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Chronic Inflammation and Iron Metabolism

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Iron is an essential component of almost all biological systems. It is required for energy production, oxygen transport and utilization, cellular proliferation, and destruction of pathogens. The biological properties of iron stem from the variability of its Fe2+/Fe3+ redox potential. Protein ligands adapt these redox potentials to meet various biological requirements. Iron containing proteins are essential to many biochemical functions including oxygen transport by the hemoproteins hemoglobin and myoglobin. Other hemoproteins include the activators of molecular oxygen; cytochrome oxidases, peroxidases, catalases, and cytochrome P450s as well as the cytochromes that transfer electrons from substrate oxidation to cytochrome c oxidase. Iron sulfur proteins are another class of iron containing proteins that mediate one electron redox processes as integral components of the respiratory chain in mitochondria. They are also involved in the control of gene expression, DNA damage recognition and repair, oxygen and nitrogen sensing, and the control of cellular iron acquisition and storage.

The vital importance of maintaining iron supply is most obvious in children. Children, unlike adults, have high iron requirements because of significant cellular metabolic demands due to the high growth rates of their developing tissues and the rapid expansion of their red cell mass. The human brain at birth is the most highly metabolic organ, consuming ~50% of the body's energy needs[1]. Highly metabolic organs need a plentiful supply of substrates, including iron, that support energy metabolism. This metabolic need is reflected in the different physiologic iron absorption requirements (per kilogram) at varying stages of development to maintain normal hemoglobin concentrations as the red cell volume expands with growth and for normal iron delivery to tissues. Per the Food and Nutrition Board of the Institutes of Medicine, the recommended dietary allowance for enteral iron starts at 0.27mg/day in the birth to 6 month age group, increases to 11mg/day in 7 to 12 month old

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infants, and then is 7mg/day in the 1 to 3 year age group. Women of child-bearing age require 18 mg/day and this value increases to 27mg/day during pregnancy[2]. Failure to maintain iron sufficiency during fetal life and in early childhood causes long-term alterations to developing organs, most importantly the brain[3]. Thus, ensuring adequate iron delivery to children during rapid growth phases is essential.

Although maintaining iron delivery to children is vital to support their growth and neurodevelopment, there exists a conundrum in that there are potential negative consequences of iron supplementation in certain contexts such as infectious states. Iron supports the growth and differentiation of other rapidly growing cells including infectious agents. Bacteria are able to form biofilms and grow more rapidly when iron is abundant[4–5]. Bacteria have evolved mechanisms to acquire iron in low iron environments that include the secretion and reuptake of iron-binding organic molecules termed siderophores. Pathogens have developed the ability to acquire iron from host iron-binding proteins like hemoglobin, lactoferrin, and transferrin[4].

The body has evolved a finely tuned mechanism to limit iron availability during infection. In the short-term, this is advantageous and promotes basic survival by protecting from overwhelming infection. In the long-term, anemia of inflammation, also known as the anemia of chronic disease, can place the child's growth and future development at risk by limiting iron availability. Given the potential for long lasting effects, we will discuss the important inter-relationships between chronic disease and iron metabolism.. Although there remain few pediatric specific examples in the literature, the mechanisms gleaned from the adult literature strongly suggest some of the same iron regulation events that take place with acute inflammation and iron metabolism apply to chronic disease in children. Therefore, we will: (1) provide background to explain the importance of the supply and demand and regulatory proteins involved in iron metabolism; (2) review how these regulation principles apply to anemia of inflammation; and (3) propose how these principles apply to anemia of chronic disease and provide clinically relevant examples.

REGULATION OF TOTAL BODY AND CELLULAR IRON

Given that the cells of various body organs are being renewed constantly, there are constant iron requirements that must be met, most significantly during periods of rapid cell growth and maturation. There are certain time periods in which iron requirements are particularly high including the fetal period, infancy and early childhood, and adolescence (especially for females). Almost 2/3 of iron in the body is found in the erythroid components (circulating red blood cells). Therefore, alterations in erythropoiesis have a dominant effect on regulation of iron through absorption, storage, and transport. The requirement for iron for vital biologic functions like erythropoiesis necessitates that an uninterrupted iron supply be available for cellular turnover. This demand, however, is balanced with the importance of preventing the potential toxic effects that would result from the presence of free iron. Thus, total body and cellular iron acquisition and iron storage are tightly regulated processes.

