STUDIES ON TREATMENT OF RENAL ANEMIA IN PATIENTS ON CHRONIC HEMODIALYSIS

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To Ingibjörg,

Arnar Bragi, Aron and Andrea

ABSTRACT

In patients with chronic kidney disease, treatment with erythropoiesis-stimulating agents (ESA) effectively corrects anemia. Most of these patients also need supplementation with regular iron injections to secure iron availability for proper erythropoiesis. Following intravenous iron injection, non-transferrin bound iron (NTBI) can appear in the circulation, capable of inducing harmful oxidative reactions. Direct measurement of free iron with the robust technique electron spin resonance (ESR) has not been used to investigate this issue in humans.

The main purposes of this thesis were to use ESR to study the levels of NTBI and oxidative stress after intravenous (IV) iron injection and to compare two commercially available IV iron formulations, low-molecular weight iron-dextran (ID) and iron-sucrose (IS), regarding this topic. In addition, the impact of two different hemodialysis modalities on iron homeostasis and the effect of modifying the ESA administration praxis on ESA requirement, were studied.

Sixty-four patients on chronic hemodialysis treatment participated in these studies. To investigate the appearance of NTBI and induction of oxidative stress, blood samples were collected before and after IV iron injections. To compare two different hemodialysis modalities, a prospective, randomized, patient-blinded study, with conventional hemodialysis (HD) and on-line hemodiafiltration (HDF), in a 2x2 months design, was conducted. Finally, a retrospective register study was performed on 18 patients, comparing periods with two different erythropoietin administration routines. After injection of IS, a parallel increase in oxidative stress and NTBI was noted, while no induction of oxidative stress was seen following injection of ID. After treatment with HDF, the levels of the iron-regulating peptide, 25-hepcidin, were in all cases within the reference interval. A change in ESA administration regimen, to less frequent dose-adjustments and no withheld doses, could be an explanation for the observed approximately 20 % reduction in ESA requirement.

In conclusion, IS injection, but not ID injection, “leaks” catalytically active iron into the blood stream, which then initiates a burst of intravascular oxidative reactions. The increased clearance of 25-hepcidin by HDF could be of benefit for the dialysis patient, bringing the pathological iron homeostasis found in this population toward a more normal state. An erythropoietin regimen with optimal frequency of dose adjustments can reduce ESA demand and thereby decrease health care cost.

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. BERGUR V. STEFÁNSSON, BÖRJE HARALDSSON, ULF NILSSON
Ascorbyl free radical reflects catalytically active iron after intravenous iron saccharate injection.
Free Radical Biology & Medicine 2008;45:1302–1307.

II. BERGUR V. STEFÁNSSON, BÖRJE HARALDSSON, ULF NILSSON
Acute oxidative stress following intravenous iron injection in patients on chronic hemodialysis: A comparison of iron-sucrose and iron-dextran.
Nephron Clin Pract 2011;118:c249–c256.

III. BERGUR V. STEFÁNSSON, MATS ABRAMSON, ULF NILSSON, BÖRJE HARALDSSON
Hemodiafiltration improves plasma 25-hepcidin levels. A prospective, randomized, participant-blinded, cross-over study, comparing hemodialysis and hemodiafiltration.
Submitted for publication.

IV. BERGUR V. STEFÁNSSON, BÖRJE HARALDSSON, ULF NILSSON
The consumption of erythropoiesis stimulating agents can be reduced by a new administration regimen.
Submitted for publication.

ABBREVIATIONS

Δ	Change
↑	Increase
↓	Decrease
AFR	ascorbyl free radical
AU	arbitrary units
BDC-LDL	baseline diene conjugation in LDL
CKD	chronic kidney disease
DFO	desferrioxamine
EPO	erythropoietin
ESA	erythropoiesis stimulating agent
ESR	electron spin resonance
ESRD	end-stage renal disease
GFR	glomerular filtration rate
Hb	hemoglobin
hCRP	high sensitivity C reactive protein
HD	hemodialysis
HDF	hemodiafiltration
HF	hemofiltration
HIF	hypoxia inducible factor
HIP	heme iron polypeptide
HMW	high molecular weight
ID	iron-dextran
IG	iron-gluconate
IM	intramuscular
IS	iron-sucrose
IV	intravenous
LMW	low molecular weight
MDA	malondialdehyde
MPO	myeloperoxidase
MW	molecular weight
NTBI	non-transferrin bound iron
PD	peritoneal dialysis
PO	per os
RES	reticuloendothelial system
SC	subcutaneous
SFP	soluble ferric pyrophosphate
TEAC	trolox equivalent antioxidant capacity

PROLOGUE

"I WILL FOLLOW that system of regimen which, according to my ability and judgment, I consider for the benefit of my patients, and abstain from whatever is deleterious and mischievous."

Hippocrates. *The Oath*; 5th Century B.C.

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INTRODUCTION

In this thesis, the etiology, pathophysiology and management of renal anemia will be reviewed with special emphasis on iron metabolism and iron treatment.

Natural history

In 1836, Richard Bright, in legendary notes on his patients, commended on the characteristic sign of renal anemia as “a progressive fading of the healthy colors of the countenance" [1]. This condition, later described as normocytic, normochromic anemia, is almost an inevitable complication of severe renal failure. It is hypoproliferative in nature, with reduced reticulocyte count [2, 3] and progress in parallel with the reduction in renal function [4-6]. However, the hemoglobin level is usually within the normal reference interval as long as the GFR is above 30 mL/min, but, in diabetes, anemia can develop earlier, or when GFR falls below 45 mL/min [7].

Definition

According to The World Health Organization (WHO), the definition of anemia in general is a hemoglobin value below 130 g/L for males and 120 g/L for non-pregnant women [8]. However, great variability exists, depending, for example, on the level of altitude inhabitation. According to American and European guidelines, the definition of anemia in the CKD population is a hemoglobin level < 120 g/L in an adult female patient and < 135 g/L in an adult male patient [9, 10]. The diagnosis of renal anemia is by exclusion and it is recommended to initiate anemia work-up when hemoglobin falls below these limits.

Etiology

The etiology of anemia in patients with renal failure is complicated and multifactorial [11]. The main cause is inadequate production of erythropoietin by the diseased kidneys [12-14] but other factors, such as derangements in iron regulation, significantly contributes to the development of renal anemia.

Erythropoietin

Interstitial cells, located in the peritubular capillary bed of the kidneys, are the main site of erythropoietin (EPO) production [15-17]. In addition, some extrarenal production can occur in certain situations, mainly by the hepatocytes [18, 19]. Normally, the production is regulated by a feedback mechanism involving an oxygen sensor that monitor the oxygen level in the vicinity of the EPO producing cell.

The key mediator in this system is hypoxia-inducible factor (HIF), a transcription factor produced in the kidney and the liver [20]. Hypoxia leads to increased level of HIF by stimulating the production and inhibiting the degradation, which in turn stimulates EPO production [21]. Furthermore, new evidence has been provided that HIF plays a more general role in cellular adaption to hypoxia, involving even the regulation of iron metabolism genes, such as the hepcidin gene, supporting a role for HIF in the coordination of EPO synthesis with iron homeostasis [20].

The mechanisms behind renal anemia are not entirely understood. The explanation is not simply that the EPO production falls secondary to cell damage, because serum EPO is often normal or slightly elevated in renal failure, even in patients with ESRD [4, 22]. However, in comparison to anemic patients with normal kidney function, EPO production in patients with renal failure does not respond adequately to the fall in hemoglobin [4, 23]. Accordingly, the EPO levels are lower than in patients with similar degree of anemia, but without renal failure [22]. Thus, it seems that the regulation of EPO production is impaired and the EPO-producing cells are unable to respond adequately to the signals triggered by the oxygen tension.

A well-known observation is that patients with renal failure, staying at high altitude, have increased EPO production and lower ESA requirement [24, 25]. This is interesting and it has been implied that hypoxia can trigger an extrarenal EPO production or activate unused production capacity of EPO in the deceased kidneys. The facts that hypoxia induces HIF

production and that a pharmacological enhancement of HIF in patients with ESRD leads to several-fold increase in EPO production, further support the theory that the inappropriately low production of EPO in renal anemia is a result of desensitization of the oxygen-sensing mechanism rather than a destruction of the cells [26].

Iron

Normally, iron homeostasis is beautifully adapted to the body's requirements. By evolution, systems regulating iron absorption, iron transport in blood and iron storage in cells have developed. On the other hand, no mechanisms for iron excretion exists [27-29], so the only way to avoid toxic iron overload is to adjust iron absorption. In the normal process of erythropoiesis, iron is delivered to the erythroblasts as needed to maintain adequate hemoglobin synthesis. In renal failure, iron regulation is disordered; the absorption is decreased and iron is often blocked in stores, which can lead to the development of absolute and/or functional iron deficiency [30]. Moreover, in patients on chronic hemodialysis, iron loss, due to frequent blood samples and sequestration of red cells in dialysis membranes and tubing, has been calculated to around 1000 mg/year [31].

Hepcidin

The discovery of hepcidin about a decade ago [32, 33] has brought a new light on the pathogenesis of iron deficiency in renal anemia. Hepcidin, a 25 amino acid peptide hormone (25-hepcidin) produced by the hepatocytes, is a key regulator of iron homeostasis [34]. It binds to and induces internalization of ferroportin, a transmembrane iron-channel present in enterocytes, macrophages and hepatocytes [35]. This hinders iron transport out of cells. Thus, increased hepcidin levels can lead to true iron deficiency by decreasing intestinal iron absorption, and to functional iron deficiency by blocking iron release from iron stores. Hepcidin production is induced by inflammation and iron overload [33, 36], two common findings in CKD patients. Further, since the kidneys normally eliminate hepcidin, a successive accumulation occurs in parallel with the fall in GFR [37] and patients reaching ESRD have high serum hepcidin concentrations [37-40]. Accordingly, abnormally high hepcidin levels can, at least in part, be the explanation for the reduced iron absorption described in both PD and HD patients [41, 42]. In addition, iron absorption in HD patients is further reduced by concurrent inflammation, which also supports the involvement of hepcidin [42]. The fundamental role of hepcidin in the pathogenesis of renal anemia is illustrated in figure 1.

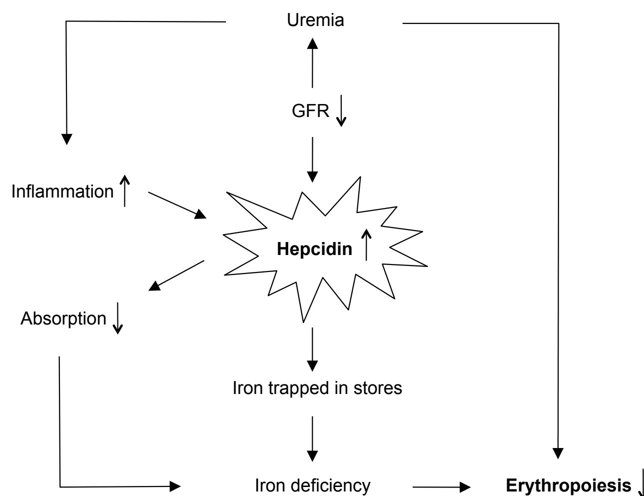


Figure 1. *The central pathogenetic role of hepcidin in the development of renal anemia.*

Uremic toxins

The retention of various waste products in uremia can, in different ways, lead to anemia. They are probably responsible for the shortened life span of red blood cells (from normal 120 to 60-90 days), which has been documented in ESRD [12, 43]. Further, serum extracted from uremic patients has been found to suppress erythropoiesis in a dose-dependent way [44, 45] and this suppression was not found with creatinine or urea. This indicates the existence of a uremic toxin, or toxins, that act as a "suppressor" in the bone marrow. In addition, increased erythropoiesis following start on dialysis supports the role of uremic toxins in the pathogenesis of renal anemia (see below). It is not clear which uremic toxins are involved but it has been suggested that they are in the middle molecular weight class and

probably involve substances such as various polyamines [7].

