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Iron intake and cardiovascular disease

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6.1 Introduction

Iron is an essential dietary component, necessary for a number of cellular functions including respiration and immune response. Since there is no physiological iron excretion, the element is reutilised in the body, and only a small fraction of the body's iron is gained or lost each day. The daily iron losses are mostly from desquamation of epithelia, such as skin and the lining of gastrointestinal tract. Greater iron losses may occur during growth in childhood, haemorrhages, menstruation and pregnancy in women.

Besides the fact that it is a vital element in life, iron may participate in diverse pathological processes. This may be due to its involvement in the production of reactive oxygen species. These species play important roles in immune response. However, they have also been shown to be involved in the pathogenesis of several diseases, such as Alzheimer's, Parkinson's, Crohn's disease, diabetes, cancer and arthritis. Furthermore, considerable evidence has supported the role of oxidative stress in atherogenesis. It has been hypothesised that iron-mediated oxidation is involved in this process (Sullivan 1981). Several epidemiological studies as well as *in vivo* and *in vitro* experiments are in favour of this iron hypothesis, although some studies have yielded conflicting results. This chapter describes the regulation of daily iron intake, the physiological, cellular and molecular metabolism of iron, the abnormal conditions of body iron balance, and the potential role of iron in the development of atherosclerosis and cardiovascular diseases.

6.2 Dietary iron intake, absorption and metabolism

Although there is no physiological means of iron excretion, a well-balanced diet containing sufficient iron is needed. Only about 10 per cent of ingested iron is absorbed in the gut. Therefore, around 10–20 mg of dietary iron intake is needed to balance the 1 or 2 mg of daily losses. The normal amount of total body iron is about 40–50 mg/kg body weight. In the body, iron is mainly needed to form the porphyrin complex of haemoglobin (30 mg/kg), myoglobin in muscle cells (4–8 mg/kg) and also iron-containing enzymes, such as cytochromes, oxidases and peroxidases. Up to 30 per cent of body iron (12 mg/kg) may be stored as ferritin and hemosiderin in the bone marrow, spleen and liver.

The efficiency of iron absorption is mainly regulated by body requirements to maintain iron homeostasis. Iron deficiency causes an increase in iron absorption, while iron overload reduces but does not eliminate absorption. Fertile women, for example, need to absorb up to 2–5 mg of iron each day to compensate for the menstrual blood loss. Many conditions causing a greater body iron demand may increase the efficiency of dietary iron absorption up to 20 per cent. A feedback mechanism exists to enhance or down-regulate iron absorption. Excretions from the liver, gall bladder and pancreas to the duodenum influence the uptake of iron.

6.2.1 Mechanism of iron uptake

Iron is mainly absorbed in the duodenum and the upper jejunum of the small intestine (Fig. 6.1). Both haeme iron and soluble complexes of iron are absorbable. Iron absorption from the gut lumen across the enterocytes to the circulation occurs in two stages: uptake across the apical membrane and transfer across the basolateral membrane (Fig. 6.2). The mechanisms of apical iron uptake from intestinal lumen to the enterocytes depend on the source of iron: Fe(II) or Fe(III) complexes. Haeme is absorbed in receptor-mediated fashion by the enterocytes. The haeme oxygenase-1 releases iron which then is reduced intracellularly. Fe(II) complexes are readily absorbed through a transporter called DMT-1. Fe(III) complexes are first reduced by a membrane-bound iron reductase called Dcytb before absorbed into the enterocytes, or bound to mucin and intracellularly reduced by either β-integrin, mobilferrin or flavin monooxygenase. The reduced intracellular iron may be stored in ferritin. The basolateral iron transfer is mediated by Ireg-1. After transfer, Fe(II) is immediately oxidised by the membrane-bound iron oxidase, hephaestin, or by the circulating oxidase, ceruloplasmin. Fe(III) is then bound to transferrin, an iron carrier protein in the circulation. Like any other cells, the enterocytes can take up transferrin by expressing transferrin receptor when iron is needed. Hepcidin is a signalling protein produced by the liver when iron level is high in the body. This molecule may regulate iron absorption through the enterocytes via an iron-sensitive protein, IRP. IRP can bind to iron-responsive element (IRE) present in transcription products of several genes including Dcytb, DMT-1, Ireg-1 and transferrin receptor regulating the expression of these corresponding proteins. HFE acts to facilitate TfR-1-mediated iron uptake from plasma into crypt cells.

