Corrado M, et al. Recombinant human hepcidin expressed in Escherichia coli isolates as an iron containing protein. Blood Cells Mol Dis 2005;35:177–81.
54. Melino S, Garlando L, Patamia M, Paci M, Petru- zzelli R. A metal-binding site is present in the amino terminal region of the bioactive iron reg- ulator hepcidin-25. J Pept Res 2005;66:65–71.
55. Tselepis C, Ford SJ, McKie AT, Vogel W, Zoller H, Simpson RJ, et al. Characterization of the transition-metal-binding properties of hepcidin. Biochem J 2010;427:289–96.
56. Schranz M, Bakry R, Creus M, Bonn G, Vogel W, Zoller H. Activation and inactivation of the iron hormone hepcidin: biochemical characterization of prohepcidin cleavage and sequential degra- dation to N-terminally truncated hepcidin isoforms. Blood Cells Mol Dis 2009;43:169 –79.
57. Valore EV, Ganz T. Posttranslational processing of hepcidin in human hepatocytes is mediated by the prohormone convertase furin. Blood Cells Mol Dis 2008;40:132– 8.
58. Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 2001;276:7806–10.
59. Kemna EH, Tjalsma H, Podust VN, Swinkels DW. Mass spectrometry-based hepcidin measure- ments in serum and urine: analytical aspects and clinical implications. Clin Chem 2007;53: 620–8.
60. Kroot JJC, Laarakkers CM, Geurts-Moespot AJ, Grebenchtchikov N, Pickkers P, van Ede AE, et al. Immunochemical and mass-spectrometry-based serum hepcidin assays for iron metabolism disorders. Clin Chem 2010;56:1570–9.
61. Suzuki H, Toba K, Kato K, Ozawa T, Tomosugi N, Higuchi M, et al. Serum hepcidin-20 is elevated during the acute phase of myocardial infarction. Tohoku J Exp Med 2009;218:93– 8.

62. Tomosugi N, Kawabata H, Wakatabe R, Higuchi M, Yamaya H, Umehara H, Ishikawa I. Detection of serum hepcidin in renal failure and inflammation by using Protein Chip System. Blood 2006;108:1381–7.
63. Peters HP, Laarakkers CM, Swinkels DW, Wet- zels JF. Serum hepcidin-25 levels in patients with chronic kidney disease are independent of glomerular filtration rate. Nephrol Dial Transplant 2010;25:848 –53.
64. Tessitore N, Girelli D, Campostrini N, Bedogna V, Pietro SG, Castagna A, et al. Hepcidin is not useful as a biomarker for iron needs in haemo-dialysis patients on maintenance erythropoiesis- stimulating agents. Nephrol Dial Transplant 2010;25:3996 – 4002.
65. Rivera S, Nemeth E, Gabayan V, Lopez MA, Farshidi D, Ganz T. Synthetic hepcidin causes rapid dose-dependent hypoferremia and is con- centrated in ferroportin-containing organs. Blood 2005;106:2196 –9.
66. Nemeth E, Preza GC, Jung CL, Kaplan J, Waring AJ, Ganz T. The N-terminus of hepcidin is es- sential for its interaction with ferroportin: structure-functio study. Blood 2006;107:328 – 33.
67. Peyssonnaux C, Zinkernagel AS, Datta V, Lauth X, Johnson RS, Nizet V. TLR4-dependent hepci- din expression by myeloid cells in response to bacterial pathogens. Blood 2006;107:3727–32.
68. Bekri S, Gual P, Anty R, Luciani N, Dahman M, Ramesh B, et al. Increased adipose tissue ex- pression of hepcidin in severe obesity is inde- pendent from diabetes and NASH. Gastroenter- ology 2006;131:788 –96.
69. Nguyen NB, Callaghan KD, Ghio AJ, Haile DJ, Yang F. Hepcidin expression and iron transport in alveolar macrophages. Am J Physiol Lung Cell Mol Physiol 2006;291:L417–25.
70. Merle U, Fein E, Gehrke SG, Stremmel W, Ku- laksiz H. The iron regulatory peptide hepcidin is expressed in the heart and regulated by hypoxia and inflammation. Endocrinology 2007;148: 2663– 8.
71. Kulaksiz H, Fein E, Redecker P, Stremmel W, Adler G, Cetin Y. Pancreatic beta-cells express hepcidin, an iron-uptake regulatory peptide. J Endocrinol 2008;197:241–9. 25. Kulaksiz H, Theilig F, Bachmann S, Gehrke SG, Rost D, Janetzko A, et al. The iron-regulatory peptide hormone hepcidin: expression and cel- lular localization in the mammalian kidney. J Endocrinol 2005;184:361–70.
72. Gnana-Prakasam JP, Martin PM, Mysona BA, Roon P, Smith SB, Ganapathy V. Hepcidin expression in mouse retina and its regulation via lipopolysaccharide/Toll-like receptor-4 pathway independent of Hfe. Biochem J 2008;411:79 – 88.
73. Isoda M, Hanawa H, Watanabe R, Yoshida T, Toba K, Yoshida K, et al. Expression of the peptide hormone hepcidin increases in cardio- myocytes under myocarditis and myocardial in- farction. J Nutr Biochem 2010;21:749 –56.
74. Ganz T, Olbina G, Girelli D, Nemeth E, Wester- man M. Immunoassay for human serum hepci- din. Blood 2008;112:4292–7.

75. Swinkels DW, Girelli D, Laarakkers C, Kroot J, Campostrini N, Kemna EH, Tjalsma H. Advances in quantitative hepcidin measurements by time- of-flight mass spectrometry. PLoS ONE 2008;3: e2706.