Other causes

Several other factors can contribute to anemia in renal patients. Malnutrition, hyperparathyroidism and chronic inflammation are best documented, conditions that are commonly found in uremia and well-known suppressors of erythropoiesis [7, 46].

Clinical manifestations

Renal anemia is associated with deprived general health, manifesting as fatigue, loss of libido, dizziness, shortness of breath, reduced exercise tolerance and poor quality of life [11, 47]. In general, these symptoms occur when hemoglobin is less than 100 mg/L [11]. Further, anemia has been found to be a risk factor for left ventricular growth [48] and heart failure [49]. These two conditions are strong predictors of mortality and are frequently present in patients starting on long-term dialysis [50]. Indeed, Foley et al found that among hemodialysis patients, anemia was independently associated with mortality [51] and it seems that a hemoglobin level around 100-110 is critical as mortality increases exponentially with fall in hemoglobin beneath this level [52].

Treatment

The core treatment of renal anemia is to fuel erythropoiesis by regular injections with erythropoiesis stimulating agents (ESAs) and to secure sufficient iron availability for proper erythropoiesis [9, 53].

Erythropoietin

Historical notes

In 1960, erythropoietin was obtained from plasma of anemic sheep [54] and a decade later from urine of anemic humans [55]. It was then purified [56] and finally cloned, making it possible to produce biologically active recombinant human erythropoietin (rHuEPO) [57]. In 1986, Winearls et al. reported that injections with rHuEPO effectively increased the hemoglobin level in patients on chronic hemodialysis, keeping them off the otherwise needed blood transfusions [58] and, in 1987, Eschbach and colleagues further confirmed this impressive response by using the first generation of ESA, epoetin alpha, in patients on chronic hemodialysis [59].

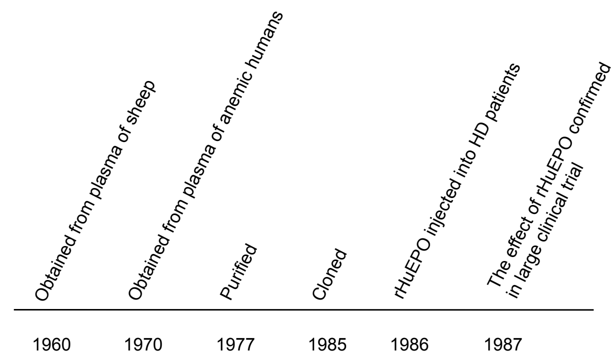


Figure 2. *An overview of the history of erythropoietin in clinical medicine.*

Mechanism of action

Endogenous erythropoietin (EPO), as well as all currently available erythropoiesis-stimulating agents (ESA), binds to the EPO receptors found on the cell membrane of colony-forming-unit erythroid cells and erythroblasts in the bone marrow [60]. By activation of these receptors, EPO prevents apoptosis of these cells, making them able to continue the differentiation toward mature erythrocytes [61-64].

Administration

The response to ESA is dose-dependent [59] but the dose-variation is huge, both between individuals [65] and within a given individual [66]. In consequence, frequent individual dose-adjustments are required to keep the hemoglobin value within the recommended interval [67], which, in patients on chronic hemodialysis treatment, is between 110 and 120 g/L [10, 68]. Several agents are on the market and differ in terms of molecular structure, receptor affinity, serum half-life, bioavailability, and potency [60]. Together, these characteristics shape distinctive dosing schedules for each agent, but otherwise, no clinically

important variations between these agents have been confirmed [10].

Numerous factors can affect ESA response [46, 69], such as the pharmacological property of the agent or health status of the patient. Thus, for ESA with short half-life, IV administration results in higher ESA demand than SC injections [70, 71]. Further, more ESA is needed in case of inflammation and infection [72, 73], iron deficiency [74], malnutrition [75] and insufficient dialysis dose [76]. Taken together, it can be stated that ESA response reflects the overall health status of the patient and, indeed, high ESA resistance is associated with increased mortality and morbidity [77].

Side effects

Treatment with ESA is generally safe and side effects are few. The most serious, but rare, complication, is the development of EPO-antibodies, which results in pure red cell anemia [11]. Hypertension has also been described [78], in particular if hemoglobin rises quickly, and is usually seen within the first 90 days of treatment [11]. In addition, large ESA doses may be hazardous, leading to increased risk of cardiovascular events [79-83].

Clinical outcomes

The benefit of ESA treatment on reducing the need for transfusions, and thereby decreasing the risk of immunologic sensitization, infections and iron overload, is well documented. Further, significant improvements in quality of life and functional parameters, such as aerobic capacity, cognitive function and sexual function, has been noted with ESA treatment [65, 84-88]. Surprisingly, no improvement in quality of life was noted in the TREAT trial [80], comparing ESA with placebo in 4038 diabetics with CKD not on dialysis. In addition, randomized clinical trials showing reduced mortality with ESA treatment are lacking [11].

The optimal hemoglobin target in CKD patients is controversial. Judging from studies on the CKD population, no clear benefit appears to be associated with hemoglobin levels higher than 110 g/L. Thus, in clinical studies, the beneficial effect of ESA on left ventricular hypertrophy was mainly seen with hemoglobin target of 100-110 g/L [89, 90] and in observational studies, mortality descended with increasing hemoglobin concentrations up to a level of 100-110 g/L [51, 52]. In attempt to answer the question if further increase in hemoglobin is beneficial, several prospective randomized trials have been conducted. Briefly, the results of these studies have been disappointing. Patients randomized to normal hemoglobin levels (130-150 g/L) have been found to have increased rate of cardiovascular complications and mortality compared to patients randomized to low hemoglobin levels (100-115 g/L) [79-82]. The reason for this is not clear, but the results from a secondary analysis on data from the CHOIR study [81], where the poor outcome in the high hemoglobin group were restricted mainly to patients with high ESA resistance [83], argue for a mechanism involving high ESA doses rather than the hemoglobin value per se. Thus, the response to ESA appears to be an indicator of the general physical condition and patients that respond to ESA, even though high doses are needed, have lower mortality risk than non-responders [91]. Still, the general recommendation is to keep the hemoglobin level beneath 120 g/L [10, 68].

Oral iron salts

Historical notes

In Greek Mythology, Melampus the seer advised Phylacus to cure his son, Iphiclus, from impotence by having him ingest iron melted from his broadsword. After this original report, many centuries escaped before iron was introduced as medicine. In 1640 Lazarus Riverius, a physician to the France king, Louis XIV, recommended, “steel dissolved in wine” as a treatment for chlorosis, a disease of young woman, considered a kind of “love sickness” but later known as iron deficiency anemia [92]. In 1832, another Frenchman was the first to introduce ferrous iron as pills for treatment of anemia [93] and thereafter, many different oral iron salts have been developed.

Side effects

Various gastrointestinal symptoms, like nausea, constipation and diarrhea, are common side effects with oral iron treatment. Mostly, this is

due to a release of the reactive ferrous iron (Fe^{2+}), which can provoke various reactions in the mucous membrane. The incidence of these side effects is high, or 47 % in healthy individuals [94] and at least 35 % in CKD patients [95, 96].

Clinical outcomes

To evaluate the effect of oral iron in renal anemia, one has to distinguish between different patient categories. In general, iron requirement increases in following order: CKD not on dialysis > PD > HD. Moreover, additional iron is often needed in patients requiring ESA.

In most CKD patients not on dialysis, oral iron, if tolerated, seems to be sufficient treatment for proper erythropoiesis [97] even though these patients require treatment with ESA [96, 98-100].

PD patients are not as well investigated as the HD population. However, when ESA is needed, treatment with oral iron is only sufficient in a minority of patients [101]. In HD patients receiving ESA treatment, short term treatment with oral iron has resulted in increased or stable hemoglobin levels, but iron stores, estimated by serum ferritin, decreased over time [96, 102-104].

Oral heme-iron

Meat and food products from blood components are rich in heme iron, an organic iron, which, in contrast to inorganic iron salts, is absorbed via a unique heme receptor in the intestinal cell [105-107]. Unlike inorganic iron, heme iron absorption is not affected by simultaneous food intake and its absorption is approximately 10 times higher than for inorganic iron salts [108].

Tablets with concentrated heme iron for human use have been produced and marketed as a nutritional supplement. One such compound, heme iron polypeptide (HIP), has been found to have a high bioavailability, 23 times better absorption than iron fumarate, and no gastrointestinal side effects [109]. One clinical trial has been conducted on HIP effects in patients on chronic hemodialysis with promising outcomes. In that study, Nissenson and colleague found that during 6 months, HIP could successfully replace IV iron in majority of patients and, interestingly, treatment with HIP was associated with lower ESA demand [110].

Intravenous iron

Historical notes

Parenteral iron was first introduced in the early 20th century, when Heath and colleague [111] injected ferric hydroxide solutions into patients with hypochromic anemia. They observed a rise in hemoglobin that was proportional to the amount of iron administered. On the other hand, severe toxic reactions were noted, likely due to the instability of the compound, permitting iron to dissociate into the circulation. In 1947, Nissim introduced an iron complex for IV injection, containing a carbohydrate shell (saccharide) around a ferric iron core and concluded that this form of iron was safer and more suitable for parenteral administration [112]. In 1954, high molecular weight iron-dextran (HMW-ID) was introduced for IM and IV use. It was found to be stable and side effects were few. However, severe anaphylactic reactions could occur, leading to the cautioning against use of parenteral iron except under extreme clinical conditions. HMW-ID was the only parenteral iron product available until the 1990s and a minor product until the introduction of epoetin alpha, the first ESA, in 1989 [65]. Since then, different iron-carbohydrate complexes have been developed, such as low molecular weight iron-dextran (LMW-ID), iron-gluconate, iron-sucrose, ferumoxytol, iron-carboxypolymaltose and iron-isomaltoside.

Chemical properties

All IV iron agents consist of a ferric iron core surrounded by a carbohydrate shell, which stabilizes the complex and slows iron release. IV iron agents differ in terms of core size and identity of the shell [113]. These dissimilarities determine not only different pharmacological properties of the agents such as stability, iron release and maximum tolerated dose but also various side effects and safety profiles [114].

Mechanism of action

After IV injection, most of the iron complex is removed from the circulation by phagocytes of the reticuloendothelial system located in the liver, spleen, and bone marrow. Within

phagocytes, iron is released and either stored in ferritin or released for extracellular transport by transferrin, which delivers iron to transferrin receptors on the surface of erythroblasts in the bone marrow [113].

Side effects

In general, treatment with IV iron agents is safe and well tolerated [115]. However, side effects can arise, either from iron released from the ferric iron core, or from the carbohydrate shell. Further, there is a possibility of long-term adverse effects due to iron overload.

Complications related to iron

Adverse reactions occur if the iron complex is unstable, allowing labile iron to appear in the circulation, which, in theory, only can happen if iron release from the complex overrides the total plasma iron binding capacity. This has been investigated in vitro, by looking at different IV iron agents and their ability to directly donate iron to transferrin. These studies showed that their stability depends on the type of the carbohydrate shell and the size of the IV iron complex [116, 117]. Thus, direct iron release was 2.5-5.6 % with the following progression IG > IS > ID. This "labile" fraction has been a matter of concern, because it can induce various oxidative reactions. Unbound ferric iron (Fe^{3+}) is potentially hazardous to the body as it could rapidly be reduced to its ferrous state (Fe^{2+}) by any bioreductants available, such as ascorbic acid. Ferrous iron is toxic and can catalyze reactions associated with oxidative tissue damage. For example, it can mediate the formation of the noxious hydroxyl radical (HO·) via the Fenton reaction [118]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-}$$

Several studies have reported unbound iron in the circulation following IV iron-sucrose injections [119, 120]. However, the nature of the iron measured is controversial, and it is a possibility that the iron assay used has in fact measured iron extracted directly from the circulating iron complex. However, various "footsteps" of catalytically active iron have been observed in the circulation following IV iron injections. Mostly, an elevation of various oxidative stress markers, such as markers of lipid peroxidation, DNA damage [121] and protein oxidation have been noted [122].