Dietary iron enters the body through absorptive intestinal mucosal cells, thereby regulating net total body iron accumulation. Iron also enters the plasma from macrophages that recycle

iron from senescent erythrocytes. Iron is delivered to the plasma and extracellular fluid by ferroportin, a transmembrane protein encoded by the *SCL40A1* gene (solute carrier family 40 member) that is expressed on the surfaces of duodenal intestinal endothelial cells and reticuloendothelial macrophages[6–7]. Ferroportin binds ferrous (Fe2+) iron, but iron transfer to apotransferrin requires an oxidation step via a multi-copper oxidase because apotransferrin has a high binding affinity for ferric (Fe3+) iron. Ferric iron that enters plasma from macrophages or the intestine is bound to plasma transferrin and is delivered to cells through the interaction of diferric transferrin and cell-surface transferrin receptor[8]. Important homeostatic mechanisms prevent excessive iron absorption in the small intestine and regulate the rate of iron release from macrophages involved in recycling. This is important because the body has no way to excrete iron in a regulated manner. Cellular iron not used by other ferroproteins accumulates in ferritin. Ferritin has a large, but ultimately limited capacity for iron and in fact, capacity may be exceeded in times of iron excess. Toxic free iron in tissues can cause significant organ damage as is seen in severe forms of hemochromatosis. These toxic effects of free iron are due to iron's ability to catalyze formation of reactive oxygen species, stimulating inflammatory responses, and allowing activity of pathogens[9].

Systemic iron homeostasis is regulated by keeping plasma transferrin-bound iron within a narrow range[10]. Iron bound to transferrin remains soluble, but is prevented from generating free radicals. Transferrin is the major carrier and vehicle for iron delivery to individual cell types (eg, erythrocytes, neurons, cardiomyocytes), which have the ability to further regulate iron import and storage. The circulating transferrin pool contains only ~ 3mg of iron at any one time, but ten times that much iron, most destined for developing red blood cells, moves through the transport system every day in an adult[11, 12].

Systemic iron homeostasis is maintained by the regulation of the rate of ferroportin-mediated iron delivery from the intestinal epithelial cell and the macrophages to circulating transferrin. The expression of ferroportin on cell membranes is regulated by hepcidin. Hepcidin binds to ferroportin causing the complex to be internalized into clathrin coated pits, phosphorylated, ubiquinated, and degraded. It functions via a negative feedback loop so that during times of iron sufficiency, hepcidin expression is increased, leading to reduced ferroportin and therefore, reduced iron absorption. Hepcidin acts as the central regulator to control iron absorption, iron recycling, and the size of the iron stores. Most hepcidin is synthesized in hepatocytes as an 84 amino acid propeptide that is processed in the Golgi apparatus into an active 25 amino acid peptide prior to secretion into the circulation. Circulating hepcidin is bound to a α2-macroglobulin. Renal excretion is the major pathway of hepcidin clearance[13–17].

Iron stores, erythropoietic activity, hypoxia, and inflammation are the most important factors that regulate hepcidin gene expression and serum protein concentration. These factors act through three interrelated pathways controlled by circulating iron concentration, hepatocellular iron stores, and inflammatory cytokines. Hepatocytes and developing erythrocytes express transferrin receptor 1 (TfR1) and a second isoform, transferrin receptor 2 (TfR2), both of which are affected by Human hemochromatosis protein (HFE). Mono- or diferric transferrin binds to both TfR1 and TfR2. It displaces HFE from TfR1. HFE is then

able to interact with TfR2 to produce a complex that induces hepcidin transcription by bone morphogenic protein-6 (BMP-6)/sons of mothers against decapentaplegic (SMAD) signaling[13, 18, 19].

