lower the serum EPO value. Hence serum EPO levels are the result of a balance between the rate of EPO production and its utilization by the erythroid marrow.

2.1.3. References

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2.2. Iron deficiency

Iron deficiency is the most important pathophysiological mechanism leading to anemia in IBD. It occurs in over one third of the patients suffering from Crohn's disease or ulcerative colitis (1). Iron metabolism is an active research field and recent advances have been made in understanding pathophysiological processes involved in the regulation of iron homeostasis.

2.2.1. Physiology of iron metabolism

Iron absorption

Iron is an essential element for most organisms from prokaryotes to mammals, being a part of numerous enzymes participating in redox reactions or oxygen delivery (2-4). The major source of iron is food, where it comes in the form of heme-bound or non-heme iron. Iron absorption occurs only in the duodenum and upper jejunum. Heme-bound iron has a considerably higher bioavailability than non-heme iron. Heme, released proteolytically from myoglobin and hemoglobin (mostly from meat), binds to the heme carrier protein HCP-1 and enters the mucosa epithelium unchanged via endocytosis, where iron is subsequently freed from the complex by the low pH; however, this process has not yet been studied in detail.

More is known about the absorption of non-heme iron. Most iron is available in the form of ferric (Fe^{3+}) ions, which do not readily pass the mucosal barrier; therefore a reduction to ferrous ions (Fe^{2+}) is needed to facilitate transport. Reducing agents are present in the food itself, the most common example being vitamin C. An additional mechanism utilizes the membrane-bound ferrireductase DcytB (duodenal cytochrome B). Ferrous iron then passes through the brush-border membrane via the divalent metal transporter DMT-1, which operates via a proton electrochemical gradient (2, 4-6).

Several factors influence iron uptake in the gut mucosa, among them the form of iron and its redox state in the food, the pH in the intestinal lumen, the presence of chelating agents in the food (e.g. phytic acid, oxalic acid) and the expression levels of DMT-1 in the epithelial cells. As a consequence, only a small percentage of the ingested iron (physiologically 1-2 mg per day) is absorbed (6).

Once iron has entered the cell, it is either used for metabolic purposes, incorporated into the iron storage protein ferritin, or is released into the circulation for the needs of the organism. The exact pathway by which iron crosses the gut epithelial cell from the apical brush border to the basolateral side has not been clarified sufficiently. Both transcytosis as well as a participation of iron chaperone proteins have been suggested to play a role in this process (7, 8). Once iron reaches the basolateral surface, it leaves the enterocyte via ferroportin and is packed into the iron transport protein transferrin to enter the circulation. Prior to binding to transferrin, the ceruloplasmin - related copper-containing membrane-bound ferroxidase hephaestin oxidises Fe^{2+} back to Fe^{3+} (6) (summarized in Figure 2.3).

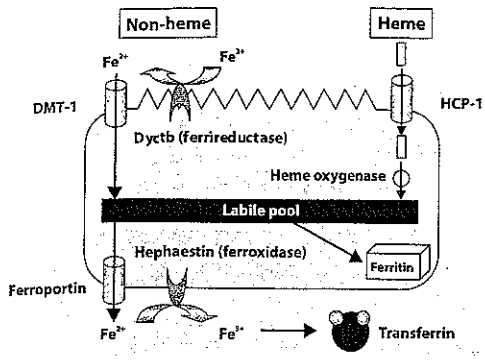

Figure 2.3: Scheme of iron absorption. Heme absorption is mediated by HCP1 (heme carrier protein 1) and ferrous iron is released after degradation of heme by heme oxygenase. After reduction to its ferrous state by the ferrireductase Dcytb, non-heme iron is imported into enterocytes by DMT1 (dimetal transporter 1 or Nramp2). Within enterocytes, iron can either be stored in ferritin (later excreted through cell exfoliation) or enter the so-called labile iron pool from where it can be actively exported by ferroportin or Ireg1. Hephaestin then oxidizes ferrous iron back to ferric iron, allowing its binding to circulating Tf.

2.2.2. Iron distribution and utilization

Almost all iron released into the circulation by the enterocytes is transferrin-bound, a transport form that can be utilized by all cell types including the developing red blood cells that have the highest iron demand of all cell types (2, 4, 5, 8). One molecule of transferrin (Tf) can carry two ferric ions. Apotransferrin, mono- and diferric transferrin are all found in the blood plasma, where the distribution of iron between these forms relates to the transferrin saturation. Tf enters the cells via endocytosis mediated by the ubiquitously expressed transferrin receptor 1 (TfR1) and iron is subsequently released from the binding by the low pH.