76. Costa E, Swinkels DW, Laarakkers CM, Rocha- Pereira P, Rocha S, Reis F. et al. Hepcidin serum levels and resistance to recombinant human erythropoietin therapy in haemodialysis patients. Acta Haematol 2009;122:226 –9.
77. Sumboonnanonda A, Malasit P, Tanphaichitr VS, Ong-ajyooth S, Sunthornchart S, Pattanakit- sakul S. et al. Renal tubular function in beta-thalassemia. Pediatr Nephrol 1998;12:280 –3.
78. Wan L, Bellomo R, Di GD, Ronco C. The pathogenesis of septic acute renal failure. Curr Opin Crit Care 2003;9:496 –502.
79. Kulaksiz H, Theilig F, Bachmann S, Gehrke SG, Rost D, Janetzko A, et al. The iron-regulatory peptide hormone hepcidin: expression and cel- lular localization in the mammalian kidney. J Endocrinol 2005;184:361–70.
80. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM. et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 2004;306: 2090 –3.
81. De Domenico I, Ward DM, Nemeth E, Vaughn MB, Musci G, Ganz T, Kaplan J. The molecular basis of ferroportin-linked hemochromatosis. Proc Natl Acad Sci U S A 2005;102:8955–60.
82. Delaby C, Pilard N, Goncalves AS, Beaumont C, Canonne-Hergaux F. The presence of the iron exporter ferroportin at the plasma membrane of macrophages is enhanced by iron loading and downregulated by hepcidin. Blood 2005;106: 3979 – 84.
83. Ramey G, Deschemin JC, Durel B, Canonne- Hergaux F, Nicolas G, Vaulont S. Hepcidin targets ferroportin for degradation in hepatocytes. Haematologica 2010;95:501– 4.
84. Sow FB, Florence WC, Satoskar AR, Schlesinger LS, Zwilling BS, Lafuse WP. Expression and localization of hepcidin in macrophages: a role in host defense against tuberculosis. J Leukoc Biol 2007;82:934 – 45.
85. De Domenico I, Zhang TY, Koening CL, Branch RW, London N, Lo E, et al. Hepcidin mediates transcriptional changes that modulate acute cytokine-induced inflammatory responses in mice. J Clin Invest 2010;120:2395–405.
86. Theurl I, Theurl M, Seifert M, Mair S, Nairz M, Rumpold H, et al. Autocrine formation of hep- cidin induces iron retention in human mono- cytes. Blood 2008;111:2392–9.
87. Keel SB, Abkowitz JL. The microcytic red cell and the anemia of inflammation. N Engl J Med 2009;361:1904 – 6.
88. De Domenico I, Nemeth E, Nelson JM, Phillips JD, Ajioka RS, Kay MS, et al. The hepcidin- binding site on ferroportin is evolutionarily con- served. Cell Metab 2008;8:146 –56.
89. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of Mammalian iron metabolism. Cell 2010;142:24 –38

90. Goswami, T., and Andrews, N.C. (2006). Hereditary hemochromatosis protein, HFE, interaction with transferrin receptor 2 suggests a molecular mechanism for mammalian iron sensing. J. Biol. Chem. 281, 28494–28498
91. Gao, J., Chen, J., Kramer, M., Tsukamoto, H., Zhang, A.S., and Enns, C.A. (2009). Interaction of the hereditary hemochromatosis protein HFE with transferrin receptor 2 is required for transferrin-induced hepcidin expression. Cell Metab. 9, 217–227.
92. Schmidt, P.J., Toran, P.T., Giannetti, A.M., Bjorkman, P.J., and Andrews, N.C. (2008). The transferrin receptor modulates Hfe-dependent regulation of hepcidin expression. Cell Metab. 7, 205–214.
93. Gao, J., Chen, J., De Domenico, I., Koeller, D.M., Harding, C.O., Fleming, R.E., Koeberl, D.D., and Enns, C.A. (2010). Hepatocyte-targeted HFE and TFR2 control hepcidin expression in mice. Blood 115, 3374–3381.
94. Feng XH, Derynck R. Specificity and versatility in tgf-beta signaling through Smads. Annu Rev Cell Dev Biol. 2005;21:659–93.
95. Andriopoulos, B., Jr., Corradini, E., Xia, Y., Faasse, S.A., Chen, S., Grgurevic, L., Knutson, M.D., Pietrangelo, A., Vukicevic, S., Lin, H.Y., and Babitt, J.L. (2009). BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. Nat. Genet. 41, 482–487.
96. Meynard, D., Kautz, L., Darnaud, V., Canonne-Hergaux, F., Coppin, H., and Roth, M.P. (2009). Lack of the bone morphogenetic protein BMP6 induces massive iron overload. Nat. Genet. 41, 478–481.
97. Grimsrud, C.D., Romano, P.R., D'Souza, M., Puzas, J.E., Reynolds, P.R., Rosier, R.N., and O'Keefe, R.J. (1999). BMP-6 is an autocrine stimulator of chondrocyte differentiation. J. Bone Miner. Res. 14, 475–482.
98. Zhang AS, West AP Jr, Wyman AE, Bjorkman PJ, Enns CA. Interaction of hemojuvelin with neogenin results in iron accumulation in human embryonic kidney 293 cells. J Biol Chem. 2005;280:33885–94
99. Babitt JL, Huang FW, Wrighting DM, Xia Y, Sidis Y, Samad TA, Campagna JA, Chung RT, Schneyer AL, Woolf CJ, Andrews NC, Lin HY. Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. Nat Genet. 2006;38(5):531-9
100. Wang, R.H., Li, C., Xu, X., Zheng, Y., Xiao, C., Zerfas, P., Cooperman, S., Eckhaus, M., Rouault, T., Mishra, L., and Deng, C.X. (2005). A role of SMAD4 in iron metabolism through the positive regulation of hepcidin expression. Cell Metab. 2, 399–409.