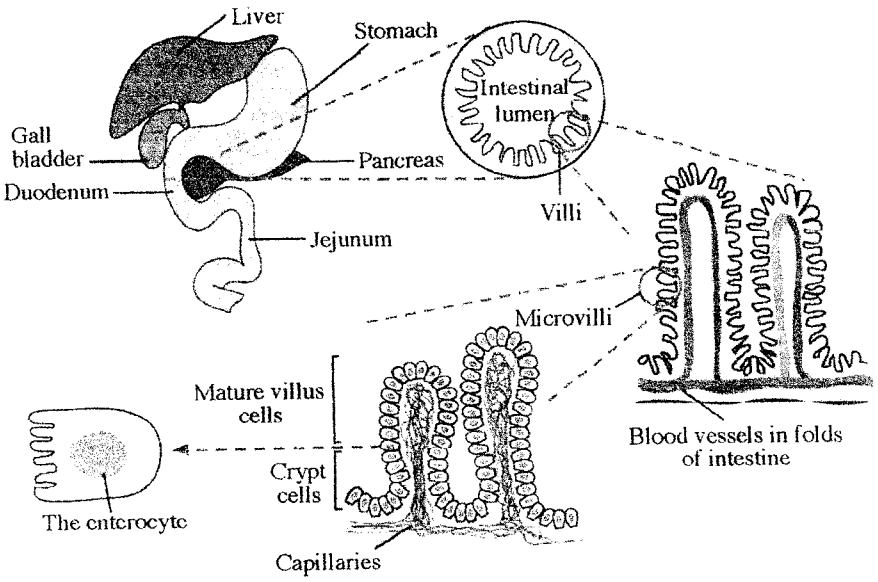

Fig. 6.1 Duodenal enterocytes within the gastrointestinal tract. Iron is absorbed from intestinal lumen, through the enterocytes lining the duodenum, to the circulation. The young enterocytes called the crypt cells move to the tip of microvilli and develop into mature villus cells. These two types of cells differentially regulate the uptake of iron.

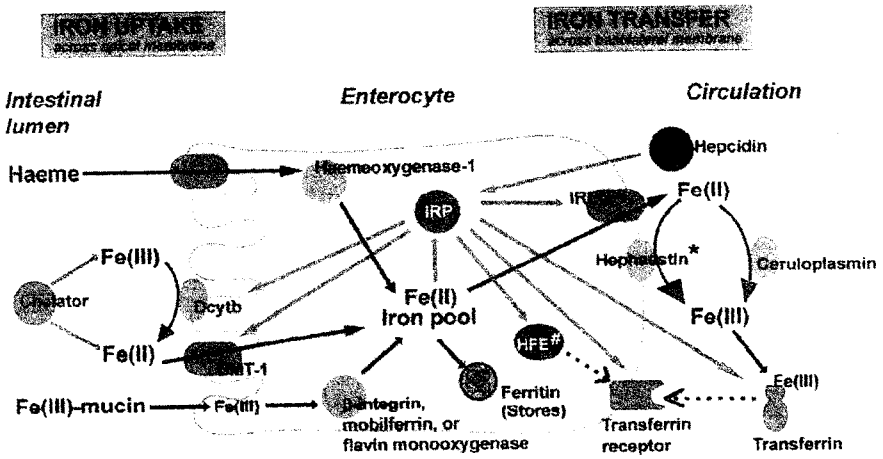

Fig. 6.2 Schematic representation of iron absorption and metabolism in normal enterocytes. # = in crypt cells only, * = mostly in mature villus enterocytes and hardly found in crypt cells. Fe(II) = divalent iron. Fe(III) = trivalent iron. Dcytb = duodenal cytochrome-b. DMT-1 = divalent metal transporter-1. IRP = iron responsive protein. IREG-1 = iron-regulated protein-1.

Apical iron uptake

The apical iron uptake is the absorption of iron from the intestinal lumen across the apical membrane to the enterocytes. The mechanisms of apical iron uptake from haeme and non-haeme iron sources are different.

Haeme iron derived from digested hemoproteins is able to interact with the membrane of the intestinal epithelial cells. This haeme is taken up by the enterocytes through a distinct receptor-mediated transport (Grasbeck *et al.* 1979). Inside the enterocytes, the haeme is broken down by haemeoxygenase-1. The released iron then enters the intracellular iron pool.

Non-haeme iron complexes can exist in two oxidation states: divalent iron, Fe(II), and trivalent iron, Fe(III). The soluble Fe(II) complexes can be taken up more easily than the almost insoluble Fe(III) derivatives by the cell membrane. However, in physiological condition, Fe(II) is rapidly oxidised to Fe(III). The presence of either endogenous chelators, such as mucin, lactoferrin or bile acids, or exogenous chelators, such as fatty acids, amino acids (e.g. asparagine, glycine, histidine) or organic acids (e.g. lactic, citric, succinic, ascorbic, malic, pyruvic) may increase the solubility and hence the uptake of iron.

Secretion of gastric acid lowers the pH of the gut. This increases the solubility and enhances the uptake of iron as well. When gastric acid production is impaired, for instance by the consumption of antacids and in the conditions of achlorhydria, iron absorption is reduced.

The soluble Fe(II) is taken up by the enterocytes through a specialised divalent metal transporter called Nramp-2, DCT-1 (divalent cation transporter) or DMT-1 (divalent metal transporter) (Gunshin *et al.* 1997). DMT-1 is highly expressed in the duodenum of normal individuals, and even strongly upregulated in anaemic conditions (Gunshin *et al.* 2001). This protein, however, is also capable of transporting other metal ions, such as zinc, lead, cadmium, manganese and copper. This lack of iron specificity may cause an increase in the absorption of toxic metals in iron deficiency.

The insoluble Fe(III) can only be taken up by the enterocytes after a reduction step catalysed by an iron-reductase. The iron-reductase, called Dcytb (duodenal cytochrome-b) has highest activity in the duodenum and lowest in the ileum (McKie *et al.* 2001), which is well-matched with the profile of iron absorption along the gut.

Mucin, a stomach glycoprotein, may also help the uptake of Fe(III) complexes in the duodenum. The mucin–Fe(III) complex, termed gastroferrin, readily traverses the mucus layer and acid microclimate at the mucosal surface. Within the enterocytes, Fe(III) is dissociated from the mucin. This released iron is readily reduced by several reductases including β-integrin, mobilferrin and flavin monooxygenase (Conrad *et al.* 1999), contributing to the intracellular iron pool.