HFE, a surface protein with homology to MHC class I molecules that physically associates with β2-microglobulin, can interfere with this process by binding to TfR1 (3). The C282Y mutation, which is typical of genetic hemochromatosis in most cases, hampers the ability of HFE to interact with β2M, with the consequences of HFE degradation and reduced cell surface expression. HFE expression is highest in hepatocytes but it is also present in Kupffer cells and enterocytes. As their binding sites on TfR1 overlap, competition of HFE with diferric transferrin has been suspected. However, the role of HFE in the regulation of iron absorption is not clear.

A truncated soluble form of the TfR1 (sTfR1) circulates in the plasma and its concentration is directly proportional to the total body mass of cellular TfR1 (9). It is therefore largely influenced by the level of erythropoietic activity (through changes in the number of erythroblasts) and to a lesser extent by iron stores (through regulation of the number of TfR1 per cell). sTfR1 is an excellent indicator of iron deficient erythropoiesis, particularly for the differential diagnosis of iron deficiency (increased sTfR1 and low ferritin) versus inflammation (normal sTfR1 and ferritin) or for detecting iron deficiency in a patient with concomitant inflammation (increased sTfR1 and normal ferritin). However, it cannot be used as a marker of iron deficiency in erythropoietic disorders or during EPO treatment.

An alternative transferrin receptor, TfR2, is expressed only on hepatocytes, duodenal crypt cells and erythroid cells (8). Unlike TfR1, it is believed to have a primarily regulative function, as described below.

Within most cells, iron stores are built up in the form of ferritin (10), which represents a molecular "nanocage" built up by 24 subunits of ferritin heavy and light chains and holding up to 4,500 ferric ions. Serum ferritin is secreted in proportion to storage iron in macrophages and hepatocytes and its levels thus represent a quantitative marker of iron stores. It is a highly specific marker, any decreased value demonstrating exhaustion of iron stores. It is however not very sensitive, because numerous conditions are associated with falsely elevated serum ferritin levels. These include hepatic cytolysis, inflammation, renal failure, hyperthyroidism, poorly controlled diabetes mellitus, some tumors and the rare hyperferritin-cataract syndrome. Indeed cutoff values for iron deficiency can be as high as 40-120 µg/l instead of the classical 12 µg/l in situations such as renal failure or inflammation.

The human body contains 30-40 mg/kg of iron, adding up to approximately 4 g in adults (11). Iron is mostly contained in hemoglobin (2.5 g), ferritin (1 g) and other heme and non-heme proteins (0.5 g), while plasma iron amounts to only 3 mg. Iron losses are limited to about 1 mg/day through gastro-intestinal and skin desquamation. A normal western diet provides 10 to 15 mg of iron daily

but only a small fraction, 1 mg in a male adult, is absorbed. Iron requirements depend on age (1.5 mg/day during puberty), sex (1.5 mg in women because of menstrual losses) and pregnancy (3.5 mg/ day and even 5 mg/day in 3rd trimester).

65 % of the total iron content is localized in the erythrocytes. Erythropoiesis is a highly dynamic process with $2x10^{11}$ new red blood cells produced each day to maintain a constant erythrocyte count (4, 5). The average lifetime of an erythrocyte is 120 days. This means that the daily amount of absorbed iron is not sufficient to cover the needs of erythropoiesis thus making iron recycling necessary. Senescent erythrocytes undergo phagocytosis by cells of the monocyte-macrophage system (MMS) mainly in the spleen and liver. In the phagosomes, the heme complexes are degraded, and iron exits in the form of ferrous ions via a different isoform of DMT-1 to be stored in the form of ferritin or be released back in the circulation via ferroportin. In this case, the copper containing protein ceruloplasmin acts as the principal plasma ferroxidase to convert Fe^{2+} to Fe^{3+} prior to transferrin binding.

