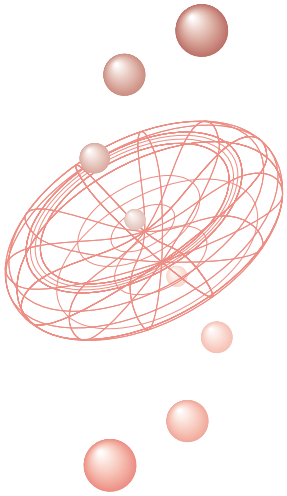
REVIEW ARTICLE



Iron Metabolism, Iron Deficiency and Anaemia

From Diagnosis to Treatment and Monitoring

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Key Words

Iron Metabolism, Iron Deficiency, Iron Deficiency Anemia, Anemia of Chronic Disease, Reticulocyte Hemoglobin Content

Received 24 December, 2003. Accepted 5 January, 2004

This review explains why iron is such an important trace element, what its main functions are and how iron is transported and used within the body. The reasons for iron deficiency and its medical consequences are described. The classical diagnostic tools to assess iron metabolism are of limited value. Alternative modern haematological markers are now available which can help to classify correctly, treat and monitor patients with iron deficiency.

Abbreviations :

ACD	= anaemia of chronic disease
DMT1	= divalent metal transporter 1
ID	= iron deficiency
IDA	= iron deficiency anaemia
IRE	= iron-responsive element
IRP	= iron regulatory protein
mRNA	= messenger ribonucleic acid
sTfR	= soluble transferrin receptor

Introduction: iron deficiency is an important international health problem

Iron deficiency (ID) is the most widespread nutritional disorder world-wide[1]. Estimations of the number of people being affected are variable, however, the figures are alarming: according to WHO estimates as many as 4-5 billion people may be iron deficient, corresponding to up to 80% of the world's population[2]. Two billion people are anaemic, mainly due to ID, which in developing countries is frequently aggravated by worm infections and malaria. It is, however, a common misbelief that ID is only a disease of the developing countries. On the contrary, it is the only nutrient deficiency which is also significantly prevalent in virtually all industrialised nations[3], especially in toddlers, in women in the child-bearing age - whether pregnant or not - and in the elderly. For example, the Third National Health and Nutrition Examination Survey 1988-1994 revealed that in the USA 9% of children aged 12-36 months had ID and 3% had iron deficiency anaemia (IDA). In non-pregnant females between 16 and 49 the prevalence was 11% for ID and 3-5% for IDA[4]. Malnutrition as the cause of ID is being addressed by a variety of international and national programmes. In many developing countries the WHO has initiated programmes for the fortification of food with iron to prevent and control ID. In some countries, for example in the UK, the fortification of flour is mandatory. To understand the clinical consequences of ID and the diagnostic strategies for detecting ID and monitoring therapy under various clinical conditions it is essential to understand the basics of iron metabolism. Iron has a large number of different functions in the body and just how essential it is for health is illustrated by the number of medical terms which contain the word iron in different languages, for example **ferrum** (Latin) or **sideros** (Greek).

Iron is everywhere in the living world

Iron is the fourth most common element in the earth's crust after oxygen, silicon and aluminium. Metallic iron

is unstable under normal environmental conditions. Iron is usually present in the form of bivalent Fe^{2+} under reducing conditions. Trivalent Fe^{3+} occurs under oxidative conditions, for example in the presence of oxygen. **Rust** consists mainly of the oxides and hydroxides of trivalent Fe^{3+}. Rocks from prehistoric times (when there were no oxygen-producing plants) sometimes contain bivalent iron. Red, Fe^{3+}-containing rocks were then later formed as a result of exposure to oxygen.
The easy transition between Fe^{2+} and Fe^{3+} plays a vital role in nature and as a result the **redox system Fe^{2+}/Fe^{3+}** is "used" in many biochemical processes in nature. Some basic anaerobic bacteria make use of the fact that the oxidation of Fe^{2+} to Fe^{3+} releases **energy**, and "feed" on bivalent iron. Such processes occur in biotopes devoid of oxygen. One example is biotopes near underwater volcanoes where organisms are able to survive at depths of several thousand metres.