In CKD patients, oxidative stress induction has been seen mainly following IS injection [121, 123-136] but also following administration of IG [137-140] and ID [130,141]. This is especially worrying because increased oxidative stress has been linked to the severe cardiovascular disease found in patients with ESRD [142-144] and, indeed, a 4 year prospective follow-up study on 94 HD patients reported that oxidized LDL was an independent predictor of mortality [145].

Other potential associations between IV iron and vascular disease have been postulated. In healthy individuals, endothelial dysfunction has been reported following IS injection [132, 146], in CKD patients, IV iron-polymaltose injection has been shown to increase fibroblast growth factor 23 (FGF-23), a pathogenic factor for arteriosclerosis [147, 148] and in iron-dextran loaded mice, accelerated thrombus formation after arterial injury has been observed[149]. Moreover, a link between labile iron and vascular calcifications, either directly or via oxidative stress, has been noted in vitro [150] and NTBI has been found to stimulate the expression of vascular adhesion molecules and promote monocyte recruitment to vascular endothelium [151-153]. These findings further support the concept of iron-induced endothelial injury.

The above-mentioned studies have evaluated the toxicity of IV iron by measuring changes in extracellular markers. Recently, a study on 10 HD patients reported increased levels of intracellular reactive oxygen species following IS and ID injection [154]. Interestingly, the same study also reported an elevation in IL-6 and TNF-α, indicating iron-induced inflammation.

Several other side effects of IV iron have been described, such as nephrotoxicity, iron overload and increased susceptibility to infections. Agarwal et al. raised concern about potential nephrotoxicity [123]. They found transient proteinuria, enzymuria and tubular damage following IV IS injection and postulated a mechanism involving increased oxidative stress. Iron overload is a possibility during long-term IV iron treatment. This is worrying because, in HD

patients, the accumulated iron dose over time, has been associated with the level of atherosclerosis [155], risk of hospitalization [156] and death rate [156-158]. Finally, a link between iron treatment and increased risk of bacterial infection has been suggested. Iron is a growth factor for bacteria [159] and high iron load in HD patients has been reported to inhibit neutrophil function [160, 161]. However, clinical evidence linking IV iron treatment to dialysis-related infections is lacking [162].

Complications related to carbohydrates

Serious, life-threatening allergic reactions may occur during treatment with iron-carbohydrate complexes. These reactions, evoked by epitopes in the carbohydrate shell, have primarily been noted with high-molecular weight ID and are rarely seen with the IV iron agents used today [115].

Clinical outcomes

In comparison with oral iron, IV iron is superior regarding hemoglobin response in patients on HD and PD [99, 163]. In this context, superior means faster hemoglobin response, higher hemoglobin value and lower ESA dose. On the other hand, in CKD patients not on dialysis and not treated with ESA, IV iron is not superior to oral iron treatment [97] and even though these patients require ESA, the benefit of IV iron compared to oral iron is small [98, 99] or non-existent [96, 100].

Treatment with vitamin C

Some efforts have been made in treating functional iron deficiency by using ascorbic acid to mobilize iron from the stores [164]. Studies on HD patients with refractory anemia and hyperferritinemia have shown that IV ascorbic acid has a beneficial effect, significantly increasing transferrin saturation and hemoglobin levels [165, 166]. Moreover, a decrease in serum ferritin and an increase in the response to ESA have been noted, suggesting iron mobilization from the tissue stores. However, controversies surrounding treatment with IV ascorbic acid exist. Some authors have not found any beneficial effect at all [167] and there is concern regarding the safety of this treatment. One potential side effect is hyperoxalatemia and another is induction of oxidative stress. Sudden high doses of intravascular ascorbic acid in a patient with high iron load may reduce the ferric iron to catalytically active ferrous iron, capable of inducing oxidative reactions. This pro-oxidative effect of ascorbic acid has been observed in vitro with plasma from HD patients [168] and in serum of iron-loaded animals [169]. Moreover, in a study in HD patients, orally administrated ascorbic acid was found to increase lipid peroxidation [170].

Renal replacement therapy

It is not surprising that renal anemia is corrected by successful kidney transplantation [23]. In a five years follow-up study after transplantation, anemia was cured in the majority of patients and even erythrocytosis occurred in 18 % [171]. However, with time, approximately 30 % of the patients developed anemia [171, 172].

When CKD patients start on dialysis, a significant improvement of erythropoiesis has been observed. This is most likely due to removal of uremic toxins (“erythroid suppressors”) by the dialysis process. Thus, in 34 peritoneal dialysis patients, DePaepe et al. found a significant increase in hemoglobin during the first 6 months after initiation of the treatment [173]. Similarly, Radtke et al. studied 42 ESRD patients and observed better hematocrit and lower serum erythropoietin levels after start of hemodialysis [174].

Before the era of ESA, it was noted that the hemoglobin level increased in patients transferred from HD to PD [175]. Further, when comparing these two dialysis modalities, a milder degree of renal anemia has been observed in the PD population [176, 177]. Thus, in a large register study, the proportion of patients requiring ESA was 25 % in PD compared to 80 % in HD and the ESA dose was 50 % lower in the PD population [176]. The possible reason for this is that blood loss is less marked and residual renal function is better preserved in PD [176, 178]. Indeed, residual renal function has been observed as an important predictor of the severity of renal anemia, both in the PD [179] and HD [180] population. The dialysis dose is also important since inadequate hemodialysis is associated with suboptimal response to erythropoietin therapy [181]. Further, increased dialysis dose has been associated with

reduced ESA need [76, 182]. On the other hand, in patients receiving hemodialysis three times a week, the benefit of increasing the hemodialysis dose is only present up to a level of approximately 1.3 in Kt/V. Increasing dialysis dose beyond this level seems to have no effect on the severity of renal anemia [69, 183]. The reason for this is not clear.

Whether different dialysis modalities, such as hemodiafiltration (HDF) and hemofiltration (HF), are superior to HD in treating renal anemia is a matter of debate. Some authors have described better anemia control with HDF [184-187] while others have failed to find such an effect [188, 189]. Thus, good evidence indicating any benefit of convectional dialysis treatments on renal anemia is lacking [190, 191].

AIMS OF THE THESIS

The general purpose of this thesis was to study three different issues in the treatment of renal anemia; (1) potentially toxic side effects of IV iron, (2) impact of convective hemodialysis on iron homeostasis and (3) ESA dose-sparing effect of change in ESA administration praxis.

The specific aims were:

- To evaluate if electron spin resonance (ESR) spectroscopy could be used to address the issue of iron toxicity by measuring free iron appearance and acute oxidative stress following IV iron injection (Paper I).
- To explore possible changes in markers of oxidative injury in the circulation following iron injection (Paper I and II).
- To investigate if any difference exists between iron-sucrose and iron-dextran regarding release of free iron and induction of oxidative reactions (Paper II).
- To answer the question if there is any clinically relevant disparity between two different hemodialysis modalities, HD and HDF, especially concerning iron homeostasis and erythropoietin or iron demand (Paper III).
- To study if the frequency of ESA dose-adjustments has effect on ESA requirements and hemoglobin response (Paper IV).

SUBJECTS AND METHODS

Subjects

All clinical data in this thesis are collected from 64 patients with end stage renal disease, of which 13 participated in more than 1 study. During the respective study periods, all patients were receiving regular dialysis treatment and their clinical condition was stable. The demographic data are described in detail in the respective studies.

Study designs

Paper I

This study is a prospective, open label, hypothesis-testing study. It was designed to investigate the release of intravascular "free" iron and acute oxidative stress following IV injection of 100 mg iron-sucrose. Two experiments were conducted, one with and one without ongoing dialysis. Blood samples were collected before, 10, 30 and 60 minutes after IV iron injections.

Paper II

This study was designed to compare two commercially available IV iron formulations regarding intravascular "free" iron release and induction of acute oxidative stress. It is an open label, prospective, non-randomized, and cross-over, explorative study. Blood samples were drawn before and 10 minutes after IV injection with iron sucrose, and four weeks later the procedure was repeated with iron-dextran.

Paper III

This study was designed to compare conventional hemodialysis and on-line hemodiafiltration regarding dialysis-related symptoms as well as various biochemical parameters. It is a prospective, randomized, participant-blinded, partially observer-blinded and cross-over explorative trial. Study visits took place before as well as after 30 and 60 days on respective treatment.

Paper IV

This is a retrospective, hypothesis-testing study. It was designed to compare ESA use and predictors of ESA requirement in two equivalent periods before and after a change in ESA administration praxis. Data from the local dialysis database were assembled for statistical analysis.

Biochemical analyzes

Routine analyzes

With the exception of NTBI, 25-hepcidin, Il-6 and markers of oxidative stress, all biochemical analyzes were performed as accredited routine clinical laboratory tests by the Central Laboratory of Sahlgrenska University Hospital.

Plasma iron

Analysis of total plasma iron was performed by a standard method at the clinical laboratory. This is a colorimetric assay based on iron binding by ferrozine [192].

Electron spin resonance spectroscopy

In Paper I and II, the technique of electron spin resonance (ESR) was used to analyze NTBI and AFR (see below). Because this is not a widespread method in clinical medicine, the basic principles will be briefly explained.

ESR, aka electron paramagnetic resonance (EPR), is a very robust and sensitive method for characterization and quantification of substances with unpaired electrons [193]. The method is based on the physical properties of an electron, being a charged particle spinning around its axis. This spinning causes the electron to behave like a small magnet, which could be compared the earth's rotation, creating a magnetic field at its poles. For any given electron in any given substance, the probabilities for clockwise and counter-clockwise rotation around the axis are equal. Thus, these two rotational energy states, or spin states, are energetically equivalent in the absence of a magnetic field. However, if a

constant external magnetic field is applied, the electrons will align either parallel or anti-parallel with that field. The parallel state is associated with a lower energy level than the anti-parallel state. The energy difference between the two levels is proportional to the field strength, B_0, of the external magnetic field according to the following equation:

$$\Delta E = g_e \mu_b B_0$$

wherein g_e (the g-factor) and μ_b (the Bohr magneton) are fundamental constants of the electron. The lower energy level (parallel spin) tends to be heavier populated than the higher one. If electromagnetic radiation with energy corresponding to:

$$h\nu = g_e \mu_b B_0$$

(the resonance condition) is introduced into the system, transitions from the lower to the higher level can be induced. These transitions, each at the prize of an amount of energy equivalent to hν, can be measured as an absorbance of energy by the system under study, and this forms the basis of the powerful quantitative and qualitative analyses performed with ESR.

It is important to note that only substances containing at least one unpaired electron, typically free radicals end certain metal complexes, can be detected by ESR. This is due to the fact that electrons have a strong tendency to form pairs with opposite spins. Such an electron pair is magnetically neutral, unaffected by the external field B_0 and, hence, ESR-silent. Since the vast majority of substances occurring in biological systems do not have any unpaired electrons, ESR is an extremely powerful tool for detecting free radicals, such as ascorbyl radicals, or metals, such as iron compounds, even in very complex systems like blood or biological tissues, without the need for extensive sample treatment. Typical magnetic fields used for the studies described herein are in the order of 0,3–0,4 Tesla, and the electromagnetic energy absorbed by the unpaired electrons is typically in the microwave range, with a frequency of 9–10 GHz.

Quantitative information about absorbing substances in the sample is gained via the amplitude of the signal. Qualitative information is gleaned from the fact that different substances present a different magnetic environment to any unpaired electron, mainly due to the magnetic fields caused by orbital motions of all other electrons in the molecule. This means that the external magnetic field is always either counteracted or augmented by a local magnetic field, varying in strength and direction for each type of molecule. Thus, in order to satisfy **the general resonance condition** (**$h\nu = g_e \mu_b B$**), where B is a constant equal to $B_{local} + B_0$, the external field B_0 has to be adjusted slightly to a unique value for each substance.