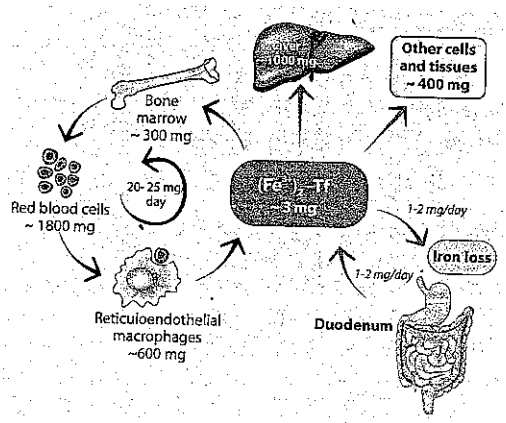

Figure 2.4: Iron distribution in the organism. The average iron content of the organs as well as the daily flow of iron through the different compartments are shown. For detailed description, see text. Modified from Cell 2004 (8).

The liver is the most important iron storage organ. When systemic iron is in excess, it is taken up by the hepatocytes and the MMS cells and is stored in the form of ferritin for future use. As soon as the body iron levels drop, iron is released by the hepatocytes via the ferroportin/ceruloplasmin system. In this way the liver is capable of balancing alterations in plasma iron concentration (4) (☞ Figure 2.4).

2.2.3. Regulation of iron homeostasis

Almost all iron used by the cells is recycled. The daily iron losses through shedding of epithelial cells and bleeding from minor injuries account for only 1-2 mg, and the same amount is taken up to maintain a body iron balance. Since no excretion mechanism is present, iron homeostasis is regulated on the level of absorption and release from the macrophage and hepatocytic iron storages (5, 7, 8).

2.2.3.1. Regulation by hepcidin

Hepcidin is the recently characterized major regulator of systemic iron homeostasis (2, 4, 7, 12, 13). This small molecule (25 aminoacids) belongs to a family of anti-microbial peptides (e.g. defensin) and is synthesized in the liver. It interferes with the iron efflux into the plasma from enterocytes, macrophages and hepatocytes by binding to ferroportin and causing its internalization and degradation. Hepcidin levels rise when the body iron levels are high, thus inhibiting iron absorption and release in a negative feedback manner. Conversely, under conditions of iron deficiency, the plasma hepcidin concentration is low, which leads to a higher iron uptake by the enterocytes and efflux from macrophages and the hepatic storage.

Furthermore, hepcidin synthesis is enhanced during inflammation, mostly under the control of IL-6 and IL-1. This leads to a redistribution of iron with iron being trapped in macrophages and hepatocytes, whereas plasma levels, mirrored by the transferrin saturation, drop. This physiological defence mechanism depletes microorganisms from iron that is needed for their growth and also contributes to the development of anemia of chronic disease, which is discussed in the following chapter. It is also known that hypoxia (via the hypoxia inducible factor-1, HIF-1, the levels of which are high in anemia) as well as enhanced erythropoiesis (rather than anemia *per se*) diminish hepcidin production (8, 12, 13) (☞ Figure 2.5). The erythropoietic regulator is stronger than the iron regulator but its nature remains elusive, as the potentially good candidate sTfR1 (9) has been excluded.