Iron in the human body: a versatile tool for many biochemical reactions

Since the reversible transition between Fe^{2+} and Fe^{3+} can be controlled better in a favourable chemical environment, nature has "created" molecules such as the **cytochromes**. In these red-coloured molecules iron is incorporated in **porphyrin rings** which, in turn, are bound to a protein. The complex formed by the porphyrin ring and the iron is called **haem**. These complex molecules are, among others, involved in the **detoxification of drugs** and in **cell respiration**, in other words, in the gentle chemical combustion of hydrogen to water in the mitochondria of the cell. Here iron assists the transport of electrons from hydrogen to oxygen. In cell respiration not only enzymes which contain porphyrin iron play an important role, but also **complexes of iron and sulphur**. These molecules, too, facilitate the reversible transition between Fe^{2+} and Fe^{3+}. Other enzymes which are involved in redox processes also contain iron; for example, **oxidases** which catalyse special syntheses and **peroxidases** which protect the body from oxidative stress. In addition, iron is indispensable for the synthesis of the DNA.
Closely associated with the easy oxidation and reduction of iron is its ability to form stable complexes with non-ionic molecules such as **oxygen**. This is based on the special structure of iron's electron shell. By using this property, nature has found a molecule which can bind oxygen at high concentrations in the bloodstream: the iron-containing blood pigment **haemoglobin**. This compound also contains iron bound to the porphyrin ring and embedded in a protein, similar to the cytochromes. This structure ensures that oxygen is transported in the bloodstream at a much higher concentration than would normally be possible based on its physical solubility. The protein in muscle cells which binds the oxygen released by haemoglobin is called **myoglobin** and is also a haem-iron protein.
The variety of biochemical processes in which iron is involved explains that ID has important multiple consequences. Let us take a look at the total amount of iron in the body and how it is distributed: Men contain 4.2 g of iron on average, women only 3.5 g, and all of it is bound to proteins. About two thirds of the iron is part of haemoglobin, 5% accounts for myoglobin, and 0.2% of the iron is part of haem containing enzymes like cytochromes and peroxidases, making haem-bound iron the most prevalent form of iron in the body. 10% of the iron pool is bound to non-haem proteins. All these forms of iron, making up 80% of the body's iron pool, can be summarised as **functional iron**, as opposed to about 20% **storage iron**, which is the sum of ferritin and haemosiderin. This distribution is valid for men. In women, storage iron accounts only for about 12%[5]. Only 0.1% of the body iron is **transport iron** which is "on the road"[6] to where it is currently needed. Transport iron is almost exclusively bound to transferrin. The distribution of iron in the body is summarised in ***Fig. 1***[7].
Iron is not only a versatile tool and catalyst for biochemical reactions. The easy transition between Fe^{2+} and Fe^{3+} is also the reason that in the presence of iron free radicals can be generated that cause oxidative damage to proteins, lipids and DNA. This is the reason why free iron would be very toxic.

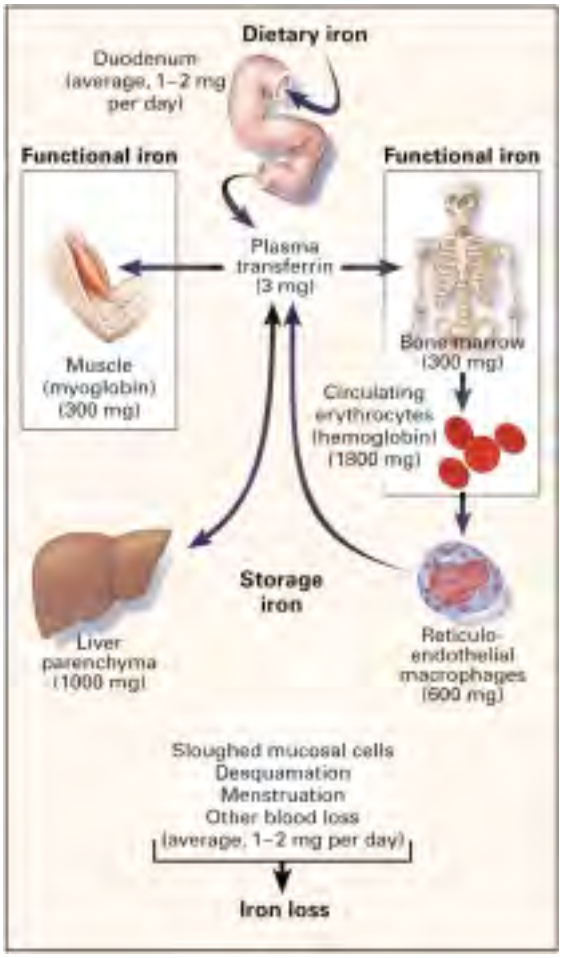

Fig. 1 The uptake, distribution and excretion of iron
Transferrin is the fulcrum of iron metabolism, distributing the iron either to the iron stores (ferritin, haemosiderin) or to the tissues where the iron is used, primarily to the bone marrow. Modified and reprinted with permission[7].

The uptake, transport, storage and excretion of iron: a quick review of iron metabolism

Iron is part of our daily food. However, not all forms of iron are absorbed equally well. More than 90% of the dietary iron intake is non-haem iron, mainly in the form of iron salts, present in plant and dairy products. The iron must be **solubilised** before it is absorbed. Solubilisation is supported by the acidic pH in the stomach. The absorption process, however, is located in the **duodenum**. Only Fe^{2+} can be absorbed. Therefore, substances like vitamin C that can reduce and complex iron even at the alkaline pH in the duodenum can significantly increase the proportion of iron being absorbed, but only if administered concomitantly. Haem iron which accounts for 30-60 % of the iron present in meat, poultry and fish is absorbed about 3 times more readily than non-haem iron. For reasons not yet well understood, vitamin A increases the absorption of iron. The fact that iron is not absorbed completely has been experienced by many: anyone who ever has taken iron tablets will have noticed the black faeces caused as a result of excess, non-absorbed iron. On average only 10% of the iron contained in the diet is absorbed. In the case of ID, however, the body can increase this value considerably. The uptake of iron does not rise proportionally to the iron concentration in the food. There are **control mechanisms to prevent excessive iron uptake** causing iron overload, however, this control is not perfect, as can be seen from cases of unintentional iron intoxication. Two grams of an iron solution can be lethal to small children.