The main drawback of ESR in a clinical setting, even though the method is powerful and sensitive, is that the equipment is sophisticated, expensive and not widely available.

Non-transferrin bound iron

NTBI was measured essentially as described by Kozlov et al. [194]. In brief, plasma was mixed with desferrioxamine (DFO) and incubated for 15 min in room temperature. During this period, NTBI in the sample is chelated by the added DFO and quantitatively oxidized to Fe(III). The samples were then filtrated to eliminate any interference from iron-containing proteins (transferrin, ceruloplasmin, etc.). Then, each sample was carefully frozen in liquid nitrogen and ESR spectra were recorded using an X-band spectrometer. The corresponding concentration of DFO-chelated iron was obtained from a standard curve consisting of different concentrations of ferrous ammonium sulfate $[(NH4)_2Fe(SO4)_2]$ (figure 3.)

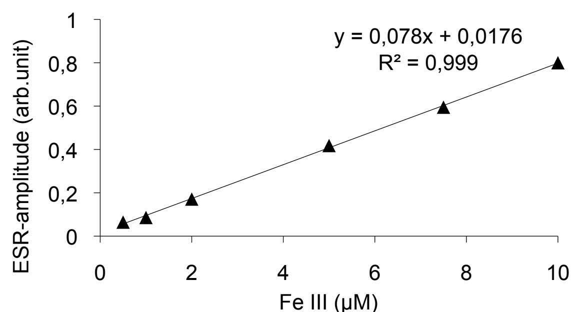


Figure 3. *The standard curve for the ESR determination of DFO-chelated iron.*

Markers of oxidative stress

Ascorbyl free radical

The measurement of plasma AFR was made by ESR. The analysis was performed on pure plasma without any additional treatment of the specimen. Immediately after thawing the sample, the AFR signal intensity was measured with an X-band spectrometer. The AFR gives a characteristic two-peak signal and the mean peak-to-peak amplitude (figure 4) was calculated to determine the AFR concentration in the sample, using 1 μM Tempol as standard.

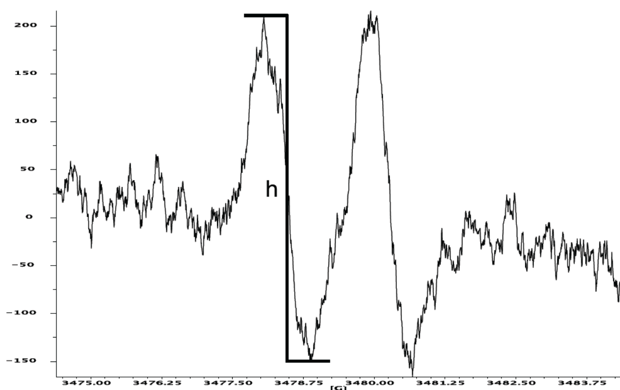


Figure 4. *The characteristic ESR spectra of AFR. The peak-to-peak amplitude (h) reflects the actual concentration in the sample.*

Other oxidative stress and inflammation markers

Estimation of lipoprotein oxidation was performed by measuring baseline levels of conjugated dienes in lipids extracted from LDL (BDC-LDL) by a spectrophotometric assay [195]. Protein oxidation was determined by measuring protein carbonyl content with a spectrophotometric technique [196]. The total antioxidant capacity was evaluated by a slightly modified spectrophotometric TEAC method (Trolox Equivalent Antioxidant Capacity) [197]. Measurement of plasma myeloperoxidase (MPO) and Il-6 were analyzed with a commercially available ELISA kit according to the manufacturer's instructions.

25-Hepcidin

Serum 25-hepcidin was measured at Hepcidinanalysis.com, Nijmegen, the Netherlands by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry [198].

Statistical methods

Statistical analyses were executed with the SPSS software package. In all Papers, T-test or non-parametric test (Wilcoxon, Mann Whitney) were used as appropriate for evaluation of differences between means, and Pearson's or Spearman's correlation was used to analyze association between two variables. In Paper I, a general linear model for repeated measures was used to explore changes in parameters over time. In Paper III, the Chi square test was used to compare frequency of symptoms between treatments. In Paper IV, a multiple regression model was used to analyze relationship between changes in predictors of ESA response and changes in ERI.

The null-hypothesis was rejected if the p value was less than 0.05.

Ethical considerations

In Paper I (experiment 2), eight patients stayed at the dialysis unit for one additional hour after ordinary dialysis treatment. In all other experiments, ordinary individual dialysis schedules were used for each patient. No changes in medication were needed to make these studies and no additional drugs were given. Regarding iron treatment, all patients were on regular iron treatment at inclusion, and in Paper II patients receiving Venofer (IS) were given Cosmofer (ID) with a potential risk of allergic reactions. However, no such reactions were noted. An additional ethical issue is the blood loss associated with extra blood samples.

SUMMARY OF MAIN RESULTS

PAPER I

Key findings

1. A measurable amount of plasma NTBI was noted in hemodialysis patients and in healthy blood donors.
2. The level of NTBI correlated significantly with the level of total iron. This was noted both pre-dialysis and following IV IS injection.
3. After IV injection of IS, a burst of oxidative stress was noted. In particular, AFR dramatically increased.
4. The increase in AFR was closely correlated to the increase in NTBI.
5. When measured in vitro,
    a. Labile iron was released from IV iron-carbohydrates, significantly more from IS than ID.
    b. Ascorbic acid was able to mobilize iron from IS but not from ID.

Non-transferrin bound iron (NTBI).

Before dialysis, all patients had a measurable level of NTBI in plasma (mean 1.6 μM) and it was closely correlated with total plasma iron (figure 5). In healthy blood donors, similar NTBI concentration was observed (mean 1.9 μM), but since total iron was not measured, a comparison of proportions of NTBI in healthy and patients was not possible.

After IV injection of 100 mg IS, the plasma concentration of NTBI increased from 1.2 to 5.7 μM and total plasma iron from 9.6 to 44.1 μM (table 1). Again, the levels of these parameters were strongly correlated (figure 6).

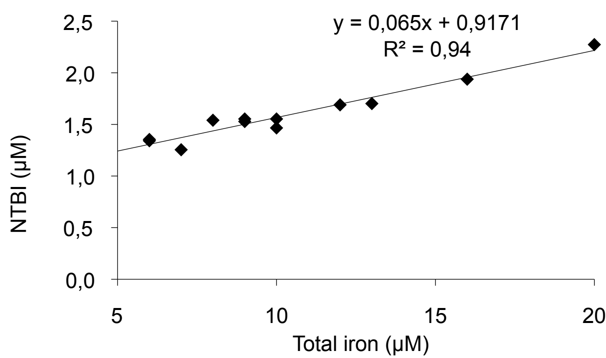


Figure 5. *The scatter plot and correlation between NTBI and total iron in pre-dialysis blood samples ($p < 0.001$).*

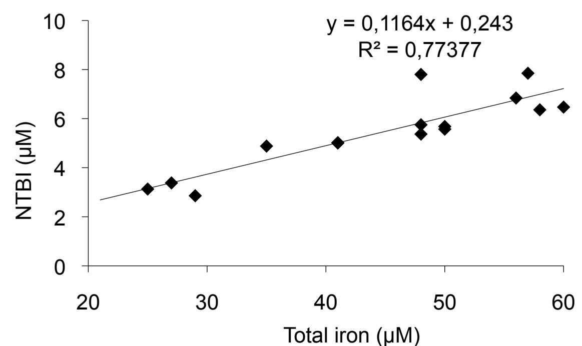


Figure 6. *The scatter plot and correlation between total iron and NTBI in plasma 10 min after intravenous IV injection of 100 mg IS ($p < 0.001$).*

Table 1. Effects of IV infusion of 100 mg IS on iron parameters and various markers of oxidative stress (mean ± SD).

	Before iron infusion	10 minutes after iron infusion	P value
p-iron (μM)	9.6 ± 4.6	44.1 ± 11.6	< 0.001
p-NTBI (μM)	1.2 ± 0.5	5.7 ± 1.4	< 0.001
p-AFR (nM)	202.5 ± 67.1	283.3 ± 108.7	< 0.001
p-MPO (ng/ml)	82.6 ± 39.0	101.1 ± 43.4	< 0.05
p-protein carbonyl (nM)	22.3 ± 5.9	27.1 ± 5.8	< 0.001
p-BDC-LDL (μM)	33.0 ± 9.4	35.7 ± 10.8	< 0.001
p-TEAC (μM Trolox)	5.13 ± 0.4	4.96 ± 0.5	n.s.

Oxidative stress markers.

Experiment 1.

Ten minutes after IV injection of 100 mg IS, a significant increase in the plasma concentrations of AFR, MPO, protein carbonyl and BDC-LDL were noted (table 1). The enhancement was 40, 22, 22, and 8 % respectively. During the same time, total antioxidant capacity (TEAC) decreased by 3.3 %, albeit not significant (p = 0.06).

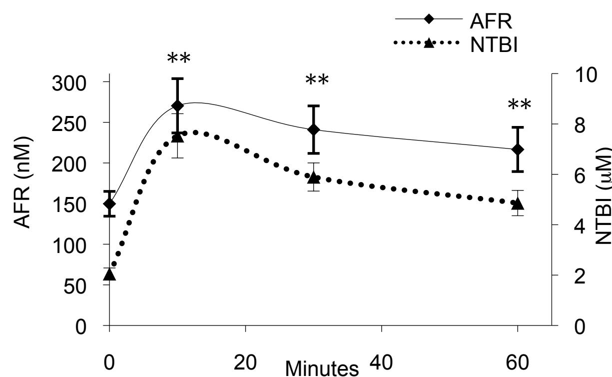


Figure 7. *The plasma concentration (mean ± SEM) of NTBI and AFR during 60 min after IV injection of 100 mg IS. ** = p < 0.01 compared to before iron injection, n=8.*

Experiment 2

Following IV injection of 100 mg IS, a parallel increase in AFR and NTBI was found (figure 7). After a peak at 10 minutes, the concentration gradually faded. However, compared to values before iron administration, a significant increase in both AFR and NTBI were still found one hour after the injection. The plasma concentrations of AFR and NTBI were very well correlated.

Iron release from IV iron formulations in vitro

During 60 minutes incubation of 500 μM IS (a concentration close to the maximum plasma concentration attained after injection of 100 mg IS) together with 10 mM DFO, 2.2 % of the iron in the IS complex was found to be loosely bound and chelated with DFO. Further, the amount of his labile iron was doubled when ascorbic acid was present. When the experiment was repeated with ID, the quantity of loosely bound iron was significantly lower (1.2%) and ascorbic acid did not mobilize iron from the ID complex.

PAPER II

Key findings

1. The iron analysis at our routine clinical laboratory directly measured 5.6 % of the iron bound in IS and 1.6 % of that bound in ID.
2. Total iron and NTBI increased significantly more after injection of IS compared to ID.
3. Acute oxidative stress was noted after IV injection of 100 mg IS but not after IV injection of 100 mg ID.

Iron measurements in vitro

The iron assay at the clinical laboratory is based on iron-chelation by ferrozine. This assay was used to measure iron in a solution of PBS and different concentrations of IS and ID. Significantly more iron was loosely bound in IS than in ID. Thus, ferrozine directly chelated 5.6 % of the iron bound in IS and 1.6 % of that bound in ID.

Iron measurements in vivo

IV injection of IS and ID significantly elevated the levels of plasma iron and NTBI (table 2). However, both increased significantly more after IS than after ID or by 27.9 vs. 7.4 µM respectively (figure 8).

Comparison between IS and ID regarding induction of oxidative stress.