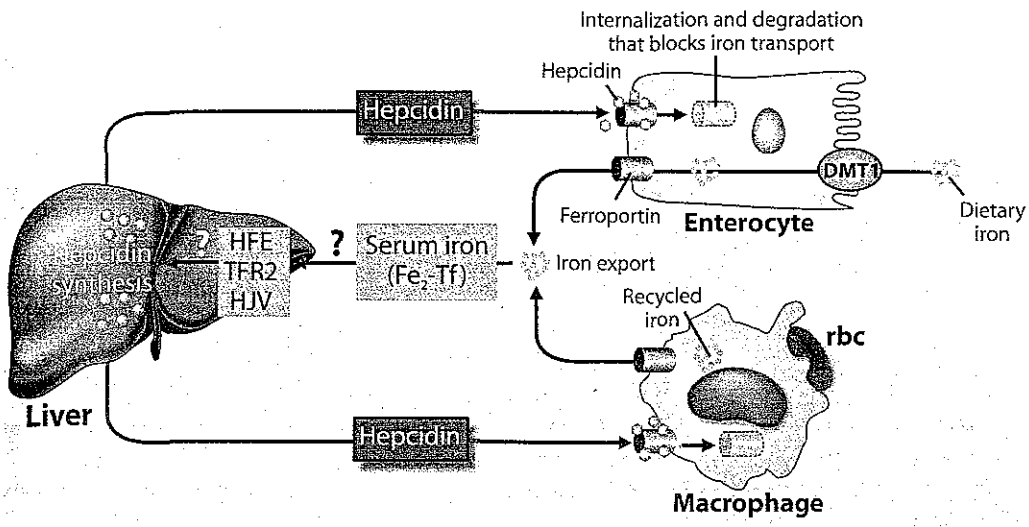

Figure 2.5: Regulation of the systemic iron homeostasis by hepcidin. High body iron levels and inflammation trigger hepatic hepcidin production, whereas hypoxia and increased iron demand keep hepcidin levels low. Hepcidin inhibits iron efflux via internalization of ferroportin in enterocytes and macrophages thus leading to a decrease in dietary iron absorption and utilization of recycled iron from the MMS. Modified from Semrin G, IBD 2006, Nemeth E, Science 2004, Vaulont, S. J Clin Invest 2005.

The exact pathway of activation of hepcidin is not known. The already discussed protein HFE as well as transferrin receptor 2 (TfR2), a homolog of the ubiquitously expressed TfR1 that may act as an iron sensor and is mutated in certain hemochromatosis forms, seem to act as non-essential upstream activators of hepcidin expression, whereas hemojuvelin (HJV), which plays a role in the development of the severe juvenile hemochromatosis, acts further downstream and is absolutely essential for hepcidin synthesis. HJV acts as a bone morphogenic protein (BMP) coreceptor and its signaling pathway has been partially discovered, but it is not known how HFE and TfR2 regulate hepcidin expression (7). Diferric transferrin could induce the signal transduction pathway to increase hepcidin expression by (1) outcompeting HFE for TfR1 binding, resulting in large numbers of unbound surface HFE molecules, and (2) stabilizing TfR2. Another possibility consists in placing the iron sensor in Kupffer cells that also express HFE and could elaborate a paracrine factor regulating hepcidin expression in hepatocytes.

2.2.3.2. Regulation of the expression of iron related genes

Iron can directly regulate the expression levels of proteins involved in iron metabolism via the so called iron responsive elements (IREs) (8). These are stem-loop sequences in the mRNAs that are recognized by the iron regulatory proteins (IRP1 and IRP2). The IRPs bind to these structures under conditions of iron deficiency. The effect of this binding is dependent on the localization of the IREs. When the IRE is situated in the 5'-untranslated region (UTR), iron deficiency leads to an inhibition of translation. This is the case with the heavy and light ferritin chains, ferroportin and some proteins of iron utilization such as 5-aminolaevulinic acid synthetase, participating in the heme synthesis, or mitochondrial aconitase. On the contrary, when IREs are localized in the 3'-UTR, IRP-binding leads to protein up-regulation due to increased mRNA stability. Such regulation has been described for TfR1 and the cell membrane-bound isoform of DMT-1.

Other non-IRE-related regulatory mechanisms also influence gene expression of iron-related genes. Such mechanisms act on transcriptional as well as on translational level. Cytokines such as IL-

6, IL-1, IFN-γ and TNF-α stimulate H-ferritin mRNA transcription but inhibit the expression of TfR1. Toll-like receptor 4 signalling downregulates the mRNA-sythesis of ferroportin. INF-γ also blocks ceruloplasmin translation (8).

2.2.4. Pathophysiology of iron deficiency

Iron deficiency occurs when iron loss exceeds the amount of iron absorbed by the gut. A situation of a negative iron balance is present when not enough iron is ingested, when iron absorption is inhibited, when the body demand of iron is elevated (e.g. during pregnancy or puberty), or because of iron loss through bleeding. Bleeding from the genitourinary tract in females or from the gastrointestinal tract in both sexes is the leading cause of iron deficiency.

Anemia in inflammatory bowel disease is mainly contributed to iron deficiency (1, 14, 15). Several of the described pathogenetic mechanisms play a role in the development of an iron deficit in IBD. Patients with IBD, especially women, have a lower daily iron intake, mainly non-heme iron, predominantly due to avoidance of high fibre Fe-rich and -fortified foods (16), which may exacerbate abdominal symptoms. Poor iron absorption due to structural changes in the mucosa is expected only in the relatively rare upper gastrointestinal form of Crohn's disease (L4 according to the Vienna classification), although iron malabsorption has been described in up to 90 % of the pediatric IBD-patients. An additional factor affecting the absorption of dietary iron is the increased level of hepcidin (as a result of chronic inflammation), leading to a diminished iron efflux from enterocytes. However, the main cause for negative iron balance in IBD remains chronic blood loss through the ulcerated intestinal mucosa (1, 14).