Haem iron is absorbed as intact metalloporphyrin. The process for ionised iron is schematically shown in ***Fig. 2***. On the apical mucosal cell surface an enzyme called ferrireductase reduces non-haem Fe^{3+} to Fe^{2+} which is then transported inside the mucosal cell by a protein called **DMT1** (divalent metal transporter 1). The iron can either be retained in the mucosal cell and be stored in the form of ferritin (see later), or it can be guided through the cell with the help of **mobilferrin** and other proteins, towards the basolateral surface of the cell. Here the iron is re-oxidised to Fe^{3+} by **hephaestin**. This protein forms a complex with another iron transporter called **IREG** (= ferroportin = MTP1), shuttling the Fe^{3+} across the cell membrane into the plasma where it is immediately bound by **transferrin**[8]. Transferrin represents the plasma pool of iron and is the fulcrum of iron metabolism. Plasma transferrin transports iron either to iron storage depots or to organs which require iron. Theoretically every cell in the body is a target cell for transferrin. Transferrin binds to the **transferrin receptor**. The complex of both is internalised - in a process termed receptor mediated endocytosis - and inside the target cell the iron is released whereas the transferrin receptor is recycled to the surface. The tissue with the highest demand for iron is the bone marrow, where iron is incorporated into haemoglobin, the blood pigment in erythrocytes. After an average life span of 120 days, the erythrocytes are eliminated by the macrophages in the spleen, and the haemoglobin is broken down into amino acids, bilirubin and iron. The iron is then fed back into the plasma pool.

Iron storage occurs in the proteins **ferritin** and **haemosiderin**, which comprises a heterogeneous group of ferritin degradation products, mainly in the parenchyma and macrophages of the liver, in muscle and in the cells of the reticuloendothelial system (RES) in the spleen and bone marrow. Liver, muscle and the RES each contain approximately a third of the stored iron. Both ferritin and haemosiderin contain about 20% iron by weight, which is sufficient to enable them to be magnetised.

The body does not possess any sophisticated mechanisms for excreting excess iron. If the level of iron in the body is sufficient, then the iron stored in mucosal ferritin is not released into the bloodstream. The mucosal cells slough off after a few days and the iron contained in them is excreted with the faeces. This accounts for more than half of the iron excreted. The rest is excreted in the urine, bile and sweat. The daily iron loss via this route amounts to 0.5 to 1 mg. Women of the reproductive age lose additional iron during menstruation. This is not always compensated by the iron uptake with the foodstuff.

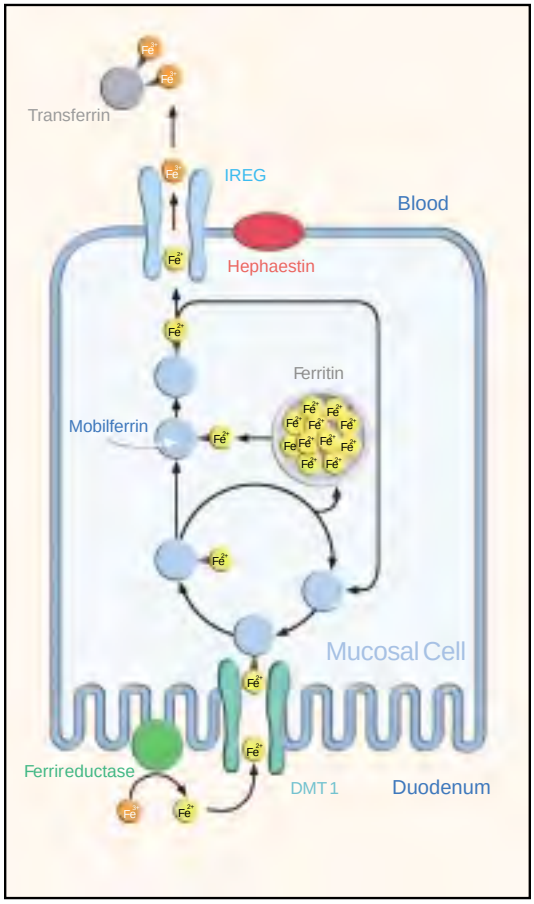

Fig. 2 ***How iron is transported from the duodenum to the blood with the help of different proteins***

Ferrireductase reduces Fe^{3+} to Fe^{2+}. DMT1 pumps the iron into the mucosal cell where it is either stored in the form of ferritin or shuttled through by mobilferrin. On the blood side the iron is re-oxidised and released into the blood by the IREG / hephaestin complex. In the blood it is immediately bound by transferrin. Adapted from[8].

A dedicated system for the excretion of iron has not evolved since there has never been selective pressure for this, as human beings were never confronted with excess iron during their evolution. This is a problem that modern medicine is now having to face: patients who require repeated blood transfusions gradually become overloaded with iron. The result is haemosiderosis which leads to the destruction of organs that store iron. A few years ago it could be shown that **hereditary haemochromatosis** is in most cases due to mutations in the **HFE gene**, causing a block of the above mentioned inhibition of iron uptake in the mucosal cells. The protein, for which the HFE gene codes, binds to the transferrin receptor and is involved in the regulation of iron uptake. The most prevalent mutation of the HFE gene is the **C282Y mutation**. It is a very common genetic defect in populations of European origin: one out of ten Europeans is heterozygous and one out of 400 is homozygous. The mutation is practically absent in populations of non-European origin. In the meantime a few other mutations of the HFE gene have been identified. The second most frequent mutation of the HFE gene is the **H63D mutation**. The other mutations are very rare. Patients having one of the homozygous phenotypes or the mixed phenotype C282Y / H63D can develop a clinical picture of iron overload, however, the penetrance of the disease is still discussed controversially. Over the years the iron deposition in hereditary haemochromatosis destroys many tissues and organs and finally leads to death if the patient is not treated (usually by repeated phlebotomies or, if the clinical condition of the patient does not permit this, by iron chelating compounds like desferoxamine or deferiprone).