The levels of oxidative stress markers in plasma before and after IV iron injection are shown in table 2. After injection with IS, the concentration of AFR and protein carbonyl increased significantly, or by 29 and 19 % respectively. No significant changes were noted after injection with ID. Other oxidative stress markers were slightly, but not significantly, increased after iron administration.

When changes in oxidative stress markers after IS were compared to changes after ID, only AFR increased significantly more after IS (figure 9).

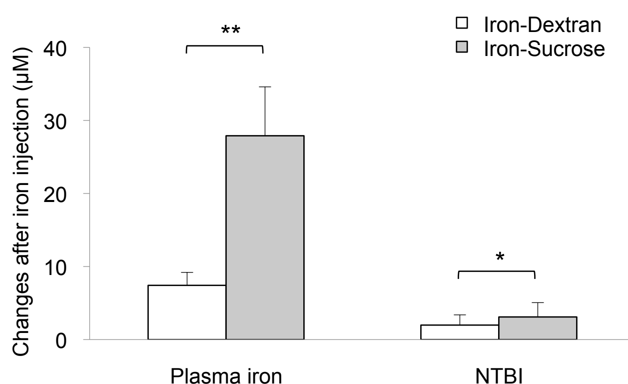


Figure 8. *Changes in levels of plasma iron and NTBI 10 minutes following IV iron injection (mean ± SD). * p < 0.05; ** p < 0.01.*

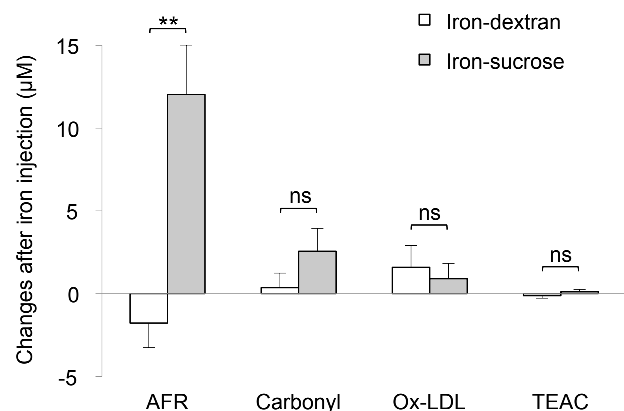


Figure 9. *Changes in plasma levels of oxidative stress markers 10 minutes after IV iron injection (mean ± SD). ** p < 0.01.*

Table 2. Iron and oxidative stress parameters before and 10 min after injection of 100 mg IS, respectively 100 mg ID (mean ± SD).

	Iron-dextran		Iron-sucrose	
	Before	After	Before	After
p-NTBI (µmol/L)	4.2 ± 1.8	6.2 ± 1.5 **	3.9 ± 1.8	7.0 ± 2.8 ***
p-iron (µmol/L)	9.5 ± 3.2	16.9 ± 2.9 ***	10.6 ± 4.7	38.5 ± 8.4 ***
p-AFR (nmol/L)	58.7 ± 32.2	56.7 ± 28.5	55.7 ± 37.4	67.7 ± 44.6 ***
p-protein carbonyl (nmol/L)	19.1 ± 3.4	19.4 ± 4.1	19.4 ± 4.8	22.0 ± 4.8 *
p-BDC-LDL (µM)	26.7 ± 10.3	28.3 ± 11.6	27.4 ± 13.0	28.3 ± 13.1
TEAC (µmol/L TROLOX)	4.5 ± 0.7	4.4 ± 0.8	4.2 ± 0.8	4.3 ± 0.7

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to before iron

PAPER III

Key findings

1. No significant differences between treatment with HD and HDF were found in:
   - Intra-dialysis complications
   - Inter-dialysis symptoms
   - Quality of life
   - Blood cell counts and hemoglobin
   - ESA and iron requirement
   - Inflammation and oxidative stress
   - Phosphate control
2. Significant differences were found in:
   - Blood pressure, which was increased by HDF
   - The level of pre-dialysis s-albumin, which decreased by HDF
   - Clearance middle-weight molecules, which was improved by HDF, exemplified by:
     i. Iohexol
     ii. ß2microglobulin
     iii. 25-hepcidin
3. A trend towards more normal iron homeostasis, with lower serum ferritin, was found with HDF.

Dialysis-related symptoms and general health

The frequency of intra-dialysis complications was similar during treatment with HD and HDF. Further, patients' answers regarding dialysis-related symptoms and general well-being were similar. Indeed, quality of life was considerably lower than in the general population in Sweden [199], but no relevant differences between HD and HDF were noted (figure 10).

Arterial blood pressure

The arterial blood pressure was slightly higher during the last week of treatment with HDF compared to the same period on HD treatment (table 3). This was noted in all blood pressure measurements, but was only statistically significant in blood pressure measured in upright position before dialysis ($p < 0.05$).

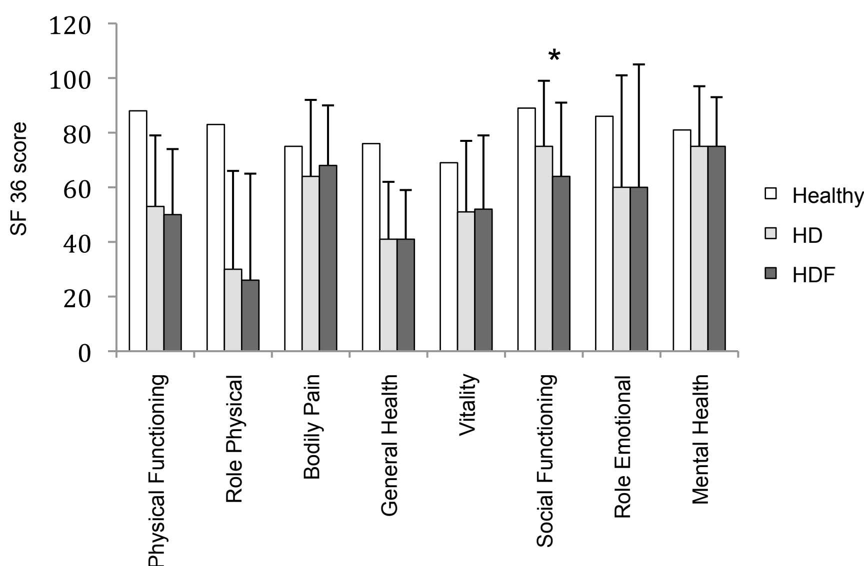


Figure 10. *Quality of life after 2 months treatment with HD and HDF (mean). In comparison, average SF-36 score in the general population in Sweden are shown.*
** p < 0.05 (HD compared to HDF)*

Table 3. Systolic and diastolic blood pressure during the last three dialysis sessions on respective dialysis modality (mm Hg, mean ± SD).

	Pre-dialysis		Post-dialysis	
	HD	HDF	HD	HDF
Systolic				
Supine	157.5 ± 26.1	161.2 ± 29.9	157.1 ± 22.8	161.6 ± 25.1
Standing	151.1 ± 26.1	156.4 ± 31.1	137.9 ± 18.4	143.0 ± 24.3
Diastolic				
Supine	86.4 ± 10.8	88.9 ± 12.6	85.3 ± 10.3	86.8 ± 12.8
Standing	86.8 ± 12.6	90.1 ± 14.0 *	81.8 ± 9.5	85.1 ± 13.4

* p < 0.05 compared to HD

Table 4. Hematological and chemical parameters at baseline, on day 30 and on day 60 in respective study period (mean ±SD).

	Day 0	Day 30		Day 60	
	Baseline	HD	HDF	HD	HDF
Hemoglobin (g/L)	116.1 ± 12.2	116.1 ± 7.5	115.6 ± 8.9	116.1 ± 8.4	115.1 ± 6.0
Mean corpuscular volume (x 10-15L)	97.1 ± 5.5	96.3 ± 5.0	97.9 ± 5.7	97.2 ± 5.7	98.0 ± 4.9
Leukocyte count (x 109/L)	8.1 ± 2.5	7.4 ± 2.1	7.5 ± 2.3	7.0 ± 1.8 a**	7.1 ± 2.3 a**
Thrombocyte count (x 109/L)	241 ± 76	232 ± 80	237 ± 75	234 ± 69	236.5 ± 83.1
s-sodium (mmol/L)	137.2 ± 3.2	137.7 ± 3.8	137.8 ± 3.3	137.3 ± 2.3	137.5 ± 2.4
s-potassium (mmol/L)	5.0 ± 0.9	4.8 ± 0.5	4.8 ± 0.6	4.9 ± 0.6	5.0 ± 0.8
s-calcium (mmol/L)	2.4 ± 0.2	2.4 ± 0.2	2.4 ± 0.2	2.4 ± 0.2	2.4 ± 0.4
s-phosphate (mmol/L)	2.0 ± 0.5	1.7 ± 0.5	1.7 ± 0.4	1.8 ± 0.4	1.7 ± 0.4
s-bicarbonate (mmol/L)	23.5 ± 2.0	24.3 ± 1.3	24.5 ± 1.7	24.2 ± 1.6	24.1 ± 2.3
s-creatinine (µmol/L)	748 ± 240	685 ± 199 a**	658 ± 209 a***, b*	688 ± 213 a**	671 ± 192 a**
s-BUN before dialysis (mmol/L)	21.9 ± 5.8	18.1 ± 4.7 a***	16.3 ± 4.5 a***	18.1 ± 4.5 a**	17.3 ± 3.8 a***
s-albumin (g/L)	34.8 ± 3.2	35.1 ± 3.0	34.9 ± 3.0	36.0 ± 2.9	34.3 ± 2.6 b**
s-ferritin (µg/L)	302 ± 141	311 ± 145	276 ± 119	311 ± 126	253 ± 95
s-hsCRP (mg/L)	11.2 ± 12.2	10.5 ± 9.5	9.3 ± 8.7	9.8 ± 10.2	9.6 ± 11.1
s-ß2microglobulin (mg/L)	30.6 ± 11.9	34.6 ± 12.0	24.9 ± 8.5 a***, b ***	34.6 ± 17.0	23.7 ± 8.1 a**, b***

a = compared to baseline b = compared to HD * p < 0.05 ** p < 0.01 *** p < 0.001

Pre-dialysis variables

The results of clinical chemistry analyses at baseline and during treatment with respective dialysis modality are shown in table 4. The only significant differences between HD and HDF after 30 and 60 days were lower level of ß2microglobulin, reflecting better clearance of this substance by HDF. At day 60, significantly lower levels of s-albumin and a trend towards lower levels of s-ferritin ($p = 0.085$) and larger MCV ($p = 0.084$) were found.

Post-dialysis variables

The results of post-dialysis serum analyses at day 60 are shown in table 5. Markers of inflammation and oxidative stress were similar between treatments. On the other hand, a significant lower concentration of 25-hepcidin was found with HDF and it was closely associated with the pre-dialysis ferritin value, as seen in figure 11.

Table 5. Post-dialysis markers of inflammation, oxidative stress and iron homeostasis on day 60 with respective treatment (mean ± SD).

	HD	HDF
s-interleukin 6 (pg/ml)	17.4 ± 17.0	18.1 ± 11.5
s-MPO (ng/ml)	90.1 ± 76.8	93.4 ± 59.9
s-protein carbonyl (nmol/l)	23.5 ± 4.4	23.1 ± 4.3
s-BD-LDL (µM)	44.4 ± 13.8	41.1 ± 13.3
s-TEAC (µM Trolox)	4.0 ± 0.6	4.1 ± 0.7
s-hepcidin (nM)	11.1 ± 5.4	6.9 ± 2.9 **

** p < 0.01 compared to HD.

ESA and iron requirements

No significant differences in ESA and iron consumption between treatment with HD and HDF were found. The variation was large and the mean doses of ESA and iron during the second month were slightly higher during treatment with HDF or 13275 ± 10850 IE/week compared to 17300 ± 16667 IE/week with HD ($p = 0.073$). The mean IV iron dose during HD was 270 ± 205 mg and 320 ± 237 mg during HDF ($p = 0.357$).