Basolateral iron transfer

The basolateral iron transfer is the transport of iron from the enterocytes across the basolateral membrane to the circulation. Ireg-1, an iron-regulated protein, also called Ferroportin-1 or MTP-1, is responsible for this transfer (McKie *et al.* 2000). The expression of the protein is localised in the duodenum and also in

several other organs, such as the macrophages and the placenta where iron transfers between maternal and fetal circulations.

6.2.2 Regulation of iron uptake

The extent of iron absorption is mainly affected by the level of body iron, the degree of erythropoiesis, the amount of iron in the diet, and the composition of the diet itself. Other conditions, such as hypoxia, pregnancy and inflammation, may also alter the absorption. Furthermore, iron absorption is inappropriately increased in primary haemochromatosis.

The iron stores regulator induces a moderate increase in iron absorption as the body iron stores fall, and vice versa. It is still an unresolved question in how the duodenal mucosa is able to sense the level and changes in demand for iron. Iron content of the enterocytes is likely to be an indicating factor for this regulator. A central role of this regulatory process is assigned to the recently discovered hepcidin, a protein that is secreted into the plasma from the liver (Nicolas *et al.* 2001).

Approximately 70 per cent of body iron is incorporated into haemoglobin. In average, an adult person produces 2×10^{11} red blood cells daily containing 2×10^{20} atoms (20 mg) of iron. To meet this daily requirement, the body develops regulatory mechanisms whereby erythropoiesis profoundly influences iron absorption. This regulator would balance the rate of erythropoiesis in the bone marrow with the duodenal iron absorption.

Iron absorption is also modulated by the amount of iron in the diet and the composition of the diet itself. When increasing amounts of iron are ingested, the relative amount of iron absorbed decreases owing to the feedback mechanism of the absorption machinery; however, the absolute amount may still increase. Several chelators present in the diet, such as citrate from citrus fruits, can promote an increase in iron absorption, by increasing the solubility of iron in the duodenum. In contrast, phytates in wheat and some other cereals, as well as tannins in teas, chelate iron but prevent its uptake. Several metal ions, such as lead, cobalt, manganese and zinc, which are taken up by the same absorption machinery, may also block the iron uptake through competitive inhibition.

Haeme iron found in meats is more readily absorbed than non-haeme iron. The absorption is independent of duodenal pH. Experimental data indicate that haeme iron absorption is less responsive to the store regulator than that of non-haeme iron. Consequently, meat is an excellent nutrient source of iron. Lack of meat in the diet can be a cause of iron deficiency.

6.2.3 Metabolism of iron

Iron absorption, plasma iron transport, iron incorporation into cells and iron storage are meticulously regulated in the body to maintain iron homeostasis. Only a small fraction of body iron actually circulates, while most of body iron is prominently represented in haemoglobin, ferritin and haemosiderin.

Organs and cells communicate their needs for iron via the plasma as the central compartment of iron metabolism. Essentially, the body contains three types of cells: (1) those that need to obtain iron from the plasma (iron-requiring cells), (2) those that need to export iron towards the plasma (iron-donor cells), and (3) those that are able to take up and release iron for the protection of other more vulnerable cells in the body (hepatocytes). Transport and storage of iron in these cells are modified in situations of iron deficiency or overload.

Iron trafficking and uptake into cells

The iron-donor cells, mainly the macrophages and the intestinal mucosal cells, release iron to the plasma as Fe(II). The majority of this iron is rapidly oxidised by hephaestin or ceruloplasmin, then bound to transferrin. Hephaestin is found in the basolateral membrane of the mature villus enterocytes along the gut, while ceruloplasmin is a humoral protein produced and secreted by the liver to the plasma. Transferrin-bound iron is offered to iron-requiring cells, with majority going to the erythroblasts in the bone marrow for haemoglobin synthesis.

Transferrin is a 80 kDa single-chain glycoprotein containing two structurally similar subunits, each with one iron binding site. Therefore, one transferrin molecule can bind two Fe(III) atoms. Upon binding to iron the subunit undergoes a rigid rotation to enclose the iron atom. A distinctive feature of transferrin is its dependence on a synergistic anion, normally carbonate or bicarbonate for Fe(III) binding. When this anion is protonated, iron will be expelled from this harbouring protein.

Normally, all the non-haeme iron in the circulation is bound to transferrin. The liver synthesises transferrin and secretes it to the plasma. Transferrins are also produced locally in the testes and the central nervous system. Only around 20–45 per cent of transferrin binding sites are occupied in the circulation, so that most of available transferrins are free from iron. Nevertheless, non-transferrin-bound iron (NTBI) can be detected in some iron-overload conditions (de Valk *et al.* 2000) and may be attached to a variety of ligands. Most NTBI is taken up by the hepatocytes via the portal venous system. Furthermore, NTBI may also enter many other cell types promoting tissue damage.

Both monoferric and diferric transferrins are internalised by receptor-mediated endocytosis. Differic transferrin binds with higher affinity than monoferric transferrin. Two transferrin receptors have been described, i.e. TfR-1 and TfR-2. TfR-1 is expressed far more abundantly in iron-requiring cells than TfR-2, while TfR-2 is constitutively expressed in the liver (Gatter *et al.* 1983).