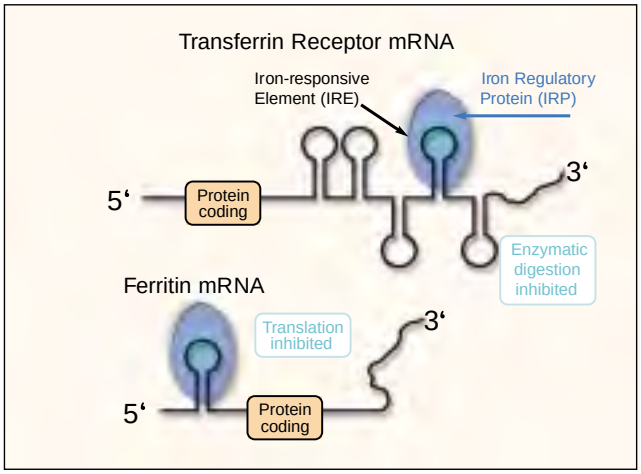

Fig. 3 How the intracellular iron concentration is controlled
The intracellular iron homeostasis is regulated by iron regulatory proteins (IRPs) that bind to iron-responsive elements (IREs) on the messenger RNAs (mRNAs) for various proteins involved in iron metabolism. In the case of the mRNA for soluble transferrin receptor the IRPs prevent the enzymatic digestion of the mRNA, in the case of the mRNA for ferritin the translation of the mRNA is inhibited.

Iron homeostasis: the main regulatory mechanisms to keep the iron content of the body constant

Iron homeostasis is controlled at different levels, involving two major sites of regulation: regulation of iron uptake and regulation of the synthesis of iron binding proteins.

Whereas iron is absorbed by the **villus cells** in the duodenum, the **uptake of iron in the mucosa** is regulated by one or more iron sensor proteins in the **crypt cells** of the duodenum. The HFE transferrin receptor complex on the basolateral surface of the crypt cells is the most promising candidate for such a sensor protein[9], however, the details of this process remain unclear. The sensor receives information from the tissues about the body's iron content and programmes the crypt cells in a way that iron uptake and transport through the cell are adjusted to the body's needs. This programming involves, in particular, the expression of the iron transport proteins. Within a few days during their life cycle the crypt cells migrate to the tips of the villi and become villus cells, having already the correct "settings" in place for optimised iron absorption.

The other principle guaranteeing iron homeostasis is regulation of the **synthesis of proteins involved in iron metabolism**[10]: When intracellular iron levels become low, the expression of transferrin receptor on the surface of the cell is upregulated and the synthesis of ferritin is downregulated by so-called **iron regulatory proteins (IRPs)** that bind directly to special loop-like binding sites of the respective messenger RNAs (mRNAs). These binding sites are termed **iron-responsive elements (IREs)**. *Fig. 3* shows that once IRPs bind to the IREs, the enzymatic breakdown of the transferrin receptor mRNA is inhibited whereas in the case of ferritin the translation of the mRNA into the protein is prevented. Also the mRNAs of some other proteins involved in iron metabolism contain IREs and are targeted by IRPs, namely the mRNAs for DMT1, IREG and ε-aminolaevulinate synthase, a key enzyme for haem biosynthesis. Not only iron, but also a low oxygen pressure and erythropoietin can activate IRPs[11].

Iron deficiency develops under a variety of conditions

The causes of ID can be divided into five groups:

Insufficient supply

As explained in the introduction this is the most common cause of ID world-wide. Whereas **malnutrition** is responsible for ID in the developing countries, an unbalanced diet causes ID also in industrialised countries. **Vegetarians** are more likely to develop ID since por-

phyrin-bound, easily absorbed iron is scarce in their diet. The **elderly**, having generally a reduced food intake, are also at risk to develop ID.

Malabsorption

If the acidic environment in the stomach, which is favourable for the solubilisation of iron, is not present due to **chronic atrophic gastritis** or due to a **surgical resection** of a part of the stomach, ID will develop. The same is true for diseases of the small intestine such as **gluten intolerance** (coeliac sprue) or inflammatory bowel disease like **Crohn's disease** where the iron-absorbing mucosa is damaged. In the presence of **calcium**, **phosphate** or iron-complexing plant constituents, like **tannins** and **polyphenols** in tea, coffee and cocoa, the bioavailability of iron is reduced. Some drugs like **tetracyclines** can react with iron, impairing its absorption. Abuse of **laxatives** is also associated with malabsorption of iron.

Increased iron requirement

Pregnant women need more iron since they have to satisfy the erythropoietic requirements of the foetus. Children in the **growth phase**, especially toddlers, are at risk for ID due to increased iron demands.

Blood loss

Fertile women lose iron during **menstruation**. Since one millilitre of blood contains approximately 0.5 mg of iron, during the course of the menstrual cycle around 20 mg of iron are lost. Blood loss during **surgical procedures** is not such a problem, since this situation is usually well controlled. Blood loss during **childbirth** often goes "unnoticed", especially in countries where few women can afford to deliver their babies in hospitals. Chronic gastrointestinal blood loss due to **gastric or duodenal ulcers** or **polyps** of the large intestine are common sources of ID. In developing countries **worm infections** often cause chronic gastrointestinal bleeding. **Blood donors** lose iron from the blood they are donating. The reasons for the blood loss of **endurance athletes** are not fully understood. However, gastrointestinal bleeding during exercise, muscle trauma and mechanical destruction of erythrocytes in the so called "runner anaemia" seem to contribute to the ID. Finally, we should not forget the iatrogenic ID due to repeated phlebotomies: A few blood samples taken from a patient within a day or two can easily make up 20 mL and contain 10 mg of iron, which is 10 times the normal daily uptake!