THE PATHOPHYSIOLOGY OF ANEMIA OF INFLAMMATION

Hepcidin contributes to innate immunity and is a major component of the anemia of inflammation. The anemia of inflammation develops in multiple clinical scenarios including infection, inflammatory disorders, trauma, and malignancies. The mechanism by which hepcidin functions likely evolved as a way to control the amount of bioavailable iron in the plasma for bacteria during acute infection. However, this non-specific mechanism for restricting bioavailable iron to all cells limits erythropoiesis as well. Thus, the anemia of inflammation is characterized by decreased serum iron concentration (hypoferremia), iron sequestration in macrophages, elevated serum ferritin concentration, and a blunted response to erythropoietin. Red cell survival can be decreased as well. Anemia of inflammation is often a mild normocytic, normochromic anemia, although it can be severe with microcytic and hypochromic red cells if the inflammation-induced restriction of iron availability is long-standing. Although the effect of limiting iron availability on erythropoiesis via this mechanism is well described, it is likely that any organ with a high cellular iron requirement, (eg, the developing brain, heart), would also be compromised by the persistent hypoferremia[22].

Hypoferremia in patients with anemia of inflammation occurs despite sufficient macrophage iron stores, indicating a block in macrophage iron recycling and return of iron to the serum. Increasing evidence suggests that the primary mediator of this block is hepcidin. Hepcidin is a component of the type II acute phase response induced by the pro-inflammatory cytokine interleukin-6. IL-6 activates the janus kinase signal transducer and activator of transcription 3 (JAK/STAT) which stimulates the hepcidin promoter. Hepcidin binding targets ferroportin to lysosomes for degradation. Without ferroportin at the macrophage surface, iron transport to the serum slows, causing accumulation within the cells. When ferroportin is removed from the basolateral surface of enterocytes, dietary iron transfer is diminished, and retained intracellular iron is lost via exfoliation of the intestinal epithelium[23–24].

CLINICAL IMPLICATIONS OF THE ANEMIA OF INFLAMMATION IN CHILDHOOD INFECTIONS: THE MALARIA EXAMPLE

Despite the abundance of elemental iron on the earth, iron deficiency remains one of the most prevalent nutritional problems worldwide. This may in part be due to the mechanisms described above that tightly regulate iron uptake and distribution. The tight regulation is necessary because of the strong oxidoreductive properties of iron which are of vital importance for biochemical pathways not only in human cells, but also in pathogens. The reduction of iron availability during the acute phase response is a major component in innate immune defences but also limits iron availability to the growing child[25].

Trials of iron fortification in the setting of frequent or chronic inflammatory conditions have stimulated the examination of the highly evolved metabolic competition for iron. It has

become increasingly clear through work in developing countries that iron deficiency is not solely accounted for by low dietary iron consumption by young children for whom therapy would center on iron supplementation. Due to concern that universal supplementation of children with iron and folic acid in the setting of high malaria transmission may be harmful, a randomized, placebo-controlled trial of iron supplementation in children from 1–35 months in Pemba, Zanzibar, Tanzania was started by Sazawal et al in 2002. The trial was halted in 2003 on the recommendation of the data and safety monitoring board after noting that those who received iron and folic acid with or without zinc were 12% more likely to die or need inpatient hospital treatment. There were also 15% more deaths in these groups. The conclusion from this trial was that routine supplementation with iron and folic acid in preschool children living in conditions with high rates of malaria can result in increased risk of severe illness and death. A subgroup analysis revealed that the risk of adverse events was increased only in children who were iron replete at baseline[26].

Prompted by the results of the Tanzania trial, in 2006, the World Health Organization (WHO) and the United Nations Children's Fund (UNICEF) released a joint statement recommending that in areas with endemic malaria, that only children with anemia and at risk of iron deficiency should receive supplements[27]. However, it was not feasible to meet population wide screening requirements in those areas of high malaria prevalence and in 2011, the WHO amended the guidelines for the control of iron deficiency among children from 6–23 months, specifically recommending home fortification of food with iron containing micronutrient powder. The provision called for iron therapy in conjunction with measures to prevent, diagnose, and treat malaria. There remained concerns, however, regarding the safety of such fortification in malaria endemic regions prompting additional work by Zlotkin et al. in 2010[28].