After binding to its receptor on the cell surface, transferrin is rapidly internalised through the formation of a clathrin-coated pit, which further develops into an endocytotic vesicle. This endosome undergoes acidification to pH 5.5 weakening the association between iron and transferrin. A membrane iron-reductase may help to completely dissociate iron from transferrin (McKie *et al.* 2001). Iron is then transported to the cytosol by DMT-1. The intact receptor-apotransferrin then recycles to the cell surface, where neutral pH promotes

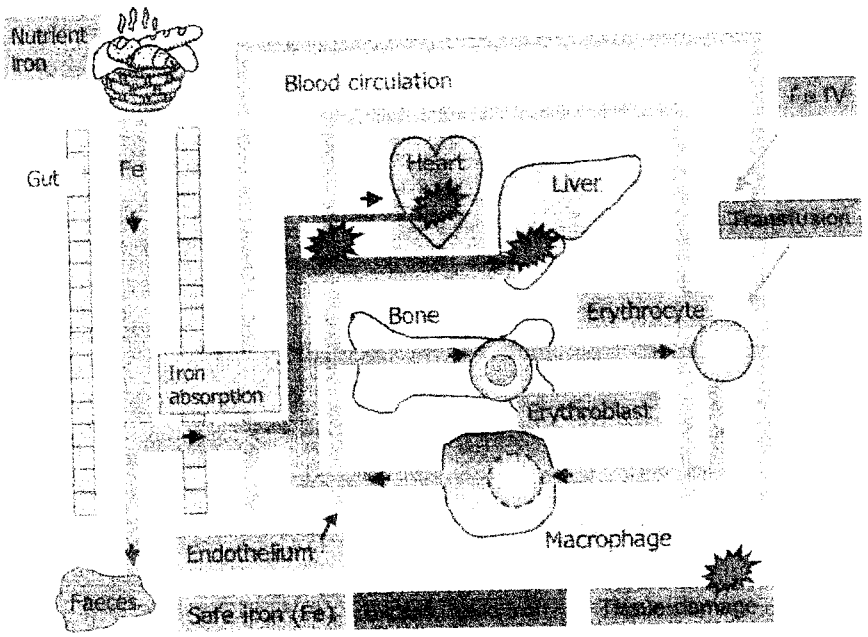

Fig. 6.3 Schematic representation of iron metabolism and its toxic potential (adapted from Marx and Hider, *Eur. J. Clin. Invest.* March 2002).

detachment of apotransferrin into the circulation. Exported apotransferrin can undergo further cycles of iron delivery into cells. The average transferrin molecule with a half-life of 8 days may be used up to one hundred times for iron delivery.

In iron overload, because of excessive iron intake, genetic defects, or repeated blood transfusions, considerable amounts of NTBI may be present in plasma. This iron can be weakly complexed to citrate, albumin, amino acids or sugars (Loreal *et al.* 2000). Most of NTBI is found in the complex form of Fe(III) to citrate, as shown by nuclear magnetic resonance (NMR) spectroscopy of serum from patients with iron overload (Grootveld *et al.* 1989). Non-haematopoietic tissues, mainly the liver, and also endocrine organs, kidneys, heart and the endothelium lining the blood vessels, preferentially take up NTBI through a transferrin-receptor independent mechanism. This mechanism may explain the continuous uptake of iron by the hepatocytes, in which iron overload has suppressed TfR1 expression beyond detectability. Furthermore, NTBI may generate toxic oxygen radicals and promote tissue damage in these organs (Fig. 6.3). Normal amount of iron, obtained from dietary iron or recycled body iron released by macrophages, is required for normal body functioning, especially for haemoglobin formation in the bone marrow. In the case of increased total body iron, either from increased dietary iron absorption, regular blood transfusion or intravenous iron injection, excess toxic iron may enter the circulation. Toxic iron is readily taken up by several organs, including the liver, the heart and the endothelium lining the blood vessels, and may cause further tissue damage in these organs.

Molecular metabolism and storage of iron

Inside the cell, iron first enters the intracellular iron pool in the form of Fe(II), which is soluble and biologically available. This iron is able to enter various intracellular locations, including mitochondria (especially for haeme biosynthesis, and ferritin (for storage).

Haeme biosynthesis occurs in all tissues, although the principal sites of synthesis are erythroid cells (~85 per cent) and hepatocytes (accounting for nearly all the rest of haeme synthesis). In hepatocytes, haeme is incorporated into cytochromes, in particular the P_{450} class which is important for detoxification. In erythroid cells, almost all of the haeme is synthesised for incorporation into haemoglobin. When the red cells mature, both haeme and haemoglobin synthesis cease. Normally after 120 days, senescent red blood cells are engulfed by macrophages. The globin is recycled or converted into amino acids, which in turn are recycled or catabolised as required. The haeme is oxidised by haemoxygenase, which results in the production of linear tetrapyrrole biliverdin, iron and carbon monoxide (CO). Most of the CO is excreted through the lungs, while the erythrocytic iron is then either stored as ferritin or released into the plasma via the iron export protein, ferroportin-1. The released iron is oxidised to Fe(III) by ceruloplasmin and is bound to circulating transferrin.

Sequestering of iron is necessary in all cells to avoid its tendency to form oxygen radicals that may damage cells. Ferritin and hemosiderin (reviewed by Harrison & Arosio 1996) are iron storage proteins that store iron within cells. Ferritin forms a hollow, spherical particle, in which 2000–4500 iron atoms can be stored as Fe(III). All ferritins are composed of 24 subunits associating to form a spherical particle. In animals, ferritin is found not only inside cells, but also circulating in the plasma. Plasma levels of ferritin are routinely been used as a measure for body iron.

Haemosiderin is another iron-storage complex. Its molecular nature is less defined than ferritin, but it is always found within cells and appears to be a complex of ferritin, denatured ferritin and other materials. Haemosiderin is most commonly found in macrophages and is especially abundant in tissues following internal haemorrhage, suggesting that its formation may be related to phagocytosis of red blood cells and haemoglobin.