Functional ID

This type of ID has attracted much attention over the recent years. The term "functional ID" describes a state where the total iron content of the body is normal or even elevated, but the iron is "locked away" so that it is unavailable for erythropoiesis. The underlying mechanisms are poorly understood. Most likely the regulation of the expression of the transferrin receptor and of ferritin is disturbed by inflammatory cytokines[12]. It is known that cytokines like IL6 or TNFα cause an increase of the plasma ferritin concentration, sequestering iron into the storage pool in the macrophages. Therefore, the macrophages that normally transport iron fail to release it at the site of delivery. Functional ID is mainly observed in patients with chronic inflammation, cancer or on chronic haemodialysis.

The clinical signs of iron deficiency are often non-specific, but the consequences can be severe

Since iron is involved in so many biochemical reactions in the body, ID has multiple adverse effects on cellular function. Only at a late stage the production of haemoglobin is impaired and anaemia occurs.

Unfortunately, the clinical symptoms of ID are very non-specific in the beginning. General fatigue, irritability, depressive mood, headache, impaired strength and work capacity and a reduced resistance to infection are often early non-specific features of ID. Pallor, loss of hair and brittle nails are also non-specific. All those symptoms might occur long before anaemia becomes visible. However, the long-term effects of ID can be severe, especially if finally erythropoiesis is affected, causing **iron deficiency anaemia (IDA)**. Iron deficient and anaemic children show an impaired cognitive and motor development and perform less well at school and in IQ tests. Even a mild anaemia can result in decreased performance and endurance in workers. Babies of women with anaemia often have a low birth weight and show increased mortality. More severe anaemia causes tachycardia as an adaptive increase of the cardiac output to compensate for the anoxia due to insufficient oxygen supply. Signs of anoxia include fatigue, vertigo, dyspnoea and if coronary heart disease is also present finally unstable angina.

Serum tests and the classical red blood count are often of limited value to assess iron deficiency

Normally the diagnosis of ID is easily made by classical laboratory tests. However, in the presence of inflammation and some other conditions ID may be difficult to detect and more advanced tests are required.

Serum iron

Although analytically simple and performed million-fold in the laboratories around the world even today, the **measurement of iron** in plasma or serum is not useful at all to assess ID! The iron concentration shows a strong intra-individual variation, depending on diurnal rhythms and on food intake[13]. Even though on average the iron concentration is low in ID, the measurement of iron is neither sensitive nor specific. In acute inflammation iron is relocated from the blood into the iron stores and the serum concentration falls.

Ferritin

A much better marker to assess the iron stores is **ferritin**. Ferritin is a large protein with a molecular mass of 470 kDa. It is composed of 2 types of subunits, L and H. Twenty-four subunits form a hollow sphere-like structure

resembling a football. In the cavity of this structure up to 4,500 Fe^{2+} ions can be stored. Ferritin does not seem to play a physiological role in the plasma and it is commonly thought that the ferritin in the plasma has leaked there from the iron storage pools. So the ferritin concentration does reflect the size of the iron storage pools. Although different international standards do exist and although different ferritin assays often give deviating results - mainly at higher concentrations - in early ID the ferritin concentration in the plasma falls and ferritin concentrations below 12 to 15 µg/L indicate that the body's iron stores are empty. Unfortunately, ferritin is an acute phase protein and normal or elevated concentrations do not necessarily reflect filled iron stores in inflammation, infection or cancer. Ferritin is further released from the liver into the blood in all kinds of hepatocellular disease, tumours or liver metastases, after prolonged alcohol abuse or following oral contraceptives. In these conditions the ferritin values alone are not helpful. The measurement of ferritin is simply done by means of immunoassays and most immunological analysers are capable of measuring it. Reference intervals are, somewhat dependent on the assay used, 35 - 300 µg/L for men and 20 - 100 µg/L for women. The upper reference limits are significantly higher in subjects above 65 years of age.

Transferrin / transferrin saturation

The normal **transferrin** concentration in plasma is 10,000- to 100,000-fold higher than the ferritin concentration. As the name suggests, transferrin transports iron to the tissues where it is needed, i.e. predominantly to the bone marrow, receiving it either from the iron stores or directly from the duodenal mucosa. Transferrin is composed of a single protein chain with two carbohydrate side chains attached. The molecular mass is 79.6 kDa. Each transferrin molecule can bind two Fe^{3+} ions. Measurement of transferrin may be performed as a turbidimetric test on clinical chemical mainframe analysers or as a nephelometric test on dedicated instruments. The reference interval is 2.0 - 3.6 g/L. The so-called **transferrin saturation** can be calculated easily using the equation:

$$\text{Transferrin saturation (\%)} = (\text{iron} \times 100) / (\text{transferrin} \times 2)$$

For iron and transferrin the molar concentrations must be entered into the equation, for example mmol/L. Under normal conditions in adults about 15 - 45% of the transferrin is loaded with iron.

In the case of ID the transferrin concentration increases and the iron concentration falls. As a consequence, the transferrin saturation is lowered. The transferrin saturation is a more sensitive marker for ID than transferrin alone. Contrary to ferritin, the transferrin concentration falls during the acute phase response. Since the iron concentration falls as well, the transferrin saturation may be normal. In pregnancy or following oral contraception the transferrin concentration is increased and the transferrin saturation is decreased. Due to these multiple influences the transferrin saturation is not a reliable marker to detect ID.

Soluble transferrin receptor

Recently the **soluble transferrin receptor (sTfR)**, a 85 kDa fragment of the intact transferrin receptor molecule, has been used to assess the iron stores. The transferrin receptor is a transmembrane molecule expressed on the surface of all cells that have a need for iron. Part of it is shed into the circulation, forming the sTfR. The concentration of the sTfR mainly reflects the iron demand of the erythropoietic tissue, i.e. both increased erythropoiesis and severe ID will produce an increase in sTfR. Like transferrin, the sTfR can be measured turbidimetrically or nephelometrically. Unfortunately, the sTfR measurement is not standardised and reference intervals vary considerably, requiring the determination of appropriate cut-off values for each individual assay.