101. Casanovas, G., Mleczko-Sanecka, K., Altamura, S., Hentze, M.W., and Muckenthaler, M.U. (2009). Bone morphogenetic protein (BMP)-responsive elements located in the proximal and distal hepcidin promoter are critical for its response to HJV/BMP/SMAD. J. Mol. Med. 87, 471–480.
102. Truksa, J., Lee, P., Peng, H., Flanagan, J., and Beutler, E. (2007). The distal location of the iron responsive region of the hepcidin promoter. Blood 110, 3436–3437.
103. Yu, P.B., Hong, C.C., Sachidanandan, C., Babitt, J.L., Deng, D.Y., Hoyng, S.A., Lin, H.Y., Bloch, K.D., and Peterson, R.T. (2008).

Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. Nat. Chem. Biol. 4, 33–41.
104. Mleczko-Sanecka, K., Casanovas, G., Ragab, A., Breitkopf, K., Müller, A., Boutros, M., Dooley, S., Hentze, M.W., and Muckenthaler, M.U. (2010). SMAD7 controls iron metabolism as a potent inhibitor of hepcidin expres- sion. Blood 115, 2657–2665.
105. Corradini, E., Garuti, C., Montosi, G., Ventura, P., Andriopoulos, B., Jr., Lin, H.Y., Pietrangelo, A., and Babitt, J.L. (2009). Bone morphogenetic protein signaling is impaired in an HFE knockout mouse model of hemochromato- sis. Gastroenterology 137, 1489–1497.
106. Kautz, L., Meynard, D., Besson-Fournier, C., Darnaud, V., Al Saati, T., Coppin, H., and Roth, M.P. (2009). BMP/Smad signaling is not enhanced in Hfe-deficient mice despite increased Bmp6 expression. Blood 114, 2515–2520.
107. Wallace, D.F., Summerville, L., Crampton, E.M., Frazer, D.M., Anderson, G.J., and Subramaniam, V.N. (2009). Combined deletion of Hfe and transferrin receptor 2 in mice leads to marked dysregulation of hepcidin and iron overload. Hepatology 50, 1992–2000.
108. Silvestri, L., Pagani, A., Nai, A., De Domenico, I., Kaplan, J., and Cam- aschella, C. (2008a). The serine protease matriptase-2 (TMPRSS6) inhibits hepcidin activation by cleaving membrane hemojuvelin. Cell Metab. 8, 502–511.
109. Truksa, J., Gelbart, T., Peng, H., Beutler, E., Beutler, B., and Lee, P. (2009). Suppression of the hepcidin-encoding gene Hamp permits iron overload in mice lacking both hemojuvelin and matriptase-2/TMPRSS6. Br. J. Hae- matol. 147, 571–581.,
110. Finberg, K.E., Whittlesey, R.L., Fleming, M.D., and Andrews, N.C. (2010). Downregulation of Bmp/Smad signaling by Tmprss6 is required for main- tenance of systemic iron homeostasis. Blood 15, 3817–3826.
111. Lee, D.H., Zhou, L.J., Zhou, Z., Xie, J.X., Jung, J.U., Liu, Y., Xi, C.X., Mei, L., and Xiong, W.C. (2010). Neogenin inhibits HJV secretion and regulates BMP-induced hepcidin expression and iron homeostasis. Blood 115, 3136–3145.
112. Kautz L, Meynard D, Monnier A, et al. Iron regulates phosphor- ylation of Smad1/5/8 and gene expression of Bmp6, Smad7, Id1, and Atoh8 in the mouse liver. Blood. 2008;112(4):1503- 1509.
113. Peyssonnaux, C., Zinkernagel, A.S., Schuepbach, R.A., Rankin, E., Vau- lont, S., Haase, V.H., Nizet, V., and Johnson, R.S. (2007). Regulation of iron homeostasis by the hypoxia-inducible transcription factors (HIFs). J. Clin. Invest. 117, 1926–1932.
114. Robach, P., Recalcati, S., Girelli, D., Gelfi, C., Aachmann-Andersen, N.J., Thomsen, J.J., Norgaard, A.M., Alberghini, A., Campostrini, N., Castagna, A., et al. (2009). Alterations of systemic and muscle iron metabolism in human subjects treated with low-dose recombinant erythropoietin. Blood 113, 6707–6715

115. Pak, M., Lopez, M.A., Gabayan, V., Ganz, T., and Rivera, S. (2006). Sup- pression of hepcidin during anemia requires erythropoietic activity. Blood 108, 3730–3735.
116. Fleming, R.E. (2007). Hepcidin activation during inflammation: make it STAT. Gastroenterology 132, 447–449.
117. Huang, H., Constante, M., Layoun, A., and Santos, M.M. (2009a). Con- tribution of STAT3 and SMAD4 pathways to the regulation of hepcidin by opposing stimuli. Blood 113, 3593–3599.
118. Vecchi, C., Montosi, G., Zhang, K., Lamberti, I., Duncan, S.A., Kauf- man, R.J., and Pietrangelo, A. (2009). ER stress controls iron metabolism through induction of hepcidin. Science 325, 877–880.