Control mechanism for iron homeostasis in cells

Iron levels may regulate the expression of iron-related proteins, such as DMT-1, Ireg-1, ferritin, transferrin and TfR-1 (Fig. 6.2). This is shown by the presence of an iron responsive element (IRE) in the transcription product of the gene, which allows an cytoplasmic iron responsive protein (IRP) to bind in response to the level of intracellular iron (Hentze *et al.* 1987). In the case of ferritin production, for example, low intracellular iron conditions allow the binding of IRP to IRE, stopping protein synthesis. High intracellular iron level, on the other hand, prevents IRP–IRE binding, which results in increased ferritin synthesis. This IRP IRE interaction provides control mechanism for intracellular iron homeostasis.

6.3 Iron homeostasis disorders: primary and secondary haemochromatosis

Disorders in the iron homeostasis may lead to either iron deficiency or iron overload. Iron deficiency is a condition where the iron intake does not meet the body's demands. Its manifestations are paleness, lethargy, palpitations and shortness of breath. Iron overload, also termed haemochromatosis, on the other hand, is characterised by a progressive increase in the total amount of body iron followed by an abnormal iron deposition in multiple organs (Fig. 6.3). In advanced cases, it also causes a bronze colour of the skin because of the deposition of iron-containing pigments in various tissues. The disease was once thought to be a singular disease with varying degrees of severity. Nowadays, it is known to be heterogeneous, resulting from defects in various genes.

6.3.1 Primary hereditary haemochromatosis

Several types of primary hereditary haemochromatosis have been described. Type-1 hereditary haemochromatosis (HH) is a common autosomal recessive disorder affecting mostly Caucasians. One in 200 (about 3.5 million) Europeans is homozygous for this, initially symptomless, chronic disease (Powell *et al.* 2000). Most individuals with primary haemochromatosis absorb excessive amount of dietary iron irrespective of the level of body iron, suggesting that the iron store regulator is dysfunctional. The excess iron accumulates over time, leading to tissue damage and organ failure. Clinical consequences include hepatic failure, liver carcinoma, arthritis, diabetes, impotence and cardiac failure.

This type-1 HH is associated with mutations in the *HFE* gene (Feder *et al.* 1996). The progression of iron overloading for this type of HH is quite slow, and affected individuals often start to have clinical symptoms only after the fifth or sixth decade of life. The initial symptoms include fatigue and joint complaints. As iron loading is progressing, patients develop skin hyperpigmentation and liver disease, which deteriorates gradually from fibrosis to cirrhosis. Cardiomyopathy and arrhythmias may develop from deposition of iron in the heart. Endocrine abnormalities, such as hypogonadism and diabetes mellitus, are also common.

HFE is strongly expressed by intestinal crypt cells and liver macrophages. The function of HFE protein itself is poorly understood. It appears to be a regulatory molecule that influences the efficiency of intestinal iron absorption, and may play an important role in iron homeostasis through its interaction with the transferrin receptor, TfR1. HFE facilitates TfR-1–mediated iron uptake from plasma into crypt cells, and its action is abrogated in *HFE*-linked HH in which there is functional loss of *HFE* protein. In the gut of HH, the cells behave as though they are relatively iron-deficient, causing an increase in intestinal iron absorption (Moura *et al.* 1998). HH patients have no iron-loading of macrophages, since wild type HFE also functions to inhibit iron release from these cells (Drakesmith *et al.* 2002), which results in increased release of low

molecular weight iron as Fe(II) from the cells to the circulation, and may further promote NTBI formation. The majority of type-1 HH patients carry a missense mutation (C282Y) in *HFE*. Other mutations and polymorphisms (H63D, S65C, I105T, G93R) have been identified, but their contributions to HH are not clearly understood. Treatment for type-1 HH is by phlebotomy, in order to keep serum ferritin levels below 50 μg/L. Initial treatment is 500 ml phlebotomy per week, followed by continuous treatment of one to four times a year. Cirrhosis usually occurs in HH patients when hepatic iron concentrations exceed 400 μmol/g dry weight liver (22.4 mg/g).

For type-2 HH, the juvenile haemochromatosis (Perkins *et al.* 1965) the responsible gene has not been identified. This type of HH is more severe than type-1 and it is characterised by rapid iron loading and clinical manifestations within the second decade of life. Cardiac and endocrine abnormalities dominate the clinical picture, although liver problems are also significant. Type-3 HH is associated with mutations in *TfR-2* (Camaschella *et al.* 2000) and is phenotypically similar to type-1 HH. Type-4 (Montosi *et al.* 2001; Njajou *et al.* 2001) and type-5 HH (Kato *et al.* 2001) are inherited in an autosomal dominant pattern. Type-4 is caused by missense mutations altering ferroportin-1. Patients accumulate large amounts of iron in the liver macrophages and have less transferrin-bound iron. However, they eventually also develop liver, heart and pancreatic complications. Type-5 HH, which has so far affected one Japanese family, affects the ferritin molecule, which in turn causes a defect in the iron-storing process.

6.3.2 Secondary haemochromatosis

Secondary haemochromatosis may be caused by several other conditions leading to iron overload. These include excess of dietary iron intake, chronic haemolysis and frequent blood transfusions. Phlebotomy is mostly impossible in these cases. The treatment most commonly used is a continuous administration of an iron-chelating agent.