Contrary to the ferritin concentration, during the course of development of ID the sTfR concentration remains stable initially. Only when the amount of iron available is so low that erythropoiesis is affected, will the sTfR concentration rise. So the sTfR is the only available serum marker reflecting iron-deficient erythropoiesis[14]. The sTfR concentration remains practically unchanged during an acute phase response and during pregnancy. The diagnostic sensitivity of the sTfR measurement is better than that of the transferrin saturation.

sTfR / log ferritin ratio

The concept of functional ID, i.e. the assumption that neither the demand for iron nor the state of the iron stores are important alone but that the relationship between the two is, has led to the creation of indices combining sTfR and ferritin. The most often used index is the so-called **sTfR / log ferritin ratio** (sTfR-F index)[15,16]. An elevated index reflects functional ID better than any of the above mentioned parameters.

The classical red blood count

The **classical red blood count (RBC)** is only affected in the later stages of ID where first the mean cell volume (MCV) of the erythrocytes decreases and later the mean cell haemoglobin (MCH), the haemoglobin concentration, the haematocrit and the erythrocyte concentration all fall, whereas the red cell distribution width (RDW) increases. The resulting hypochromic microcytic anaemia is typical of IDA, however, it is not specific. Other causes of anaemia such as that due to inflammation, chronic renal failure or a β-thalassaemia trait, can cause the same haematological pattern.

Hematologic indices are the new "gold standard" to assess iron deficiency and to monitor the treatment

Biochemically speaking, it can be easily understood from the last chapter that the detection of ID is particularly difficult when accompanied by an acute phase reaction. The situation is even more complex since we know today that the cytokines released during the course of inflammation, infection or cancer (like IL6, IL-1β, TNFα, and interferon-γ) do not only disturb the iron metabolism but also affect the erythropoiesis directly by suppressing the

synthesis of erythropoietin. In addition, TNFα induces apoptosis in the erythroid progenitor cells[17]. This condition is characterised by **hypoproliferative erythropoesis** and the clinical manifestation of these processes is called the **anaemia of chronic disease (ACD)**. IDA and functional ID in ACD or a combination of the two are hard to distinguish by serum markers. The power of sTfR and the sTfR-F index alone to identify IDA correctly is decreased in ACD.

To find a solution to this diagnostic problem we need to return to the red blood cells. In ID, be it due to empty iron storage pools or be it due to saturated pools and the iron being unavailable, the bone marrow produces hypochromic erythrocytes. Also their precursors, the reticulocytes, are hypochromic. With certain modern haematological instruments it is now possible to determine the **percentage of hypochromic erythrocytes** and to measure the haemoglobin content in each reticulocyte, calculating an average **reticulocyte haemoglobin content**[18]. The percentage of hypochromic erythrocytes is increased and the reticulocyte haemoglobin content is decreased in ID, irrespective of the presence or absence of inflammation. Since erythrocytes have a lifetime of about 120 days, the percentage of hypochromic erythrocytes represents a long-term average value. On the contrary, reticulocytes exist only for a few days before they develop into erythrocytes. So the reticulocyte haemoglobin content is like a "snapshot" of the acute condition and therefore a much more useful tool to immediately assess the success or failure of treatment. Recently, the use of a diagnostic / therapeutic plot has been suggested to classify anaemic patients with supposed ID (***Fig. 4***)[19]. On the abscissa of the plot the sTfR-F index (representing storage iron) is plotted against the reticulocyte haemoglobin content (representing functional iron) on the ordinate. Using appropriate cut-off values for both parameters, four different areas can be distinguished in the plot: patients in **area 1** have full iron stores and show normal erythropoiesis. Their anaemia is due to suppression of erythropoietin synthesis in patients with chronic disease, cancer or on chronic haemodialysis. They can be treated with recombinant erythropoietin. On the contrary, patients in **area 3** have full-blown IDA with empty iron stores and decreased haemoglobinisation of the reticulocytes. These patients require oral iron supplementation. Many patients in **area 2** are "coming" from area 3 and are "on the way" to area 1 during iron replenishment. They stay "in transition" in area 2 for four to six weeks.