A trial was conducted in a highly malaria-endemic area of Ghana, West Africa, to test whether iron administered in a micronutrient powder would increase the risk of clinical malaria while simultaneously working to prevent, screen for, and treat malaria. Nearly 2000 children from 6 months to 35 months in age were randomized to receive micronutrients with or without iron. All received insecticide-treated bed nets as well as malaria treatment when indicated. The malaria incidence was significantly lower in the iron group compared with the no iron group during the intervention period. However, when adjusting for baseline iron deficiency and anemia status overall, these differences were no longer significant[28, 29]. This contrasts with the finding that moderate iron deficiency and anemia are associated with reduced risk of malaria in children and pregnant women[30, 31].

On secondary exploration of the Ghana trial, Zlotkin et al found that the protective association of iron administration appeared significant within a subset of children with iron deficiency and moderate anemia, and for malaria cases with high parasite counts. This apparent contradiction may be due to the important link between iron sufficiency and healthy immune capacity, including adequate T-cell and neutrophil function. There are cells of the innate immune system including monocytes, macrophages, microglia, and lymphocytes that combat bacterial via control of iron fluxes. There are also a variety of effector molecules, eg. NF-kB, hypoxia factor-1, and toll-like receptors with the capability to mobilize anti-bacterial cytokines, neurotrophic factors, and reactive oxygen species that

are unable to respond effectively in times of iron depletion[32]. Iron deficiency, impaired immune function and the anemia itself impairing growth and development may explain some contradictory findings. Anemia had some protection against *Plasmodium* infection due to the merozoite preference for and improved growth in reticulocytes and young RBCs. More limited invasion and replication may then be protective against malaria. Because resolution of anemia requires enhanced reticulocytosis, there may be an unavoidable "transient malarial susceptibility"[33]. In the trial by Zlotkin et al, there was minimal improvement in anemia; therefore the increased malaria risk may not have been demonstrated. There may also be a theoretical possibility of non-transferrin bound iron contributing to the mechanism for adverse infectious morbidity; however studies have not yet conclusively determined this mechanism.

The dynamic interaction between malaria infection and iron metabolism is complex, but is at the heart of a major world-wide public health care problem. Iron supplementation may increase susceptibility to malaria infections in endemic areas, and malaria infections activate hepcidin, reduces iron absorption and exacerbates malarial anemia with additional anemia of inflammation and more severe of iron deficiency anemia. The anemia of malaria is multifaceted, with contributions from hemolysis of parasitized red blood cells, clearance of both parasitized and non-parasitized red blood cells by reticulo-endothelial macrophages, and impaired bone marrow activity. Iron that would normally be recycled from degraded red blood cells and transported to the bone marrow for erythropoiesis is instead sequestered in reticulo-endothelial stores under the influence pro-inflammatory cytokines IL-1, IL-6, TNF-α, IFN-γ. These are the same cytokines responsible for the alterations in iron metabolism characteristic of the anemia of inflammation/chronic disease. Hepcidin, the important regulator of iron metabolism, plays a key role in these inflammatory changes primarily throught the effects of IL-6[34]. Multiple studies have confirmed that hepcidin concentrations increase with *P. falciparum* infection and that relatively small increases in serum hepcidin can lead to profound disturbances in iron metabolism, including marked hypoferremia, increased serum ferritin, and reduced incorporation of hemoglobin into reticulocytes[35]. Independent of iron and hepcidin, these same inflammatory cytokines also have direct effects leading to decreased erythropoiesis adding to the complexity of malarial infection and anemia.

Supplementation strategies in the developing world remain controversial given the high prevalence of both iron deficiency and inflammation/infection as co-morbid conditions. In research evaluating the conditions under which iron will be absorbed and incorporated into the red blood cells, Prentice et al. addressed the question of whether inflammation or iron deficiency was a more powerful predictor of the body's response. This work was done in Gambian children with postmalarial or non-malarial anemia given oral iron supplementation for 30 days with stable iron isotopes. They found that under conditions of competing/ conflicting signals including anemia, iron deficiency, and infection that inflammation consistently trumped iron deficiency and that hepcidin was the most consistent predictor of erythrocyte iron isotope incorporation[36].