119. Oliveira, S.J., Pinto, J.P., Picarote, G., Costa, V.M., Carvalho, F., Rangel, M., de Sousa, M., and de Almeida, S.F. (2009). ER stress-inducible factor CHOP affects the expression of hepcidin by modulating C/EBPalpha activ- ity. PLoS ONE 4, e6618.
120. Weizer-Stern, O., Adamsky, K., Margalit, O., Ashur-Fabian, O., Givol, D., Amariglio, N., and Rechavi, G. (2007). Hepcidin, a key regulator of iron metabolism, is transcriptionally activated by p53. Br. J. Haematol. 138, 253–262.
121. Nicolas G, Chauvet C, Viatte L, et al. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin Invest. 2002; 110(7):1037-1044.
122. Nicolas G, Viatte L, Bennoun M, Beaumont C, Kahn A, Vaulont S. Hepcidin, a new iron regulatory peptide. Blood Cells Mol Dis. 2002;29(3):327-335
123. Kearney SL, Nemeth E, Neufeld EJ, et al. Urinary hepcidin in congenital chronic anemias. Pediatr Blood Can- cer. 2007;48(1):57-63.
124. Origa R, Galanello R, Ganz T, et al. Liver iron concentrations and urinary hepcidin in beta-thalassemia. Haematologica. 2007;92(5):583-588.
125. Pinto JP, Ribeiro S, Pontes H, et al. Erythropoietin mediates hepcidin expression in hepatocytes through EPOR signaling and regulation of C/EBPalpha. Blood. 2008; 111(12):5727-5733.
126. Vokurka M, Krijt J, Sulc K, Necas E. Hepcidin mRNA levels in mouse liver respond to inhibition of erythro- poiesis. Physiol Res. 2006;55(6):667-674.
127. Tanno T, Bhanu NV, Oneal PA, et al. High levels of GDF15 in thalassemia suppress expression of the iron regu- latory protein hepcidin. Nat Med. 2007;13(9):1096-1101.
128. Tanno T, Porayette P, Sripichai O, et al. Identifica- tion of TWSG1 as a second novel erythroid regulator of hepcidin expression in murine and human cells. Blood. 2009;114(1):181-186.
129. Oelgeschläger M, Larraín J, Geissert D, De Robertis EM. The evolutionarily conserved BMP-binding protein Twisted gastrulation promotes BMP signalling. Nature. 2000;405(6788):757-763.
130. Ross JJ, Shimmi O, Vilmos P, et al. Twisted gastru- lation is a conserved extracellular BMP antagonist. Nature. 2001;410(6827):479-483.

131. Chang C, Holtzman DA, Chau S, et al. Twisted gastrulation can function as a BMP antagonist. Nature. 2001;410(6827):483-487.
132. Xu J, Kimball TR, Lorenz JN, et al. GDF15/MIC-1 functions as a protective and antihypertrophic factor re- leased from the myocardium in association with SMAD protein activation. Circ Res. 2006;98(3):342-350.
133. Hsiao EC, Koniaris LG, Zimmers-Koniaris T, Se- bald SM, Huynh TV, Lee SJ. Characterization of growth- differentiation factor 15, a transforming growth factor beta superfamily member induced following liver injury. Mol Cell Biol. 2000;20(10):3742-3751.
134. Eling TE, Baek SJ, Shim M, Lee CH. NSAID activated gene (NAG-1), a modulator of tumorigenesis. J Biochem Mol Biol. 2006;39(6):649-655.
135. Roe MA, Spinks C, Heath AL, et al. Serum prohep- cidin concentration: no association with iron absorption in healthy men; and no relationship with iron status in men carrying HFE mutations, hereditary haemochromatosis pa- tients undergoing phlebotomy treatment, or pregnant women. Br J Nutr. 2007;97(3):544-549.
136. Kemna E, Pickkers P, Nemeth E, van der Hoeven H, Swinkels D. Time-course analysis of hepcidin, serum iron, and plasma cytokine levels in humans injected with LPS. Blood. 2005;106(5):1864-1866.
137. Murphy AT, Witcher DR, Luan P, Wroblewski VJ. Quantitation of hepcidin from human and mouse serum using liquid chromatography tandem mass spectrometry. Blood. 2007;110(3):1048-1054.
138. Murao N, Ishigai M, Yasuno H, Shimonaka Y, Aso Y. Simple and sensitive quantification of bioactive peptides in biological matrices using liquid chromatography/selected reaction monitoring mass spectrometry coupled with trichlo- roacetic acid clean-up. Rapid Commun Mass Spectrom. 2007;21(24):4033-4038.
139. Kobold U, Dülffer T, Dangl M, et al. Quantification of hepcidin-25 in human serum by isotope dilution micro- HPLC-tandem mass spectrometry. Clin Chem. 2008;54(9): 1584-1586.
140. Grebenchtchikov N, Geurts-Moespot AJ, Kroot JJ, et al. High-sensitive radioimmunoassay for human serum hepcidin. Br J Haematol. 2009;146(3):317-325.
141. Koliaraki V, Marinou M, Vassilakopoulos TP, et al. A novel immunological assay for hepcidin quantification in human serum. PLoS One. 2009;4(2):e4581.
142. Kroot JJ, Kemna EH, Bansal SS,et al. Results oft he first international round robin for the quantification of urinary and plasma hepcidin assays: need for standardization. Haematologica. 2009;94(12):1748-1752.
143. Weiss G, Theurl I, Eder S, et al. Serum hepcidin concentration in chronic haemodialysis patients: associa- tions and effects of dialysis, iron and erythropoietin therapy. Eur J Clin Invest. 2009; 39(10):883-890.