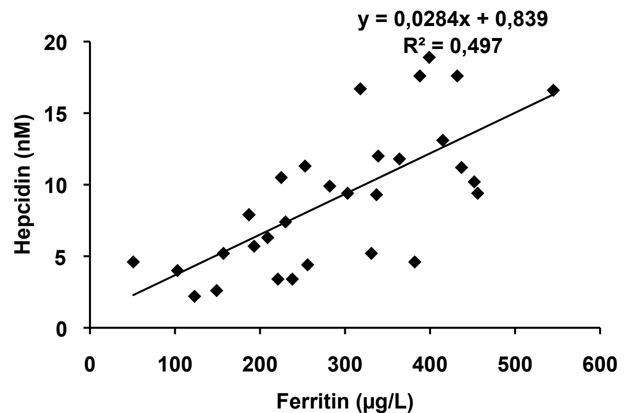


Figure 11. *Post-dialysis serum hepcidin plotted against pre-dialysis serum ferritin (p < 0.001).*

PAPER IV

Key findings

1. After a change in ESA administration praxis, a 22.5 % decrease in ESA demand and 19.9 % decrease in ERI were noted.
2. In a multivariate regression model, the drop in ESA demand could not be explained by changes in well-known predictors of ESA response.

ESA consumption and hemoglobin

Figure 12 illustrates the mean ESA dose per week and the pre-dialysis hemoglobin level in each month during the entire follow-up. As seen, the drop in ESA dose after change of the ESA administration praxis did not have any considerable effect on the hemoglobin value.

The mean ESA dose and ERI were significantly reduced in Period 2 compared to Period 1 (table 6). At the same time, mean pre- and post-hemoglobin values dropped by 1.1 and 2.4 g/L, however, this reduction did not reach stitistical significance (p = 0.42 and 0.09).

Predictors of ERI

The levels of well-known predictors of ESA response are summarized in table 7.
The transferrin saturation and dialysis dose, measured as dialyzed blood volume per dialysis session, was significantly increased in Period 2. The variation of other parameters before and after the change in ESA administration praxis was similar. When testing for associations between changes in ERI and changes in these predictors, a significant inverse correlation with changes in s-transferrin saturation, dialyzed blood volume and s-ferritin, was found (table 8).
However, in a multivariate regression model with Δ ERI as outcome and Δ CRP, Δ albumin and Δ dialyzed blood volume as independent variables, none of these factors significantly predicted changes in ERI (table 9). S-ferritin and s-transferrin were excluded in the model because of bidirectional relationship with ERI.

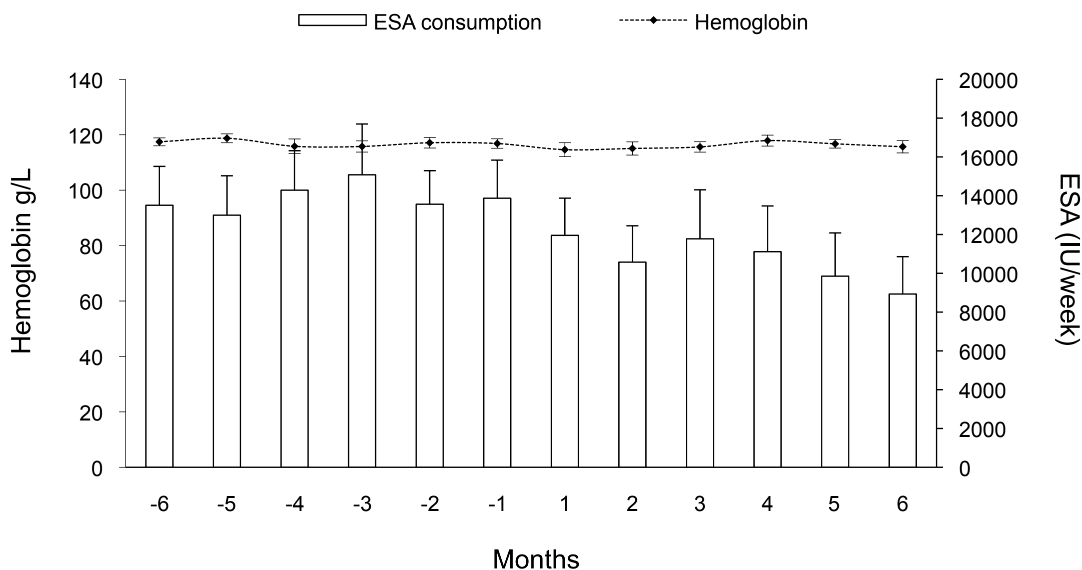


Figure 12. *Hemoglobin concentration and ESA consuption during follow up (mean ± SEM), n =18.*

Table 6. Erythropoiesis parameters during respective study period (Mean ± SD).

	Period 1	Period 2	p value
Hemoglobin pre-dialysis (g/L)	117.0 ± 4.8	115.9 ± 5.1	ns
Hemoglobin post-dialysis (g/L) #	125.1 ± 4.7	122.7 ± 5.4	ns
IV iron consumption (mg per day)	5.1 ± 4.5	5.0 ± 3.9	ns
ESA dose (IU/kg/week)	186.5 ± 117.8	144.6 ± 126.4	< 0.01
ERI (IU/kg/week/g/dL)	15.1 ± 9.9	12.1 ± 11.0	< 0.01

estimated hemoglobin according to ref. [200]

Table 7. The levels of well-known predictors of ESA response in respective study period (Mean ± SD).

	Period 1	Period 2	p value
Number of dialysis sessions	76.8 ± 2.7	75.9 ± 6.4	ns
Dialyzed blood volume (L/kg/session)	0.90 ± 0.20	0.98 ± 0.18	< 0.01
s-Transferrin saturation (%)	22.2 ± 5.1	27.6 ± 9.4	< 0.05
s-Ferritin (µg/L)	470 ± 189	528 ± 243	ns
s-CRP (mg/l)	9.9 ± 7.2	12.6 ± 11.1	ns
s-Albumin (g/L)	35.8 ± 2.2	36.2 ± 3.5	ns
s-PTH (ng/L)	419 ± 384	353 ± 210	ns

Table 8. The association between changes in ERI and changes in predictors of ESA response (bivariate Spearman's rho).

	rs	p value
Δ Dialyzed blood volume (L/kg/session)	-0.55	0.017
Δ s-Transferrin saturation (%)	-0.55	0.018
Δ s-Ferritin (µg/L)	-0.48	0.042
Δ s-CRP (mg/l)	0.44	0.065
Δ s-Albumin (g/L)	0.00	1.000
Δ s-PTH (ng/L)	-0.19	0.453

Predictors of hemoglobin concentration

Changes in transferrin saturation and changes in serum ferritin were correlated with changes in post-dialysis hemoglobin (p=0.02 and 0.04), while changes in ESA dose per week were not. In a multiple regression analysis, Δ post-dialysis hemoglobin as outcome and Δ transferrin saturation, Δ dialyzed blood volume, Δ CRP, Δ s-albumin and Δ PTH as predictors, only changes in transferrin saturation turned out as an independent predictor (beta = 0.414, p value = 0.019).

Table 9. The table shows the results of a multiple linear regression model with Δ ERI as outcome and changes in factors influencing erythropoiesis as predictors. Transferrin saturation and ferritin were omitted from the model because they are modified by ESA.
Δ = mean changes between the periods (Period 2 - Period 1).

	Beta	95 % CI interval		p value
Predictors		lower	higher	
Δ Dialyzed blood volume (L/kg/session)	-17.44	-40.71	5.84	0.130
Δ s-CRP (mg/l)	0.037	-0.34	0.42	0.838
Δ s-Albumin (g/L)	0.015	-1.13	1.16	0.979
Δ s-PTH (ng/L)	0.001	-0.01	0.01	0.712

DISCUSSION

The multifactorial etiology of renal anemia makes its treatment a challenge for the practicing nephrologist. The goal of the treatment is simple, a hemoglobin value between 110 and 120 g/L [10, 68]. On the other hand, keeping the hemoglobin level inside this tight interval is not easy. Further, an emphasis should be laid on "how to reach the target hemoglobin" rather the target itself, as this is probably of greatest relevance. As illustrated in figure 13, the hemoglobin value in patients on dialysis is determined by multiple factors that have to be considered in the management of anemia in this population.

In the studies included in this thesis, only a few aspects of renal anemia management have been touched upon; a safety issue with IV iron treatment, an option of attacking a fundamental pathogenetic factor and a possibility of reducing ESA dose by changing the administration praxis.

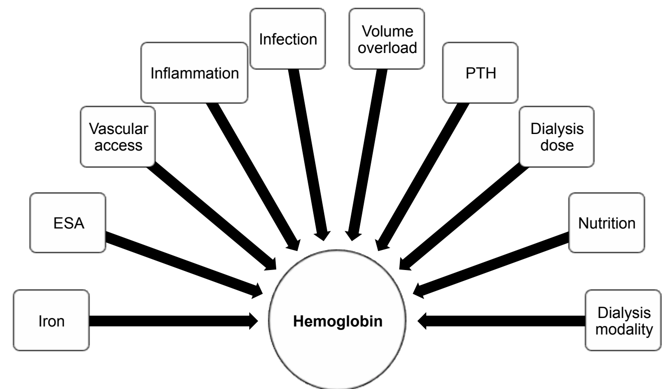


Figure 13. *The multifactorial predictors of hemoglobin production in patients on dialysis treatment.*

Evaluating the potential toxicity of IV iron

Iron is essential for all living organisms [201]. Due to its marvelous ability to both gain and release electrons it can exist in various oxidative states, giving it a crucial role in numerous biological actions, such as transporting oxygen to tissues via hemoglobin as well as functioning as a cofactor in a number of enzyme systems [202]. Because of the reactivity, iron is toxic and therefore, by nature, iron is never 'free' or unbound in biological systems. Accordingly, iron absorption, transport and storage are firmly regulated, preventing iron contact with substances not participating in iron-related biological actions or iron homeostasis. When iron is injected directly into the blood stream, the natural way of iron to enter the body is bypassed and large amounts of iron suddenly emerge in the circulation. This constitutes a risk for the appearance of labile and potentially harmful iron. Indeed, 2 - 6% of iron bound in the most common iron-carbohydrate complexes is labile and directly chelated by transferrin [117]. If this iron isn't properly taken care of by transferrin and other iron binding molecules, a hazardous redox reaction can occur. In Paper I and II this issue was investigated in two ways: directly, by measuring non-transferrin bound iron (NTBI) following IV iron injection, and indirectly, by measuring "footprints" of catalytically active iron, such as markers of oxidative stress.

Non-transferrin bound iron (NTBI)

The nature of NTBI (aka "free", labile or bleomycin-detectable iron) is not entirely clear. It is thought to represent a small native iron "pool", with nonspecific catalytic activity, bound to other plasma components than transferrin, such as albumin, citrate and phosphates [203, 204]. Moreover, the NTBI pool appears to be related to the total iron content in plasma. This is illustrated in figure 5, which shows a significant correlation between total iron and NTBI in plasma from HD patients before hemodialysis treatment. Moreover, this correlation was still seen after IV iron injection (figure 6), which is interesting as it indicates that the NTBI pool increase in spite of available transferrin binding capacity.

Quantifying NTBI in plasma is quite difficult, mainly owing to the presence of much larger pools of physiologically bound iron (e.g., transferrin, hemoglobin, and other iron-containing proteins). In Paper I and II, an ESR method, based on iron chelation by desferrioxamine (DFO) was adapted and used for this purpose. By this method, ferric iron, not bound to transferrin, is detected. The sensitivity is good, making it possible to quantify low levels of NTBI, even in healthy individuals. This is

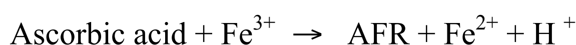
shown in Paper I, where the mean level of NTBI in blood donors was 1.9 μM, compared to 1.6 μM in HD patients. Further, applying the method on plasma following IV iron injection (Paper I), a rapid increase in plasma NTBI was noted (figure 7). This is in line with several other reports on appearance of NTBI after intravenous IS infusion, both in hemodialysis patients [117, 119, 120, 131] and in healthy volunteers [132].