Chronic anaemia such as aplastic anaemia, sickle cell anaemia, and thalassaemia cause iron overload mostly because of frequent blood transfusions. Each 250 ml transfused red cells adds about 250 mg elemental iron to the body. Frequent transfusions may promote diabetes mellitus and cardiac failure when iron concentrations exceed 268 μmol/g dry weight liver (15 mg/g). The end-organ manifestations of iron overload, such as cirrhosis, cardiac failure, hepatocellular carcinoma, diabetes mellitus and hypopituitarism resemble the manifestations in hereditary haemochromatosis patients.

6.4 The role of iron in cardiovascular disease

In significant parts of the modern world, iron overload is found in the population more often than iron deficiency. Consequently, the potential hazards of iron

excess are gaining more attention. Excessive iron may promote cardiomyopathy, arthropathy, infection, liver fibrosis, diabetes mellitus and malignancy, as well as endocrine and neurodegenerative disorders. A relatively new hypothesis has been postulated by Jerome Sullivan in 1981 (Sullivan 1981) that iron may play an important role in atherosclerosis and related cardiovascular diseases. Despite significant controversy and the negative results of several studies, de Valk and Marx (1999) concluded a strong epidemiological evidence for this iron hypothesis.

6.4.1 Epidemiological studies in favour of the iron hypothesis

Serum ferritin concentration as a measure of body iron has been shown to significantly correlate to the risk of myocardial infarction or carotid artherosclerosis (Haidari *et al.* 2001; Kiechl *et al.* 1994; Kiechl *et al.* 1997; Klipstein-Grobusch *et al.* 1999b; Salonen *et al.* 1992; Salonen *et al.* 1998; Tuomainen *et al.* 1997b, 1998). Ultimately, Lauffer (1991) showed significant correlation between iron stores, measured by liver biopsy, and cardiovascular mortality. Additionally, low prevalence of CHD has been observed in areas with high prevalence of iron deficiency (Sullivan 1981).

The lower incidence of coronary heart disease in premenopausal women compared with men of the same ages and with postmenopausal women was shown to be due to the lower total body iron caused by menstrual blood loss (Sullivan 1989). In men, body iron assessed by ferritin concentration, rose after adolescence, while in women, ferritin began to rise only after the age of 45 years (Burt *et al.* 1993). The Framingham study showed that the risk of heart disease in women increased equally by natural or surgical menopause (Gordon *et al.* 1978; Hjortland *et al.* 1976; Kannel *et al.* 1976). In heterozygotes of familial hyperlipoproteinaemia, the premenopausal women had a lower risk of coronary heart disease than men (Ascherio & Hunter 1994; Slack 1969; Stone *et al.* 1974).

The iron hypothesis may also explain the association between frequent blood donations and reduced risk of myocardial infarction (Meyers *et al.* 2002, Salonen *et al.* 1998, Sullivan 1991, Tuomainen *et al.* 1997b). Additionally, a community-based prospective cohort study showed that haem iron intake was positively associated with the total body iron and the risk of cardiovascular diseases (Ascherio *et al.* 1994; Klipstein-Grobusch *et al.* 1999a; Salonen *et al.*1992; Snowdon *et al.* 1984; Tzonou *et al.* 1998).

Recent studies have identified carriers of hereditary haemochromatosis (*HH*) gene to have significantly higher catalytically active iron than the normal population (de Valk *et al.* 2000). Some reports, moreover, demonstrated an association between heterozygous HFE gene mutation and the risk of cardiovascular events (Battiloro *et al.* 2000; Hetet *et al.* 2001; Rasmussen *et al.* 2001; Roest *et al.* 1999, Tuomainen *et al.* 1999). As for β-thalassemia, the clinical evidence of vascular complications has been shown to match with higher levels of oxidative modification of LDL compared with healthy controls (Livrea *et al.* 1998).

6.4.2 Epidemiological studies weakening the iron hypothesis

A number of studies failed to correlate serum ferritin and the risk for cardiovascular diseases (Auer *et al.* 2002; Eichner *et al.* 1998; Frey & Krider 1994; Manttari *et al.* 1994; Moore *et al.* 1995; Solymoss *et al.* 1994). Several other studies used serum iron, transferrin saturation or total iron binding capacity (TIBC) as a measure for body iron stores and did not find a correlation with cardiovascular disease. Since there is a poor correlation between transferrin saturation in the normal range and the total body iron (Beaton *et al.* 1989; Cook *et al.* 1976), their arguments opposing the iron hypothesis are weakened.

Some studies failed to reveal an increased risk of atherosclerosis in heterozygous and homozygous haemochromatosis (Candore *et al.* 2003; Fox *et al.* 2002; Franco *et al.* 1998; Nassar *et al.* 1998; Powell *et al.* 1994). Blood donation also did not appear to correlate with reduced risk for cardiovascular disease in one study (Ascherio *et al.* 2001).

Furthermore, some authors found no correlation between dietary iron intake and carotid atherosclerosis (Rauramaa *et al.* 1994), CHD (Liao *et al.* 1994), or myocardial infarction (Morrison *et al.* 1994). However, these studies did not differentiate between haeme and non-haeme iron intake. A lack of correlation between non-haeme iron intake and cardiovascular diseases suggests that dietary non-haeme iron does not contribute to an increased cardiovascular risk, except perhaps among patients with haemochromatosis (Ascherio & Hunter 1994).