144. Kato A, Tsuji T, Luo J, Sakao Y, Yasuda H, Hishida A. Association of prohepcidin and hepcidin-25 with erythro- poietin response and ferritin in hemodialysis patients. Am J Nephrol. 2008;28(1):115-121.

145. Swinkels DW, Wetzels JF. Hepcidin: a new tool in the management of anaemia in patients with chronic kidney disease? Nephrol Dial Transplant. 2008;23(8): 2450-2453.
146. Moreno, F., et al., Increasing the hematocrit has a beneficial effect on quality of life and is safe in selected hemodialysis patients. Spanish Cooperative Renal Patients Quality of Life Study Group of the Spanish Society of Nephrology. J Am Soc Nephrol, 2000. 11(2): p. 335-42.
147. Littlewood, T.J., et al., Effects of epoetin alfa on hematologic parameters and quality of life in cancer patients receiving nonplatinum chemotherapy: results of a randomized, double-blind, placebo-controlled trial. J Clin Oncol, 2001. 19(11): p. 2865-74.
148. Swaak, A., Anemia of chronic disease in patients with rheumatoid arthritis: aspects of prevalence, outcome, diagnosis, and the effect of treatment on disease activity. J Rheumatol, 2006. 33(8): p. 1467-8.
149. Eschbach, J.W., et al., Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. Results of a combined phase I and II clinical trial. N Engl J Med, 1987. 316(2): p. 73-8.
150. Pettersson, T., et al., Successful treatment of the anemia of rheumatoid arthritis with subcutaneously administered recombinant human erythropoietin. Slower response in patients with more severe inflammation. Scand J Rheumatol, 1993. 22(4): p. 188-93.
151. Hertel, J., et al., Darbepoetin alfa administered every other week maintains hemoglobin levels over 52 weeks in patients with chronic kidney disease converting from once-weekly recombinant human erythropoietin: results from simplify the treatment of anemia with Aranesp (STAAR). Am J Nephrol, 2006. 26(2): p. 149-56.
152. Ludwig, H., et al., Management of disease-related anemia in patients with multiple myeloma or chronic lymphocytic leukemia: epoetin treatment recommendations. Hematol J, 2002. 3(3): p. 121-30.
153. Adamson, J.W., The anemia of inflammation/malignancy: mechanisms and management. Hematology Am Soc Hematol Educ Program, 2008: p. 159-65.
154. Revicki, D.A., et al., Health-related quality of life associated with recombinant human erythropoietin therapy for predialysis chronic renal disease patients. Am J Kidney Dis, 1995. 25(4): p. 548-54.
155. B.P. Teehan, S.K., W.A. Stone et al. , Double-blind, placebo-controlled study of the therapeutic use of recombinant human erythropoietin for anemia associated with chronic renal failure in predialysis patients. The US Recombinant Human Erythropoietin Predialysis Study Group. Am J Kidney Dis, 1991. 18(1): p. 50-9.
156. Brookhart, M.A., et al., Comparative mortality risk of anemia management practices in incident hemodialysis patients. JAMA, 2010. 303(9): p. 857-64.
157. KDOQI Clinical Practice Guidelines and Clinical Practice Recommendations for Anemia in Chronic Kidney Disease. Am J Kidney Dis, 2006. 47(5 Suppl 3): p. S11-145.

158. Pfeffer, M.A., et al., A trial of darbepoetin alfa in type 2 diabetes and chronic kidney disease. N Engl J Med, 2009. 361(21): p. 2019-32.
159. Drueke, T.B., et al., Normalization of hemoglobin level in patients with chronic kidney disease and anemia. N Engl J Med, 2006. 355(20): p. 2071-84.
160. Gasche, C., et al., Prediction of response to iron sucrose in inflammatory bowel disease-associated anemia. Am J Gastroenterol, 2001. 96(8): p. 2382-7.
161. Coyne, D.W., et al., Ferric gluconate is highly efficacious in anemic hemodialysis patients with high serum ferritin and low transferrin saturation: results of the Dialysis Patients' Response to IV Iron with Elevated Ferritin (DRIVE) Study. J Am Soc Nephrol, 2007. 18(3): p. 975-84.).
162. Henry, D.H., et al., Intravenous ferric gluconate significantly improves response to epoetin alfa versus oral iron or no iron in anemic patients with cancer receiving chemotherapy. Oncologist, 2007. 12(2): p. 231-42.
163. Hedenus, M., et al., Addition of intravenous iron to epoetin beta increases hemoglobin response and decreases epoetin dose requirement in anemic patients with lymphoproliferative malignancies: a randomized multicenter study. Leukemia, 2007. 21(4): p. 627-32.
164. Steensma, D.P., et al., Phase III, randomized study of the effects of parenteral iron, oral iron, or no iron supplementation on the erythropoietic response to darbepoetin alfa for patients with chemotherapy-associated anemia. J Clin Oncol, 2011. 29(1): p. 97-105.
165. D. P. Steensma, B.J.S., J. A. Sloan, D. Tomita, C. L. Loprinzi; Dana-Farber Cancer Institute, Boston, MA; Amgen Inc., Thousand Oaks, CA; Mayo Clinic, Rochester, MN, The relationship between serum hepcidin levels and clinical outcomes in patients with chemotherapy-associated anemia treated in a controlled trial., in 2011 ASCO Annual Meeting. 2011.
166. Daoud, E., E. Nakhla, and R. Sharma, Q: Is iron therapy for anemia harmful in the setting of infection? Cleve Clin J Med, 2011. 78(3): p. 168-70.