In another example demonstrating the complexity of iron balance between host and pathogen, Zimmermann et al evaluated the gut microbiotia in the setting of iron

fortification[37]. Because iron is known to be essential for the growth and virulence of many pathogenic enterobacteria, the effects of providing increased colonic iron to gut microbiota were studied in a group of 139 African children. At baseline, there were increased numbers of pathogenic fecal enterobacteria in comparison with favorable lactobacilli. Although the iron fortification was not effective in decreasing anemia in this group, there was a significant increase in the number of enterobacteria and decrease in lactobacilli in the iron group. There was also an increase in the markers of gut inflammation (fecal calprotectin) with the increase in fecal enterobacteria. Iron fortification in this at risk population produced an alteration in the gut microbiota profile favouring pathogenic organisms and increasing gut inflammation. Whether the subjects were truly iron deficient was not clear; therefore, the question of whether fortification may represent a pro-inflammatory effect of unabsorbed iron remains[37].

CHILDREN WITH CHRONIC DISEASES

The chronic diseases in children associated with the anemia of inflammation are varied and include infections by all types of pathogens, malignancies including hematologic and solid tumors[38], autoimmune diseases such as rheumatoid arthritis[39], systemic lupus erythematosus, and vasculitis [40], as well as chronic kidney disease with inflammation[41]. All of these disease entities produce an immune driven (ie, pro-inflammatory cytokine) response to induce changes in iron homeostasis. Although the anemia of inflammation has been best described in adults, these chronic diseases in the pediatric population have a similar pathophysiology to their adult counterparts.

As an example, iron deficiency, defined as a microcytic anemia, is common in adult patients with cystic fibrosis and has been linked to chronic inflammation[42, 43]. Recently, Uijterschout et al. found that iron deficiency is present in 17% of a relatively healthy, well-nourished pediatric cystic fibrosis population. Ferritin concentrations are elevated and are positively associated with the age of the child (ie, the duration of the disease). The elevated ferritin concentrations likely index a state of inflammation, rather than iron status as evidenced by their relationships to reductions in pulmonary function, increased numbers of pulmonary exacerbations, and higher colonization rates with *Pseudomonas aeruginosa*. The degree of iron deficiency reported may be an underestimation given that ferritin increases as an acute phase reaction during inflammation[44]. This example highlights the diagnostic and treatment difficulty for clinicians; absolute iron deficiency in a growing child requires supplementation, and functional iron deficiency due to inflammation likely will not respond to iron supplementation because of the high hepcidin state. Instead treatment of the underlying inflammatory disorder is indicated with subsequent reassessment of iron status in the non-inflamed state.

A clinically relevant example of using anti-inflammatory therapy to improve iron absorption was utilized in severely anemic Zanzibari children chronically at risk for malarial infection. In work by Cusick et al, the provision of vitamin A supplementation in conjunction with antimalarial treatment resulted in the rapid reduction of inflammation as measured by CRP concentration. Vitamin A was shown to assist with mobilization of iron stores and stimulate the production of new erythrocytes[45]. Evaluation of vitamin A as a regulator of immune

function has also suggested that adequate vitamin A is important for maintaining a proper balance of regulated T-cell functions and for preventing excessive inflammatory reactions[46]. Further evaluation of other nutritional or pharmacologic anti-inflammatory therapies and subsequent reductions in hepcidin may be warranted.

Elevated hepcidin concentrations and anemia of inflammation are also present in adults with rheumatoid arthritis[47]. Cangemi et al demonstrated similar findings in children with juvenile rheumatoid arthritis with subsequent positive responses to anti-inflammatory therapy[39]. Other conditions in pediatrics characterized by a high rate of anemia might also have a previously unsuspected component of the anemia of inflammation. For example, anemia of prematurity is commonly diagnosed in the newborn intensive care unit and is typically thought to be due to a complex mixture of developmental erythropoetic immaturity and phlebotomy-induced losses of hemoglobin and iron[48]. Although data are scarce, it is possible that the frequent inflammatory insults common to ill neonates like sepsis, surgical procedures, and bronchopulmonary dysplasia, induces the pathophysiologic process of anemia of inflammation and contributes to the common clinical picture of a low reticulocyte anemia with the increased serum ferritin concentration in these infants.