Possible confounding factors are important to be taken into account before concluding any results from epidemiological studies. For atherosclerosis studies, it is necessary to involve an older population, since atherosclerosis progresses with age and the effect of iron on the incidence of severe atherosclerotic events can only be expected in such a population. In addition, excess iron alone as a risk factor may not be sufficient to demonstrate statistically significant different in cardiovascular events, as atherosclerosis is a multifactorial disease. The most recent study (Wolff *et al.* 2004) identified a relationship between serum ferritin levels and carotid atherosclerosis, and this correlation was even stronger when low-density lipoprotein (LDL)-cholesterol levels were taken into account.

6.4.3 Pathogenic mechanisms of iron-induced atherosclerosis

Atherosclerosis is a slow disease, starting in childhood, and progressing during ageing. It is due to a chronic inflammatory process coupled with dyslipidaemia. Two major mechanisms initiating the plaque formation include the oxidation of LDL-cholesterol and the transendothelial migration of leucocytes to the intima underneath the endothelial layer of the arterial vessels. Other processes involved in atherogenesis include T-cell and monocyte-mediated inflammation reactions, macrophage foam cell formation and proliferation of smooth muscle cells (Dzau *et al.* 2002). There are several possible mechanisms of iron involvement in atherosclerosis (Fig. 6.4)

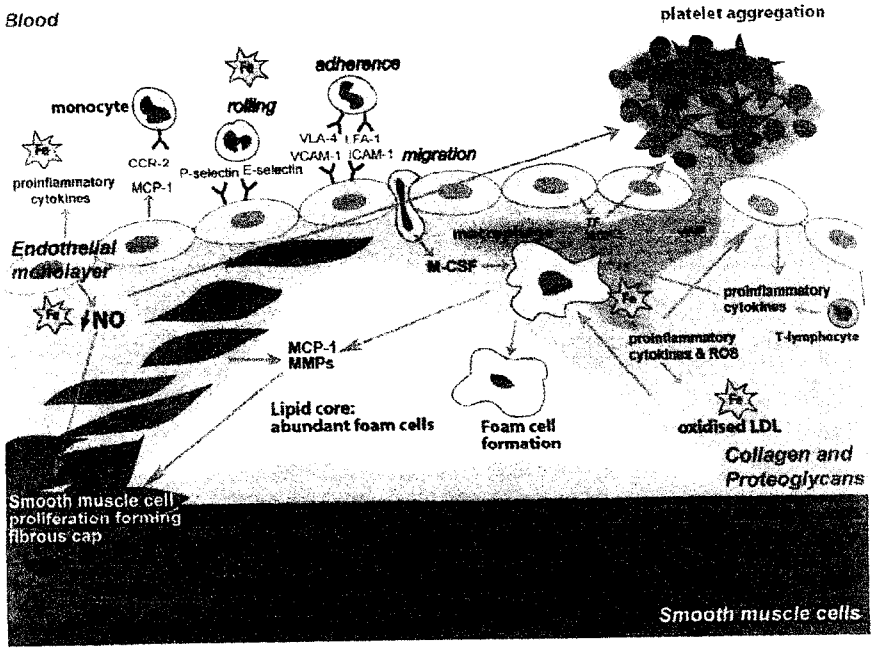

Fig. 6.4 Illustration of the cellular process and possible action of iron in the development of atherosclerosis. Fe = iron. P-selectin = platelet selectin. E-selectin = endothelial selectin. VLA-4 = very late activation antigen-4. VCAM-1 = vascular cell adhesion molecule-1. LFA-1 = lymphocyte function-associated antigen-1. ICAM-1 = intercellular adhesion molecule-1. MCP-1 = monocyte chemoattractant protein-1. CCR-2 = CC chemokine receptor-2. M-CSF = macrophage colony-stimulating factor. TF = tissue factor. vWF = von Willebrand factor. MMP = matrix metalloproteinases. NO = mononitrogen oxide. ROS = reactive oxygen species. LDL = low-density lipoprotein. These cellular processes may include: rolling, adherence and transendothelial migration of leucocytes, macrophage and T-cell mediated inflammation reaction, LDL oxidation, foam cell formation, decreased NO production, smooth muscle cell proliferation and platelet aggregation.

In vitro *studies*

The mechanism by which iron may stimulate atherogenesis is unclear. It is suggested that the catalytic role of iron in lipid peroxidation may influence the formation of atherosclerotic lesions. Iron-catalysed free radical formation may cause oxidation of LDL (Heinecke *et al.* 1984). The oxidised LDL is recognised by scavenger-receptors on macrophages, leading to accumulation of LDL in the cells. This is followed by the formation of foam cells, which are characteristic for the fatty-streak lesions of early atherosclerosis. Oxidised LDL also has chemotactic capacity providing recruitment of monocytes and macrophages to the site of lesions. The oxidised LDL also has cytotoxic capacity that induces changes in the endothelial cells with loss of endothelial integrity.

Transendothelial migration of leucocytes is also a fundamental inflammatory mechanism in atherogenesis (Gerrity 1981, Ross 1999). This process is partly

mediated by chemokines and the interaction between endothelial adhesion molecules and their ligands on monocytes (Meerschaert & Furie 1995; Navab *et al.* 1994; Shang & Issekutz 1998). Monocyte chemoattractant protein-1 (MCP-1) attracts monocytes bearing the chemokine receptor CCR-2 (Springer 1994). Several adhesion molecules have been shown to be present in human atherosclerotic plaques, including two members of the immunoglobulin superfamily of adhesion receptors, ICAM-1 (O'Brien *et al.* 1996; Printseva *et al.* 1992; van der Wal *et al.* 1992), VCAM-1 (O'Brien *et al.* 1993; O'Brien *et al.* 1996), as well as a member of the selectin family, E-Selectin (O'Brien *et al.* 1996; van der Wal *et al.* 1992). A significant correlation has been found between the degree of macrophage infiltration and endothelial ICAM-1, VCAM-1 and E-selectin expression in atherosclerotic lesions (O'Brien *et al.* 1996).