167. Sazawal, S., et al., Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital and mortality in preschool children in a high malaria transmission setting: community-based, randomised, placebo-controlled trial. Lancet, 2006. 367(9505): p. 133-43.
168. Leavy O. Therapeutic antibodies: Past, present and future. Nat Rev Immunol2010; 10: 297.
169. Sasu BJ,Cooke KS,Arvedson TL. Anti-hepcidin antibody treatment modulates iron metabolism and is effective in a mouse model of inflammation-induced anemia. Blood2010; 115: 3616–3624.
170. Xiao JJ,Krzyzanski W,Wang YM. Pharmacokinetics of anti-hepcidin monoclonal antibody Ab 12B9m and hepcidin in cynomolgus monkeys. AAPS J2010; 12: 646–657.

171. Leung DDM,Swanson BA,Tang Y,Luan P,Witcher DR. Anti-Hepcidin Antibodies and Uses Thereof. Patent # 7820163. Indianapolis, IN: Eli Lilly and Company; 2011
172. Lily. Clinical Development Pipeline; 2011. Available from:http://www.lilly.com/SiteCollectionDocuments/FlashFiles/PipeLine/Clinical%20Development%20Pipeline/13.html. Accessed on 14 October, 2011.
173. Alnylam Pharmaceuticals. ALN-HPN: Refractory Anemia; 2011. Available from:http://www.alnylam.com/Programs-and-Pipeline/Alnylam-5x15/Refractory-Anemia.php. Accessed on 27 September, 2011.
174. Wang J,Lu Z,Wientjes MG. Delivery of siRNA therapeutics: Barriers and carriers. AAPS J2010; 12: 492–503.
175. Akhtar S,Benter IF. Nonviral delivery of synthetic siRNAs in vivo. J Clin Invest2007; 117: 3623–3632.
176. Xenon, Isis and Xenon Collaborate to Develop Antisense Drugs Against Hemojuvelin and Hepcidin; 2010. Available from: http://www.xenon-pharma. com/2010/11/isis-and-xenon-collaborate-to-develop-antisense-drugs-against-hemojuvelin-and-hepcidin/. Accessed on 28 September, 2011.
177. Graham MJ,Crooke ST,Monteith DK. In vivo distribution and metabolism of a phosphorothioate oligonucleotide within rat liver after intravenous administration. J Pharmacol Exp Ther1998; 286: 447–458.
178. Flower DR. The lipocalin protein family: Structure and function. Biochem J1996; 318(Pt. 1): 1–14.
179. Schlehuber S,Skerra A. Lipocalins in drug discovery: From natural ligand-binding proteins to “anticalins”. Drug Discov Today2005; 10: 23–33.
180. Hohlbaum A,Trentmann S,Gille H, et al., Podium #92. Exploiting lipocalin biochemistry for the treatment of anemia: Discovery and characterization of an anti-hepcidin therapeutic. In: Fourth Congress of the International BioIron Society (IBIS) Biennial World Meeting (BioIron 2011), May 22–26, 2011. Vancouver, BC, Canada . Am J Hematol 2011;86(9).Hohlbaum A,Trentmann S,Gille HAm J Hematol2011; 86: E48.
181. Pieris. Preclinical Pipeline PRS-080; 10/19. 2011. Available from:http://www.pieris-ag.com/pipeline/prs-080.php. Accessed on 3 November, 2011.
182. Vater A,Klussmann S. Toward third-generation aptamers: Spiegelmers and their therapeutic prospects. Curr Opin Drug Discov Devel2003; 6: 253–261
183. Pendergrast PS,Marsh HN,Grate D. Nucleic acid aptamers for target validation and therapeutic applications. J Biomol Tech2005; 16: 224–234.
184. Noxxon. NOX-H94; 2011. Available from:http://www.noxxon.com/index.php?option=com_content&view=article&id=88&Itemid=100. Accessed on 17 October, 2011.
185. Schwoebel F,Sell S,Maasch C, et al. Podium #91. NOX-H94, In vitro and in vivo characterization of a hepcidin blocking spiegelmer. In:

Fourth Congress of the International BioIron Society (IBIS) Biennial World Meeting (BioIron 2011) May 22–26, 2011. Vancouver, BC, Canada: Am J Hematol 2011;86(9):E47Schwoebel F,Sell S,Maasch CAm J Hematol2011; 86: E47.
186. Maasch C,Vater A,Buchner K. Polyetheylenimine-polyplexes of Spiegelmer NOX-A50 directed against intracellular high mobility group protein A1 (HMGA1) reduce tumor growth in vivo. J Biol Chem2010; 285: 40012–40018.
187. Kulkarni O,Eulberg D,Selve N. Anti-Ccl2 Spiegelmer permits 75% dose reduction of cyclophosphamide to control diffuse proliferative lupus nephritis and pneumonitis in MRL-Fas(lpr) mice. J Pharmacol Exp Ther2009; 328: 371–377.
188. Ninichuk V,Clauss S,Kulkarni O. Late onset of Ccl2 blockade with the Spiegelmer mNOX-E36-3′PEG prevents glomerulosclerosis and improves glomerular filtration rate in db/db mice. Am J Pathol2008; 172: 628–637.
189. Bilik KU,Erguven E,Klussmann S. In-vitro and in-vivo antagonistic action of an anti-amylin Spiegelmer. Neuroreport2007; 18: 1855–1859.
190. Kulkarni O,Pawar RD,Purschke W. Spiegelmer inhibition of CCL2/MCP-1 ameliorates lupus nephritis in MRL-(Fas)lpr mice. J Am Soc Nephrol2007; 18: 2350–2358.