Measurements of NTBI following IV iron injection can overestimate the actual level of NTBI in the circulation, because the assay used can, in fact, measure iron from the iron-carbohydrate complex [205]. This was evaluated in Paper I, where the ESR method was found to be able to measure 1.2 respective 2.2% of iron bound in ID and IS in vitro. Thus, there is a possibility that a part of the NTBI, measured after IV iron injections in Paper I and II, comes from the iron-carbohydrate complex. However, it seems unlikely, because transferrin, a strong iron chelator, should bind the majority of this iron during the time from injection to sample collection (10 minutes). Taken together, in studies measuring NTBI in blood following IV iron injection, an assay interference with the iron complex can never be excluded.

Whatever the origin of NTBI, the crucial question is whether it is catalytically active and capable to induce oxidative reactions.

Ascorbic free radical (AFR)

AFR (semidehydroascorbate) is an intermediate when ascorbic acid is oxidized in one-electron steps into dehydroascorbate [206]. It is nontoxic and relatively stable compared to the "primary" free radicals encountered in biological systems and it is easily detectable by ESR. Normally, low steady-state concentrations of AFR are found in plasma, conceivably reflecting generation of AFR from the reaction of ascorbic acid with free radicals. AFR can also be generated by oxidation of ascorbic acid in the presence of transition metals, such as iron, which upon reduction become catalytically activated as shown by following equation:



$$\text{Ascorbic acid} + Fe^{3+} \rightarrow \text{AFR} + Fe^{2+} + H^{+}$$

Thus, in theory, if ferric iron (Fe^{3+}) is released from the iron-carbohydrate complex, it is rapidly reduced to ferrous iron (Fe^{2+}) by ascorbic acid, which in turn is oxidized to AFR. Hence, an increase in AFR following IV iron injection indicates an appearance of Fe^{2+}, which is generally toxic in biological systems and can catalyze many reactions associated with oxidative tissue damage.

In Paper I and II, it was investigated if the reaction described above occurs when iron is injected into the circulation. A huge and abrupt increase in AFR was found after IV injection of 100 mg IS (figure 7) and a significant elevation was still seen 1 hour after the injection. This supports the theory that ferrous iron has, indeed, appeared.

AFR has been suggested to be an indicator of total oxidative burden [207] and in vitro studies have shown increased AFR levels in plasma during induced free radical stress [208] and iron load [209]. In vivo, Galleano reported increased level of AFR in iron-overloaded rats [210] and the results reported in Paper I-II are the first on this topic in humans. On the other hand, this is not the first time ESR is used to evaluate oxidative stress after IV iron injection. Rooyakkers et al. used this technique to evaluate the generation of superoxide in whole blood following IV IS injection. A dramatic increase in superoxide was noted [132].

Iron induced oxidative stress

The fate of the iron-carbohydrate complex after IV injection is illustrated in figure 14. As can be seen, there is a possibility of oxidative reactions if labile iron appears in the circulation. Indeed, in vitro, the most commonly used IV agents; ID, IS, IG, have been found to release catalytically active iron and induce redox reactions [211]. Further, in vivo, numerous studies have noted an elevation in oxidative stress markers after IV IS injections, as compiled in Paper II [122]. On the other hand, not all authors have noted increased oxidative stress following IS injection [212-216] making this issue a matter of controversy. In Paper I, after IS injection, a parallel increase in AFR and NTBI was observed (figure 7) and the strong correlation between the NTBI and AFR indicates a causal relationship. Previously, one

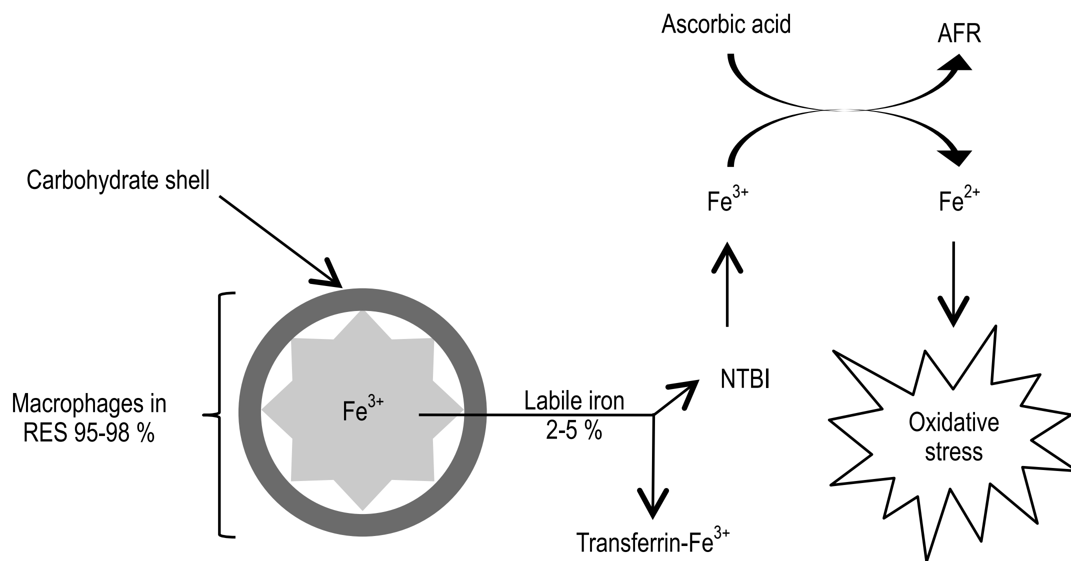


Figure 14. *The pathways for iron after IV injection. Oxidative reactions can occur if labile iron appears. Ascorbic acid is an exemple of a bioreductant existing in the circulation, many others may be involved.*

author has reported a significant correlation between NTBI and an oxidative stress marker following IS injection (MDA) [131].

Comparison of iron-sucrose and low-molecular weight iron-dextran

At the time of the studies in this thesis, two IV iron formulations were indicated for treatment of renal anemia in patients on chronic hemodialysis in Sweden; iron-sucrose (Venofer®) and low-molecular weight iron-dextran (Cosmofer®). In Paper I, possible differences in stability between these complexes were investigated in vitro. During incubation with DFO over 60 minutes, a continuous release of iron from the IS complex was noted. Thus, 2.2% of the total iron bound in the IS complex was chelated by DFO during this time. Further, the dissociation was augmented by 75% by ascorbic acid. On the other hand, the ID complex was significantly more stable than IS. During 60 minutes, DFO chelated 1.2% of the iron bound in the ID complex and ascorbic acid did not significantly increase iron dissociation from ID. The finding of superior stability of ID compared to IS is not new [117, 217]. In general, larger iron-carbohydrate complexes are more stable and the MW of LMW-ID was recently reported as 410 kDa compared to 252 kDa for IS [218]. In the same study, free iron release from different IV iron agents was investigated with similar results as presented in Paper I. Further, in recent study, NTBI increased significantly more after IV IS than ID injection, both in HD patients and healthy volunteers [154].

In Paper II, intravascular iron release and induction of oxidative stress after IV administration of IS and ID was compared. The NTBI “pool” enhanced significantly more after IS or by 86%, compared to 45% after ID. Interestingly, in spite of the increase in NTBI after ID, no increase in AFR was noted, while AFR rose by 29% following IS. However, ID has much longer half live than IS [113] so there is a possibility of higher cumulative intravascular iron release from ID over time, and an increase in AFR beyond 10 minutes can not be ruled out.

The lack of AFR formation following ID indicates that during the time between IV iron injection and sample collection (10 min), iron released from ID was properly bound to transferrin and other iron-binding molecules (NTBI) and no fraction was catalytically active. On the contrary, the amount and/or speed of iron release from IS was overriding the entire plasma iron-binding capacity and a fraction of it appeared, at some moment, in unbound form, inducing undesired redox reactions. The reason why increased oxidative stress was not seen following ID is not clear, but one explanation is

that ID and IS complexes interact differently with iron-binding molecules in blood and that ID in some way "delivers" iron directly to these molecules protected from the surroundings.

Previously, it has been believed that transferrin "oversaturation" is necessary before iron can appear as "free" and catalytically active. For instance, this has been noted by using the bleomycin assay to analyze NTBI [120]. This phenomenon was not seen in the present studies. Transferrin saturation were over 100 % in 10 out of 20 patients in Paper I and 4 out of 20 in Paper II, but the elevation in AFR was not only seen in these patients. Instead, AFR increased at any level of transferrin saturation, which, in theory, could be caused by too fast iron release from the IS complex. Methodological aspects could explain the discrepancy between the results presented here and those from studies conducted with the bleomycin assay, as the sensitivity of the ESR method is much higher. For example, the bleomycin assay is not able to measure NTBI levels in patients before IV iron injection [120].

Iron overload

The issue of harmful iron overload in HD patients has been evaluated in several studies. Two studies have reported a significant correlation between the level of atherosclerosis and annual IV iron dose [155, 219], Feldman et al. described increased hospitalization and mortality with IV iron doses exceeding 1000 mg over 6 months [156], Brookhart et al. reported elevated mortality risk with greater iron use [158] and increased mortality has been associated with ferritin levels above 800 µg/L [157] as well as levels above 600 µg/L independently of the C-reactive protein level [220]. In addition, in two large cohort studies, IV iron doses above 400 - 450 mg per month were significantly correlated with higher death rates [157, 221]. The patients participating in this thesis had an average ferritin level of 350 µg/L (assemblage of all four observation periods) and it can be concluded that these patients were not suffering from severe iron overload.

The diagnosis of iron overload is not easily performed in clinical praxis because ferritin, the marker of total iron stores, is difficult to interpret because of its behavior as an acute phase reactant, increasing secondarily to non-iron related factors such as inflammation and malnutrition. As these conditions are frequently found in CKD patients, [222], ferritin levels above normal have been considered safe in this patient population. In recent study, using non-invasive MRI technology, serum ferritin was useless in predicting liver iron content [223]. Instead, liver iron was strongly correlated to cumulated iron dose and time on dialysis. This is in contrast to an earlier, non-invasive study that measured liver iron concentration in HD patients with a noninvasive magnetic method, called SQUID (superconducting quantum interference device). In this study Canavese et al. reported a good correlation between hepatic iron content and ferritin levels in HD patients without ongoing inflammation [224]. Interestingly, both studies found iron overload even at ferritin levels that are generally considered safe. On the other hand, no data exists on a possible relationship between high iron load in ESRD and tissue damage [225].

High iron load not only leads to high serum ferritin levels, but also to high levels of serum hepcidin [34, 37]. Indeed, a significant correlation between those two parameters has been described [37, 39, 226, 227] and the results in Paper III are in line with this finding. High levels of hepcidin lead to decreased iron absorption and locking of iron in stores. This is a normal physiological response to iron overload and even during infections, as iron is important for the virulence of the microorganisms and can impair neutrophil function [120, 160, 161]. On the opposite, the hepcidin levels decrease when iron levels are low or when more iron is required for erythropoiesis, as happens with ESA treatment [227, 228].