A shortage of extracellular iron leads to a decrease in apoferritin synthesis and subsequently to a fall of serum ferritin concentration. This drop in depot iron is the first sign of iron deficiency (☞ Table 2.1). In the initial stages of iron deficiency, the transferrin saturation may be within the normal range, and erythropoiesis may function well. However, if the negative iron balance persists, the transferrin saturation becomes lower (deficiency in transport iron), and the body iron reserves are no longer sufficient to sustain a normal hemoglobin synthesis. The consequence is the development of a hypochromic microcytic anemia. The full-blown picture of iron deficiency presents with a decrease of plasma ferritin, decrease in transferrin saturation, a compensatory rise in apotransferrin, an over-expression of transferrin receptor 1 and its soluble counterpart (sTfR1). However, iron deficient erythropoiesis may occur even if iron stores are not exhausted or are even elevated. This is called functional iron deficiency that occurs when macrophage iron mobilization is not sufficient to match iron needs in the bone marrow. This can be encountered if iron needs are increased when marrow erythroid activity is stimulated (e.g. by Epo therapy) or if iron release by macrophages is impaired (e.g. in the anemia of chronic disease) (☞ Table 2.1).

- Stage 1 : Iron depletion
  - Ferritin <30 µg/l
- Stage 2 : Iron deficient erythropoiesis
  - Serum iron <60 µg/dl
  - Transferrin saturation <20 %
  - Hypochromic RBC >5 %
  - Reticulocyte hemoglobin content (CHr) <28 pg
  - Soluble transferrin receptor >7 mg/l
  - Erythrocyte protoporphyrin >70 µg/dl
- Stage 3 : Iron deficiency anemia
  - Hemoglobin <12 g/dl (female) or <13 g/dl (male)
  - Hematocrit <36 % (female) or <39 % (male)
  - RBC number normal, later decreased
  - MCV <80 fl (microcytosis)
  - MCH <28 pg (hypochromia)
- Functional iron deficiency
  - Same as above except for ferritin >30 µg/l

Table 2.1: Laboratory findings in iron deficiency.

A different pathogenetic mechanism that may contribute to the scarcity of iron for the erythropoiesis in IBD relates to a disorder of iron distribution due to high concentrations of pro-inflammatory cytokines. The latter induce expression of hepcidin, which, as we already described, inhibits iron release from the storage compartment (cells of the

monocyte-macrophage system and hepatocytes) leading to high plasma ferritin but low transferrin saturation. Again, TfR1 and also sTfR1 may be up-regulated as a sign of insufficient erythropoiesis caused by iron deficiency. The constellation of high iron depots (indicated by plasma ferritin) and low transport iron (indicated by transferrin saturation) is the most characteristic feature of anemia in chronic disease, which will be discussed in the following chapter.

Iron deficiency is the leading cause of anemia in IBD. A negative iron balance is mainly contributed to bleeding from the ulcerated gut mucosa, although insufficient nutritional iron uptake, absorption deficit as well as inhibited iron release from the storage compartments due to hepcidin action also contribute to the pathogenesis of iron deficiency. Recent advances in the research of iron homeostasis improve our understanding of the processes leading to the development of anemia in IBD.

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2.3. Anemia of chronic disease

Anemia of chronic disease (ACD) is the most frequent anemia in hospitalized patients, and after iron deficient anemia (IDA) the second most frequent anemia in the world. ACD occurs in patients with chronic diseases involving acute or chronic immune activation (1, 2). ACD is thus found in patients suffering from severe acute and chronic infections, malignancies and auto-immune disorders including IBD. The prevalence of anemia in the latter condition was estimated between 20-60 % (3). The prevalence and severity of anemia is positively associated with an advanced stage of the underlying disease.

2.3.1. Pathophysiology

ACD is an immune-driven condition in which cytokines and acute phase proteins alter body iron homeostasis, erythroid progenitor cell proliferation, erythropoietin production and red cell life span.

Iron retention within cells of the reticuloendothelial system (RES) leads to limitation of iron availability for erythroid progenitor cells, and thus to an iron-restricted erythropoiesis. This can be referred to an increased iron acquisition by cells of the RES, such as macrophages, while at the same time iron re-distribution from macrophages is reduced (☞ Figure 2.6). Macrophages have multiple