191. Bouchard PR,Hutabarat RM,Thompson KM. Discovery and development of therapeutic aptamers. Annu Rev Pharmacol Toxicol2010; 50: 237–257.
192. Bragdon B,Moseychuk O,Saldanha S. Bone morphogenetic proteins: A critical review. Cell Signal2011; 23: 609–620.
193. Camaschella C. BMP6 orchestrates iron metabolism. Nat Genet2009; 41: 386–388.
194. Zhou G,Myers R,Li Y. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest2001; 108: 1167–1174.
195. Cuny GD,Yu PB,Laha JK. Structure–activity relationship study of bone morphogenetic protein (BMP) signaling inhibitors. Bioorg Med Chem Lett2008; 18: 4388–4392.
196. Yu PB,Deng DY,Lai CS. BMP type I receptor inhibition reduces heterotopic [corrected] ossification. Nat Med2008; 14: 1363–1369.
197. Theurl I,Schroll A,Sonnweber T. Pharmacologic inhibition of hepcidin expression reverses anemia of chronic disease in rats. Blood2011.
198. Steinbicker AU,Sachidanandan C,Vonner AJ. Inhibition of bone morphogenetic protein signaling attenuates anemia associated with inflammation. Blood2011; 117: 4915–4923.
199. Vogt J,Traynor R,Sapkota GP. The specificities of small molecule inhibitors of the TGFss and BMP pathways. Cell Signal2011; 23: 1831–1842.

200. Babitt JL,Huang FW,Xia Y. Modulation of bone morphogenetic protein signaling in vivo regulates systemic iron balance. J Clin Invest2007; 117: 1933–1939.
201. Corradini E,Schmidt PJ,Meynard D. BMP6 treatment compensates for the molecular defect and ameliorates hemochromatosis in Hfe knockout mice. Gastroenterology2010; 139: 1721–1729.
202. Gitelman SE,Kobrin M,Lee A. Structure and sequence of the mouse Bmp6 gene. Mamm Genome1997; 8: 212–214.
203. Poli M,Girelli D,Campostrini N. Heparin: A potent inhibitor of hepcidin expression in vitro and in vivo. Blood2011; 117: 997–1004.
204. Walenga JM,Frenkel EP,Bick RL. Heparin-induced thrombocytopenia, paradoxical thromboembolism, and other adverse effects of heparin-type therapy. Hematol Oncol Clin North Am2003; 17: 259–282,viii–ix.
205. Ohtake T,Saito H,Hosoki Y. Hepcidin is down-regulated in alcohol loading. Alcohol Clin Exp Res2007; 31(1 Suppl): S2–S8.
206. Gerjevic LN,Liu N,Lu S. Alcohol activates TGF-beta but inhibits BMP receptor-mediated smad signaling and Smad4 binding to hepcidin promoter in the liver. Int J Hepatol2012; 2012: 459278.
207. Harrison-Findik DD. Is the iron regulatory hormone hepcidin a risk factor for alcoholic liver disease?World J Gastroenterol2009; 15: 1186–1193.
208. Song SN,Tomosugi N,Kawabata H. Down-regulation of hepcidin resulting from long-term treatment with an anti-IL-6 receptor antibody (tocilizumab) improves anemia of inflammation in multicentric Castleman disease. Blood2010; 116: 3627–3634.
209. Hashizume M,Uchiyama Y,Horai N. Tocilizumab, a humanized anti-interleukin-6 receptor antibody, improved anemia in monkey arthritis by suppressing IL-6-induced hepcidin production. Rheumatol Int2010; 30: 917–923.
210. van Rhee F,Fayad L,Voorhees P. Siltuximab, a novel anti-interleukin-6 monoclonal antibody, for Castleman's disease. J Clin Oncol2010; 28: 3701–3708.
211. Lang VR,Englbrecht M,Rech J. Risk of infections in rheumatoid arthritis patients treated with tocilizumab. Rheumatology (Oxford) 2011.
212. Edwards CJ. IL-6 inhibition and infection: Treating patients with tocilizumab. Rheumatology (2011) doi: 10.1093/rheumatology/ker311.
213. Caceres-Cortes JR. A potent anti-carcinoma and anti-acute myeloblastic leukemia agent, AG490. Anticancer Agents Med Chem2008; 8: 717–722.
214. Turkson J,Ryan D,Kim JS. Phosphotyrosyl peptides block Stat3-mediated DNA binding activity, gene regulation, and cell transformation. J Biol Chem2001; 276: 45443–45455.
215. Zhang X,Yue P,Fletcher S. A novel small-molecule disrupts Stat3 SH2 domain-phosphotyrosine interactions and Stat3-dependent tumor processes. Biochem Pharmacol2010; 79: 1398–1409.

216. Fatih N,Camberlein E,Island ML. Natural and synthetic STAT3 inhibitors reduce hepcidin expression in differentiated mouse hepatocytes expressing the active phosphorylated STAT3 form. J Mol Med2010; 88: 477–486.
217. Holick MF. The cutaneous photosynthesis of previtamin D3: A unique photoendocrine system. J Invest Dermatol1981; 77: 51–58.
218. Reichel H,Koeffler HP,Norman AW. The role of the vitamin D endocrine system in health and disease. N Engl J Med1989; 320: 980–991.
219. Perlstein TS,Pande R,Berliner N. Prevalence of 25-hydroxyvitamin D deficiency in subgroups of elderly persons with anemia: Association with anemia of inflammation. Blood2011; 117: 2800–2806.