CLINICAL DIAGNOSIS

Clinically differentiating between the anemia of inflammation and iron deficiency anemia can be difficult because there can be overlap between the two conditions. Both diseases are characterized by anemia, low reticulocyte counts, decreased serum iron concentration and transferrin saturation. Iron deficiency is distinguished from anemia of inflammation by microcytosis. The anemia of inflammation is typically normocytic, unless the inflammation has been long-standing enough to result in total body iron deficiency. Most importantly, iron deficiency results in low serum ferritin concentrations, whereas anemia of inflammation is characterized by elevated ferritin levels that reflect the process of iron sequestration in the reticulo-endothelial system. Nevertheless, ferritin concentrations can be difficult to interpret during inflammation because a child with low iron stores may have a serum ferritin concentration in the normal range due to induction of the acute phase response by pro-inflammatory cytokines[49]. Some have recommended that the ratio of soluble transferrin receptor (sTfR)/log ferritin may be useful, but this has not yet been widely available for clinical use in children[50]. Current laboratory testing for acute phase reactants associated with inflammation often includes erythrocyte sedimentation rate (ESR) and C - reactive protein (CRP). Although these markers are widely available, there are other tests such as alpha-1-acid glycoprotein (AGP) that may identify the inflammatory processes that alter iron-status indicators. In work by Beard et al. in African American school-age children in Detroit, the association between ferritin and AGP was stronger than that between ferritin and CRP. This study concluded that AGP is likely a better identifier than CRP of elevations in ferritin due to inflammation[51]. Ultimately, measurement of hepcidin levels in various pediatric inflammatory conditions will provide more differentiation because hepcidin will be elevated in anemia of inflammation, but low in pure iron deficiency. Again, there are limitations, as in the case of iron refractory iron deficiency anemia. Currently, low-cost commercial serum hepcidin assays for wide-spread clinical use are not available but are likely to be in the near future.

As diagnostic approaches to the anemia of inflammation are refined, additional discussions of potential therapeutic options are warranted given the difficulty in treating many underlying inflammatory diseases. The crucial role of hepcidin in macrophage iron retention, hypoferremia, and anemia brings into question the potential for hepcidin inhibition as a novel treatment modality. In a rat model of anemia of chronic inflammation, endogenous hepcidin production was blocked pharmacologically resulting in mobilization of iron from the reticuloendothelial system, stimulation of erythropoiesis, and correction of anemia[52]. There are several hepcidin-targeted therapeutic agents under development for different iron disorders and some that have entered human trials[53]. Given the need to prevent iron overload as evidenced by disorders of hepcidin insufficiency such as hereditary hemochromatosis, it will be important to monitor for unintended consequences of hepcidin inhibition.

Parenteral iron therapy has also been advocated as an approach to bypass the hepcidin-mediated block at the level of the enterocyte. Although parenteral iron is occasionally necessary in those unresponsive or intolerant to oral iron therapy, there are significant issues surrounding the widespread use of intravenous iron. Iron dextran, although available for more than 50 years, has significant safety concerns with anaphylaxis and severe allergic reactions leading to fatalities. Two newer parenteral iron products, ferric gluconate and iron sucrose, have improved safety profiles, although their costs are substantially higher and multiple infusions may be necessary.

CONCLUSION

The regulation of iron absorption and distribution in humans requires tight control in order to balance the immediate survival benefit from avoiding toxic effects of iron or providing an important substrate for invading pathogens versus allowing iron to be utilized for vital biologic functions including erythropoiesis and organ development in the rapidly growing child. Knowledge of the pathogenesis of the anemia of inflammation has progressed rapidly in the past decade with the understanding of the central role of hepcidin as the iron-regulatory hormone and a mediator of innate immunity. As more is understood about the interplay between iron metabolism and host defense, clinically useful advances can be made in diagnosis and therapy for common clinical entities characterized by either chronic disease or acute inflammation.