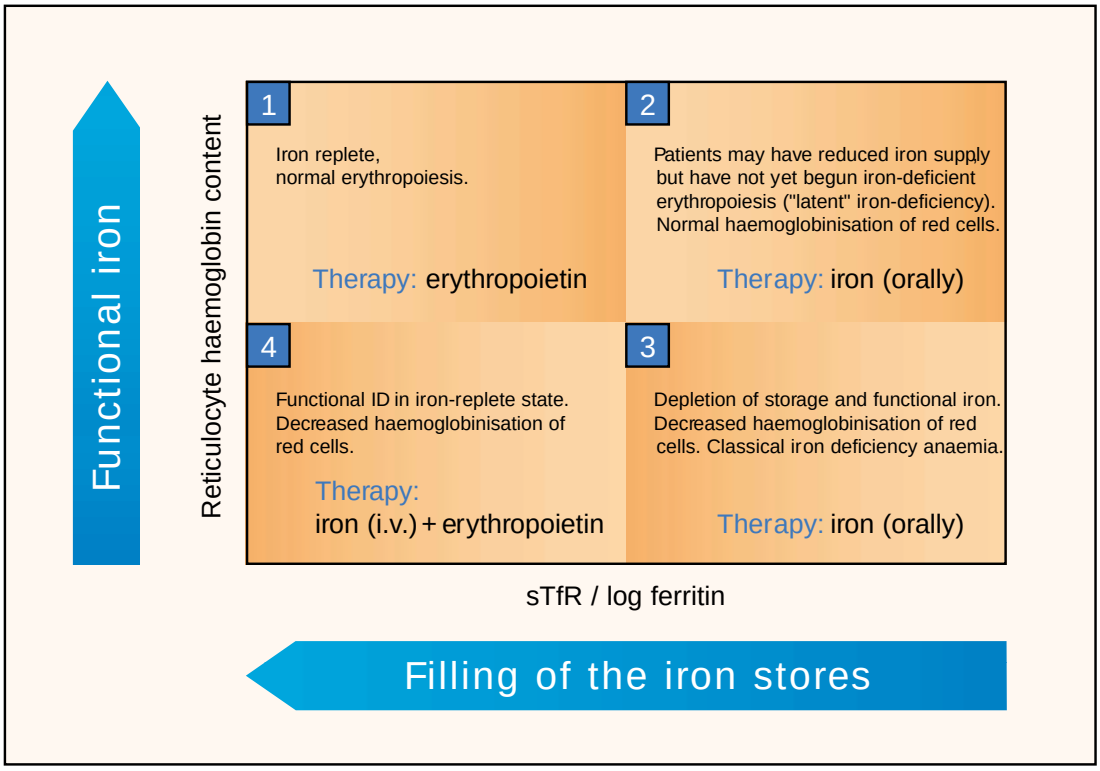

Fig. 4 *A diagnostic plot permits classification of patients with hypochromic anaemia by using classical biochemical markers and the reticulocyte haemoglobin content. This plot allows the selection of appropriate therapy for the patient and the monitoring of progress on this treatment. Adapted from*[19].

Patients in this area may also come from area 1 during the course of their disease with developing ("latent") ID where the storage iron is going down, but is still high enough to permit normal haemoglobinisation of the reticulocytes. Thirdly, these patients can show hyperproliferative erythropoiesis, for example after acute haemorrhage, in haemolytic anaemia or in late pregnancy. **Area 4** represents functional ID, for example in patients with ACD, cancer or on chronic haemodialysis. These patients have a suppressed erythropoietin synthesis and full iron stores, but they are unable to make use of the iron. In order to "move them up" into area 1 it is necessary to give them (intravenous) iron plus erythropoietin at a time. Patients with a β-thalassaemia are also found predominantly in area 4.

This plot is particularly useful since not only does it classify patients but also permits recommendations for the therapy and provides a tool to monitor progress. The cut-off value for the reticulocyte haemoglobin content is somewhat instrument specific in the order of 28 pg. The cut-off value for the sTfR-F index is largely dependent on the assays used for the determination of sTfR and ferritin. In addition, the sTfR-F index is influenced by the rise of ferritin during an acute phase response and it has been suggested that different cut-off values are required for patients with normal C-reactive protein (≤ 5 mg/L) and elevated C-reactive protein (> 5 mg/L).

The reticulocyte haemoglobin content has first been introduced by Bayer who called it **CHr** on the H*3 and on the ADVIA series. It is calculated from the product of the volume and haemoglobin concentration of single cells[20]. The haemoglobin concentration in turn is not measured directly but derived from the scattering properties of the reticulocytes. How do SYSMEX instruments measure the reticulocyte haemoglobin content? On the SYSMEX XE-2100 the RNA in the reticulocytes is stained with a fluorescent dye and the reticulocytes are identified and counted as a cluster of cells in a graph ("scattergram") where the fluorescence is plotted against the forward scatter of the cells. (The average forward scatter is called RET-Y on SYSMEX XE and XT instruments). It has been shown that the forward scatter is highly correlated with the haemoglobin content of the cell[21]. So the reticulocyte haemoglobin content can be derived from the forward scatter. Since this, too, is not a direct measurement of the reticulocyte haemoglobin content, the parameter is tentatively termed **RET-H_e** standing for the average **reticulocyte haemoglobin equivalent**. RET-H_e and CHr are clinically equally effective for distinguishing ID from ACD and the combined state of functional ID / ACD[22,23]. ***Fig. 5 (a)*** schematically shows the different cell types seen in the reticulocyte channel of the SYSMEX XE-2100. The turquoise cell cluster is representing the thrombocytes, the blue cluster the erythrocytes, the purple cluster the "older" reticulocytes, and the red cluster contains the "younger" reticulocytes. The utility of RET-H_e can be seen immediately in the following example: ***Fig. 5 (b)*** is the scattergram of a 7-year-old child with severe hypochromic anaemia. ***Fig. 5 (c)*** shows the scattergram 3 days after i.v. iron and erythropoietin substitution. Not only the concentration of reticulocytes, but also the average reticulocyte haemoglobin content has increased, as can be seen from the upward shift of the