220. Saab G,Young DO,Gincherman Y. Prevalence of vitamin D deficiency and the safety and effectiveness of monthly ergocalciferol in hemodialysis patients. Nephron Clin Pract2007; 105: c132–c138.
221. Kumar VA,Kujubu DA,Sim JJ. Vitamin D supplementation and recombinant human erythropoietin utilization in vitamin D-deficient hemodialysis patients. J Nephrol2011; 24: 98–105.
222. Lac PT,Choi K,Liu IA. The effects of changing vitamin D levels on anemia in chronic kidney disease patients: A retrospective cohort review. Clin Nephrol2010; 74: 25–32.
223. Bacchetta J,Zaritsky J,Lisse TS, et al. Poster #FR-PO1560. Vitamin D as a new regulator of iron metabolism: Vitamin D suppresses hepcidin in vitro and in vivo. J Am Soc Nephrol2011; 22: 474A.
224. Aucella F,Scalzulli RP,Gatta G. Calcitriol increases burst-forming unit-erythroid proliferation in chronic renal failure. A synergistic effect with r-HuEpo. Nephron Clin Pract2003; 95: c121–c127.
225. Panichi V,De Pietro S,Andreini B. Calcitriol modulates in vivo and in vitro cytokine production: A role for intracellular calcium. Kidney Int1998; 54: 1463–1469.
226. Fernandes A,Preza GC,Phung Y. The molecular basis of hepcidin-resistant hereditary hemochromatosis. Blood2009; 114: 437–443.
227. Fung E,Hsu J,Damoiseaux R, et al. Poster #198. High throughput screen identifies two classes of small molecules as hepcidin antagonists. In: Fourth Congress of the International BioIron Society (IBIS) Biennial World Meeting (BioIron 2011) May 22–26, 2011. Vancouver, BC, Canada. Am J Hematol 2011;86(9):E140Fung E,J.H.,Damoiseaux RAm J Hematol2011; 86: E140.
228. Leung DDM,Luan P,Menetta JV,Tang Y,Witcher D. Anti-Ferroportin 1 Monoclonal Antibodies and Uses Thereof. International Patent # PCT/US2009/066187. Indianapolis, IN: Eli Lily and Company; 2010.
229. Yokozawa T, Zheng PD, Oura H et al. Animal model of adenine-induced chronic renal failure in rats. Nephron 1986; 44: 230–234
230. Hamada, Y., et al., Alteration of mRNA expression of molecules related to iron metabolism in adenine-induced renal failure rats: a possible

mechanism of iron deficiency in chronic kidney disease patients on treatment. Nephrol Dial Transplant, 2008. 23(6): p. 1886-91.
231. Okada, H., Kaneko, Y., Yawata, T., Uyama, H., Ozono, S., Motomiya Y. and Hirao Y., Reversibility of adenine-induced renal failure in rats. CLINICAL AND EXPERIMENTAL NEPHROLOGY, 1999. 3(2): p. 82-88.
232. Simon, Z., et al., Heterotopic bone formation around sintered porous-surfaced Ti-6Al-4V implants coated with native bone morphogenetic proteins. Implant Dent, 2006. 15(3): p. 265-74.
233. Yokozawa, T., et al., Renal function and urinary prostaglandins in rats given an adenine diet. Nihon Jinzo Gakkai Shi, 1989. 31(6): p. 671-5.
234. Anderson, S., et al., Control of glomerular hypertension limits glomerular injury in rats with reduced renal mass. J Clin Invest, 1985. 76(2): p. 612-9.
235. Garcia, D.L., et al., Anemia lessens and its prevention with recombinant human erythropoietin worsens glomerular injury and hypertension in rats with reduced renal mass. Proc Natl Acad Sci U S A, 1988. 85(16): p. 6142-6.
236. Kawamura, A., et al., Effect of purified recombinant human erythropoietin on anemia in rats with experimental renal failure induced by five-sixth nephrectomy. Biotherapy, 1990. 2(1): p. 77-85
237. Babitt, J.L. and H.Y. Lin, Molecular Mechanisms of Hepcidin Regulation: Implications for the Anemia of CKD. Am J Kidney Dis, 2010.
238. Ataka, K., et al., Effects of erythropoietin-gene electrotransfer in rats with adenine-induced renal failure. Am J Nephrol, 2003. 23(5): p. 315-23.
239. Besarab, A. and D.W. Coyne, Iron supplementation to treat anemia in patients with chronic kidney disease. Nat Rev Nephrol, 2010. 6(12): p. 699-710.
240. Hayat, A., D. Haria, and M.O. Salifu, Erythropoietin stimulating agents in the management of anemia of chronic kidney disease. Patient Prefer Adherence, 2008. 2: p. 195-200.
241. Nurko, S., Anemia in chronic kidney disease: causes, diagnosis, treatment. Cleve Clin J Med, 2006. 73(3): p. 289-97.
242. Singh, A.K., et al., Correction of anemia with epoetin alfa in chronic kidney disease. N Engl J Med, 2006. 355(20): p. 2085-98.
243. Pfeffer, M.A., et al., A trial of darbepoetin alfa in type 2 diabetes and chronic kidney disease. N Engl J Med, 2009. 361(21): p. 2019-32.
244. Coyne, D.W., A. Sims, and B. Bingel, Results of an anemia management program to reduce high epoetin doses by targeted use of i.v. ferric gluconate. Nephrol Nurs J, 2008. 35(6): p. 583-7.
245. Artunc, F. and T. Risler, Serum erythropoietin concentrations and responses to anaemia in patients with or without chronic kidney disease. Nephrol Dial Transplant, 2007. 22(10): p. 2900-8.