In theory, there is a possibility that repeated IV iron injections create a vicious circle (figure 15). When high amounts of iron are injected into the circulation, the body interprets this as acute iron load and, in defense, promptly increases the level of hepcidin [37, 40, 229]. Keeping in mind that high hepcidin levels and functional iron deficiency are common in uremia, replicated IV iron loadings could, in spite of temporary elevation in transferrin saturation, could aggravate this condition by maintaining high hepcidin levels and low transferrin saturation over time. Low transferrin saturation indicates treatment with more IV iron and the process can

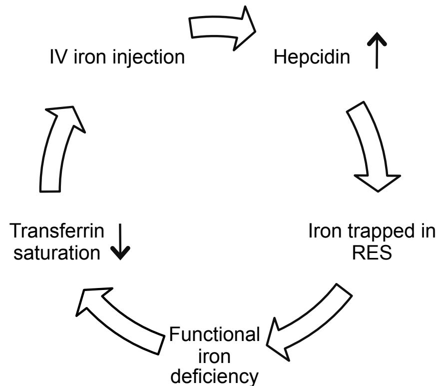


Figure 15. *A theory of a vicious circle, created by repeated IV iron injections.*

continue, finally resulting in iron overload. Judging from this hypothesis, treatment of functional iron deficiency with additional iron injections, as has been advocated by the DRIVE study [230], should be executed with caution.

Taken together, the risk for iron overload with long term IV iron treatment cannot be neglected. In US, the mean IV iron dose administrated to HD patients has been gradually increasing, and was, in 2006, 4.2 g/year [231], which is in great excess of the predicted iron loss of 1-2 g/year [31]. As a result, the serum ferritin levels have gradually increased, and in 2007 the mean levels in the HD population in US and Sweden were approximately 550 µg/L [232]. Moreover, 30% of the US patients had serum ferritin above 800 µg/L [233], which are levels that have been associated with increased mortality risk [157]. In spite of the controversial significance of ferritin in judging iron status, this trend is of concern and a recent observation supports that HD patients are given iron in excess of what is needed [234]. In this report, 26 iron-replete HD patients (mean ferritin = 503 µg/L) were able to maintain stable hemoglobin and ESA dose despite that all iron treatment was withheld for 16 weeks.

Attacking hepcidin

In Paper III, a broad comparison of two dialysis modalities, low-flux hemodialysis (HD) and hemodiafiltration (HDF), was performed. The overall results of this study were negative. In other words, no differences in clinically relevant parameters were seen. On the other hand, serum hepcidin levels were within the normal reference interval in all cases after treatment with HDF and 38 % lower than after treatment with HD. Even though the corresponding pre-dialysis levels were not measured, this indicates that HDF eliminates hepcidin more effectively than HD.

Studies on elimination of hepcidin by dialysis are sparse. Surprisingly, Ashby et al. did not observe any significant elimination of hepcidin with low flux HD [37, 235] while Weiss et al. found a significant elimination of hepcidin by both low flux HD and HDF [227]. Further, in a recent study, using a new high-flux dialyzer (Gambro Revaclear®), which effectively removes middle molecular weight substances, a 50% reduction in serum hepcidin levels was found [236].

The clinical benefit of lowering hepcidin levels by HDF remains to be investigated. However, as described earlier, patients on dialysis often suffer from functional iron deficiency, mainly caused by pathologically high levels of circulating hepcidin [237]. Therefore, it seems reasonable that efforts in lowering the level of hepcidin will bring the iron homeostasis towards a more normal state. The finding in Paper III, of a trend towards lower ferritin levels and increased MCV after 2 months treatment with HDF, is in line with this hypothesis, indicating iron mobilization from the stores. This is further supported by another study reporting lower ferritin levels and improved iron utilization with HDF [185]. Taken together, it is rational to include treatment strategies attempting to lower hepcidin level in CKD patients. This can be achieved by lowering total iron load, increasing elimination by dialysis, or, hopefully in the future, specifically decrease the hepcidin levels by some kind of hepcidin-antagonists [237].

The schedule of ESA administration

In Paper IV, an explanation to the observed 22.5% drop in ESA utilization was sought. This decrease was noted after a change in the ESA administration practice and the hypothesis was that the new routine, with fewer dose adjustment and no withheld doses, could save ESA. Such an assumption can only be made by ruling out other possible explanations, such as changes in factors that are known to affect erythropoiesis. We systematically evaluated these factors and found two other theoretical explanations; increased dialysis dose and higher transferrin saturation.

An inverse correlation has been described between the adequacy of dialysis and changes in ERI [76]. This was observed in Paper IV, where change in dialysis dose was negatively correlated to changes in ERI, albeit, in a multiple regression model, dialysis dose was not a significant independent predictor of ERI. However, there is a possibility of type-2 error here, as CI for beta was wide and predominantly negative. In such case, increased dialysis dose could explain a decrease in ESA need. If true, this is in contrast to previous studies in patients on hemodialysis thrice weekly, reporting that the beneficial effect of dialysis dose on erythropoiesis vanishes at Kt/V > 1.3 [69, 183].

Surprisingly, s-transferrin saturation was significantly higher after the change in ESA administration praxis. This was not explained by increased iron dose and if true, this could explain lower ERI because changes in transferrin saturation were found to predict changes in hemoglobin. However, most likely, higher transferrin saturation was due to a shorter interval between IV iron injection and measurement of iron parameters in the study period with the new ESA administration routine. Another possible explanation is that more patients were treated with iron-dextran in this period, while iron-sucrose was predominantly used before the change. Iron-dextran has longer half-life [113] and it is possible that a more long-lasting elevation of transferrin saturation occur after injection with iron-dextran than iron-sucrose. Further, withholding ESA doses, which was frequently practiced before the routine change, can affect iron homeostasis in various ways through factors like neocytolysis (see below), iron utilization and hepcidin.

The major limitation of Paper IV is that the study is small and observational. Optimally, the results should be confirmed by prospective randomized trials that can give answers to the following questions raised by our work: Does neocytolysis occur when ESA dose are withheld? Is there any difference between iron-sucrose and iron-dextran regarding transferrin saturation over time and response to ESA? Can we reduce ESA need in patients already receiving adequate dialysis dose (Kt/V > 1.3) three times a week by increasing the dialysis dose?

Taken together, the reason for the lower ESA requirement after the change in ESA administration practice is not clear. However, it is fair to conclude that ESA administration praxis, where ESA doses are withheld, does not reduce ESA demand. Instead, such practice could be associated with higher ESA requirement over time and, indeed, there is a possibility of neocytolysis when ESA doses are withheld.

The theory of neocytolysis

Neocytolysis is a normal physiological reaction that occurs when the body needs to adapt rapidly to an inappropriately high red-cell mass. In this process, young erythrocytes (neocytes) are selectively removed from the circulation by hemolysis in the spleen. The mechanism is not entirely elucidated, but the process is thought to start by a sudden drop in erythropoietin production [238]. Macrophages in the spleen recognize neocytes by adhesion molecules on their cell surface and it is believed that erythropoietin, in some way, disturbs the interaction between the neocytes, spleen endothelial cells and the macrophages, impeding the phagocytosis [239]. It is not clear if it is a nadir or if it is the sudden drop in erythropoietin concentration that initiates the process [240], but it has been postulated that neocytolysis can contribute to anemia in HD patients when EPO doses are withheld [241]. Moreover, erythropoietin prevents apoptotic death of erythroid precursor cells [61-63] so another potential consequence of withholding ESA dose is more pronounced loss of these cells, resulting in fewer mature erythrocytes.

Treating renal anemia in the future

Heme iron.

Heme iron, a natural nutrition supplement with excellent absorption and no side effects, should, in theory, be the iron-treatment of choice in renal anemia. Indeed, one study in patients on chronic hemodialysis supports this [110]. Another study, on peritoneal dialysis patients [242] is on the horizon, but more data are urgently needed before such oral treatment with concentrated heme iron can be generally recommended in renal anemia.

Contemporary IV iron agents

In recent years new IV iron formulations with better safety profiles have been developed [114]. Currently, three are on the market; iron-carboxymaltose (Ferinject®), and iron isomaltoside 1000 (Monofer®) in Europe and ferumoxytol (Feraheme®) in the US. All these agents are very stable with low risk of spontaneous iron release, which means that they can be given in very high doses. Further, all have very low immunogenic activity indicating minimal risk of anaphylactic reactions. Taken together, the safety profile of these new agents is promising, but more studies are needed before we can state that induction of oxidative stress and the frequency of hypersensitivity reactions is lower compared to older IV iron agents.

Prolyl hydroxylase inhibitors

During the last decade, several prolyl hydroxylase inhibitors (PHI) have been developed. By inhibiting this enzyme, the degradation of hypoxia inducible factor (HIF) is suppressed, resulting in enhanced HIF bioavailability. The consequences of increasing HIF are twofold; EPO production is stimulated [243] and hepcidin production is down-regulated [20]. This sounds ideal, as opposite changes in EPO and hepcidin production are fundamental etiological factors in renal anemia.

Few studies have been conducted so far, evaluating the effect of PHI on erythropoiesis. An oral PHI (FG-2216) has been found to stimulate EPO production and increase the hemoglobin level in healthy and anemic monkeys [243] and recently, in a study on HD patients using the same substance, a several-fold increase in serum EPO levels were noted [26]. Interestingly, a 15-fold increase in serum EPO was found in anephric HD patients, arguing for an extrarenal production. These findings are exciting, but we have to bear in mind that more than 70 HIF target genes have been identified, which may limit the specificity of this approach [244]. Without doubt, we are going to learn more about PHI in the future and, if well tolerated and safe, these agents could become an alternative to ESA and IV iron in the treatment of renal anemia.

Erythropoietin-mimetic peptide

In recent years, erythropoiesis-stimulating peptide (Hematide) has been developed. It has a unique amino acid sequence, totally different from native erythropoietin or available ESAs, [245]. Despite that, it binds to the EPO receptor and stimulates EPO production in a dose-dependent way. Hematide has been safely administrated to healthy volunteers and to CKD patients, and following a single IV injection a boost in hemoglobin, lasting for more than 1 month, was seen [246, 247]. Because of the dissimilarity to erythropoietin, anti-EPO antibody production, leading to pure red cell anemia, is not seen. Instead, this dreaded condition can be successfully treated with Hematide [248], which in such case is the only treatment available.

Ferric pyrophosphate

A new approach for iron supplementation in patients on chronic hemodialysis is currently under investigation. Soluble ferric pyrophosphate (SFP) is a low MW iron salt (~1000 Da) that can be delivered to the patient's circulation via the dialysate. By this approach, iron diffuses slowly from the dialysate to the circulation during the hemodialysis treatment [249]. It is then rapidly taken up by circulating transferrin [250] and phase II trials, have reported adequate erythropoiesis [251]. Unlike other IV iron agents, SFP has no carbohydrate shell, which minimizes the risk of allergic reactions. Moreover, the complex is very stable with low risk for appearance of labile iron. In addition, pyrophosphate is a potent antioxidant [252] and inhibitor of vascular calcifications [253, 254], properties that could be of benefit in patients with ESRD. Thus, SEP can be an interesting option for iron treatment in HD patients in the future.

CONCLUSIONS

- ESR is a robust, reliable and sensitive technique for measuring NTBI and AFR in plasma.
- A NTBI "pool" is found in both healthy and HD patients and its size is proportional to the total iron content in plasma.
- A burst of oxidative stress occurs in the circulation after IV injection of iron-sucrose. The magnitude of the oxidative reaction is closely correlated to the amount of iron released from the IS complex. A risk of oxidative injury to hemodialysis patients repeatedly receiving this type of intravenous iron supplementation cannot be disregarded.
- Due to the simplicity and reliability, measuring AFR should be the method of choice in monitoring the appearance of catalytically active iron following intravenous iron injection.
- Following IV injection of low-molecular weight iron-dextran, iron release is significantly lower than after iron-sucrose and no direct induction of oxidative stress is seen.
- The level of serum hepcidin is significantly lower after treatment with hemodiafiltration than low-flux hemodialysis. Keeping in mind that an increase in circulating hepcidin is a fundamental abnormality in renal anemia, efforts in lowering this level could bring the iron homeostasis towards a more normal state.
- Frequent dose adjustments and an ESA administration praxis where ESA doses are withheld do not reduce ESA demand. Instead, such practice could be associated with higher ESA requirement over time.

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