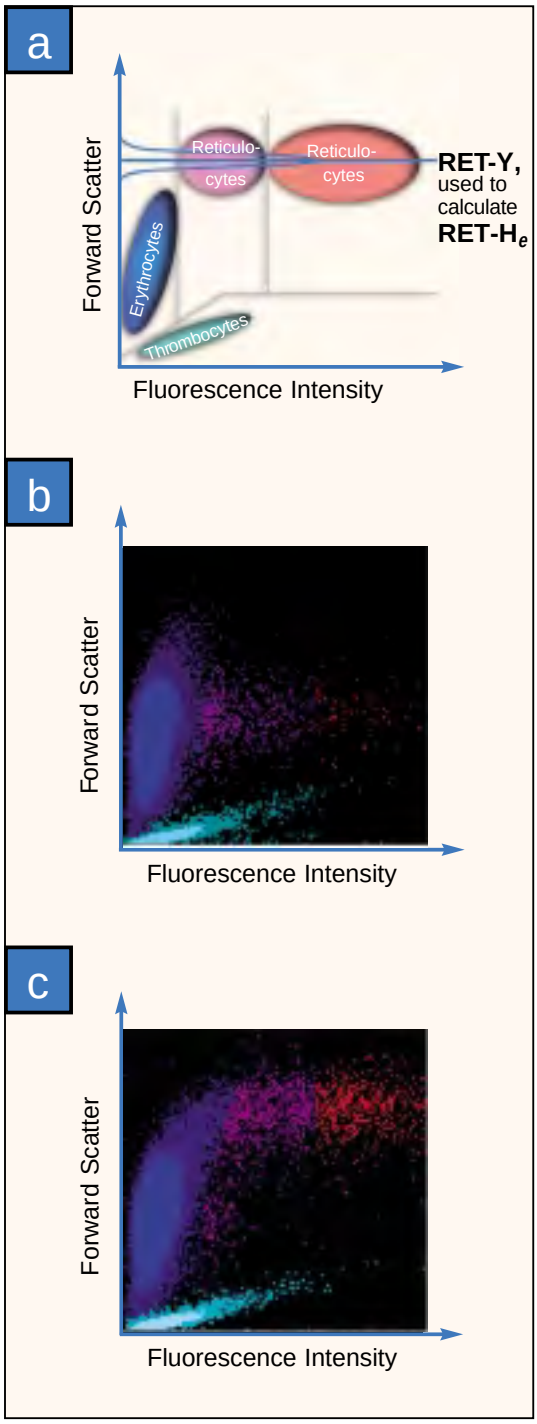

Fig. 5 ***Reticulocyte measurement in iron deficiency***
(a) schematically shows the different cell types seen in the reticulocyte channel of the SYSMEX XE-2100. (b) is the scattergram of a 7 year old child with severe hypochromic anaemia. (c) shows the scattergram 3 days after i.v. iron and erythropoietin substitution. Not only the reticulocyte concentration, but also the average reticulocyte haemoglobin content (RET-H_e) is increased, as can be seen from the upshift of the purple and red reticulocyte clusters, respectively, whereas the blue erythrocyte cluster has not changed substantially.

purple and red reticulocyte clusters, respectively, whereas the blue erythrocyte cluster has not changed substantially. Significant changes in erythrocyte count and haemoglobin in this patient were only detected 6 days after the start of therapy, demonstrating that RET-H_e can detect the start of the bone marrow response much earlier. Why not simply take the increasing **reticulocyte concentration** as a measure of the success of the therapeutic intervention? This parameter tells us that the bone marrow is starting to respond, but it does not tell us whether the iron treatment and the erythropoietin are sufficient to guarantee the production of reticulocytes of normal size and haemoglobin content, leading eventually to erythrocytes of normal size and haemoglobin content.

Iron deficiency in early childhood

Since the number of toddlers with ID is so high even in the industrialised countries, a simple and cheap routine parameter with a high predictive value would be desirable for screening. It has been demonstrated in a study with children 3 years of age on average that the reticulocyte haemoglobin content is superior to other haematological and serum markers in predicting ID and IDA[24]. The author concludes: "This study has raised the possibility that diagnosis of ID in early childhood could be achieved exclusively on the basis of haematological indices, without the need for biochemical studies[25]."

Some problems in the diagnosis of iron deficiency are finally solved

As we have seen, new reticulocyte parameters like RET-H_e are excellent tools to assess various states of ID in situations where the classical serum parameters fail or are too expensive, i.e. when inflammation or cancer are present, in patients on chronic haemodialysis or for the screening for ID in toddlers. Other applications can be expected to come soon. Are we on the edge of a new era in the diagnostics of ID?

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Key points:

- Iron is ubiquitous in life. Due to the easy transition between Fe^{2+} and Fe^{3+} iron is indispensable for many redox processes in most cells and for the reversible binding of oxygen in erythrocytes. Free iron can be very toxic, generating free radicals.
- To maintain iron homeostasis the uptake of iron in the mucosa is controlled, as is the synthesis of cellular iron-binding proteins.
- All iron in the body is protein-bound. Three functional iron compartments can be distinguished: functional iron (haemoglobin, myoglobin, enzymes), storage iron (ferritin, haemosiderin) and transport iron (transferrin).
- Iron deficiency can be due to insufficient supply, malabsorption, increased requirement and blood loss or the state of functional iron deficiency can exist. Iron deficiency is the most common nutritional disorder. Although the initial signs are non-specific and may seem harmless, iron deficiency has severe medical and social consequences.
- Functional iron deficiency describes a state where the iron stores are filled but the iron is not available for haemoglobin synthesis and other biochemical tasks. This is mainly the case for patients with chronic inflammation, cancer or on chronic haemodialysis.
- In simple cases iron deficiency can be easily detected by measuring the classical serum markers like ferritin or transferrin saturation, however, these markers are prone to misinterpretation if inflammation is present and cannot detect functional iron deficiency.
- Functional iron deficiency can be distinguished from pure iron deficiency by measuring the average reticulocyte haemoglobin content and the serum transferrin receptor - ferritin index. With the help of a diagnostic / therapeutic plot patients with hypochromic anaemia can be classified and scheduled for the appropriate therapy: administration of erythropoietin or iron or both.
- The reticulocyte haemoglobin content is also an inexpensive screening test for iron deficiency in toddlers. This test turns out to be superior to the classical serum markers.

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