Abbreviations

IRP	Iron regulatory protein
TfR	Transferrin receptor
HFE	Human hemochromatosis protein
BMP	Bone morphogenic protein
SMAD	Sons of mothers against decapentaplegic protein
HJV	Hemojuvelin

HIF	Hypoxia inducible factor
HEPC	Hepcidin anti-microbial peptide
JAK/STAT	Janus kinase signal transducer and activator of transcription 3

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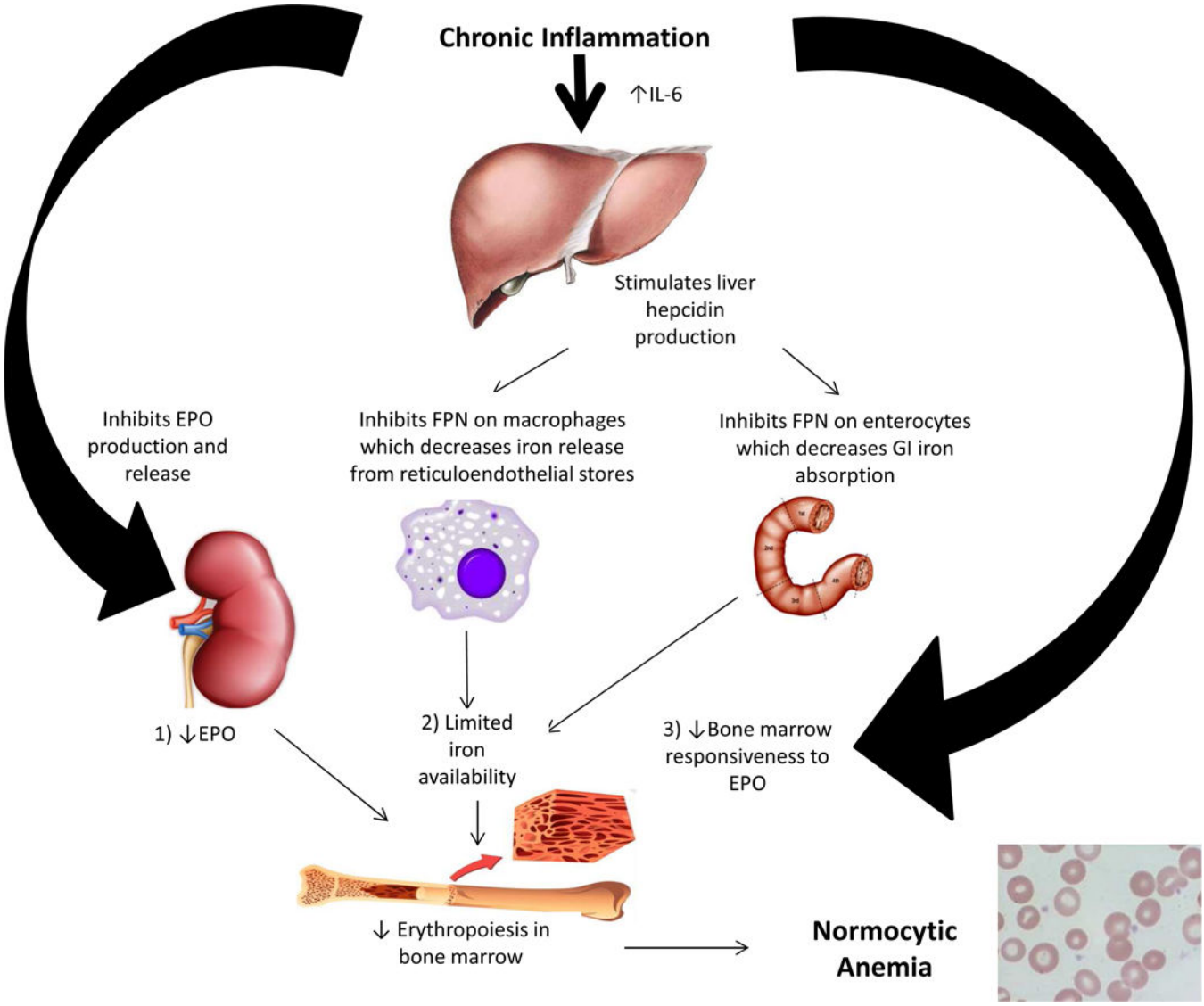

Figure 1. Etiology of normocytic anemia due to chronic inflammation

Chronic inflammation results in the development of normocytic anemia via a combination of 1) decreased erythropoietin (EPO) production in the kidney, 2) hepcidin-mediated decreased iron availability from macrophages and the duodenal enterocytes, and 3) decreased bone marrow responsiveness to EPO.