As a redox active metal, iron is capable of catalysing the formation of hydroxyl radicals in the Fenton reaction (Marx & van Asbeck 1996). Several antioxidants have been shown to protect against the endothelial dysfunction associated with atherosclerosis (Diaz *et al.* 1997). The oxygen radicals may involve in the regulation of nuclear factor KB (NF-$\kappa\beta$) DNA binding (Baldwin 2001), important for the transcription of a large number of genes, including the endothelial adhesion molecules (Collins *et al.* 1995; Neish *et al.* 1992).

The infiltration of leucocytes consists of consecutive adhesion-mediated events (Butcher 1991). The first step of adherence involves binding of selectins to carbohydrate ligands, which triggers tethering of the leucocytes to the activated endothelium along the vessel wall. After rolling and arrest, a firm adhesion of the leucocytes on activated endothelial cells may occur depending on the activation of the integrins including VLA-4 and LFA-1 (Adams & Shaw 1994; Luscinskas *et al.* 1994; Ross 1995; Springer 1994, 1995). Such activation may involve signalling initiated by inflammatory cytokines or signalling through binding of the integrins to their receptors (Adams & Lloyd 1997; Dedhar 1999; Ebnet *et al.* 1996; Ebnet & Vestweber 1999; Gahmberg 1997; Tuomainen *et al.* 1997a). Iron *in vitro* upregulates interleukin-6 (IL-6) production by endothelial cells (Visseren *et al.* 2002), while iron chelators inhibit the tumor necrosis factor-α (TNF-α mediated up regulation of endothelial adhesion molecules (Koo *et al.* 2003, Zhang & Frei 2003). Expression of IFN-κ-inducible genes in monocytic cells was affected by iron and iron-chelation (Oexle *et al.* 2003). Moreover, iron was shown to increase secretion of TNF-α (Lopez *et al.* 2003) and IL-1 (Szkaradkiewicz 1991) by monocytes.

In vivo *studies*

The *in vitro* experimental studies on generation of oxidised LDL by iron are supported by observation of the atherosclerotic lesions. The interior of advanced human atherosclerotic lesions is a highly pro-oxidant environment containing redox-active iron and copper ions which may induce lipid peroxidation (Smith *et al.* 1992). Ferritin was also found to be highly expressed in the atherosclerotic lesions (Pang *et al.* 1996). The iron is colocalised with ceroid, an insoluble complex of oxidised lipid and protein, extracellularly and also intracellularly in

the foam cells and smooth muscle cells (Lee *et al.* 1998). Iron deposits causing stimulation of macrophage infiltration to the atherosclerotic lesions and furthermore plaque rupture have recently been shown (Kolodgie *et al.* 2003). Further study by means of scanning and transmission electron microscopy revealed that erythrocytes containing haemoglobin were present in atherosclerotic lesions. Erythrophagocytosis by macrophages also occurred in the lesions (Lee *et al.* 1999a).

One study showed that patients with genetic haemochromatosis had significant eccentric hypertrophy of the radial artery, although none of them had arterial hypertension or evidence of cardiovascular diseases (Failla *et al.* 2000). The structural alteration leading to functional problems (stiffening), was largely reverted by iron depletion (Failla *et al.* 2000). Iron was also shown to induce early functional and structural vascular abnormalities due to endothelial dysfunction (Rooyakkers *et al.* 2002) which is associated with subsequent induction of oxidative stress. The radical species may also impair the mononitrogen oxide (NO) production, leading to the condition of arterial stiffness (Cheung *et al.* 2002). The vascular condition could be improved after administration of iron chelator (Duffy *et al.* 2001), which may indicate a reduced risk of cardiovascular events.

6.4.4 Animal studies

Several animal studies show the involvement of iron in the development of atherosclerosis and related cardiovascular diseases. Iron overload was shown to stimulate the formation of atherosclerotic lesions in hypercholesterolaemic rabbits (Araujo *et al.* 1995). Iron overload also increases the susceptibility of rat hearts to oxygen reperfusion damage (van der Kraaij *et al.* 1988; Voogd *et al.* 1992). Several studies showed a protective effect of iron chelators in the post-ischaemic cardiac injury period in animals, indicating that iron plays a role in reperfusion injury in tissues after ischaemeic insult (Badylak *et al.* 1987; Bolli *et al.* 1987; Reddy *et al.* 1989; van der Kraaij *et al.* 1988, 1989; Williams *et al.* 1991). Dietary iron restriction protected the apoE-deficient mice from developing the lesions (Lee *et al.* 1999b), and from having plaque rupture (Lee *et al.* 2003). Finally, iron chelation in experimental rabbits showed antiatherosclerotic effect by reducing plaque formation (Minqin *et al.* 2003).

6.5 Measuring iron toxicity

In the early course of iron overload, numerous homeostatic mechanisms prevent damage from accumulating iron. These include increased ferritin production needed to sequester the labile iron, and increment in individual antioxidants and/or antioxidant enzymes to protect against radical damage promoted by iron. However, these mechanisms might fail as more iron accumulates.

Measurement of iron toxicity is crucial for diagnosis and management of patients with iron overload from such disorders as hereditary haemochromatosis, thalassemia major, sickle cell disease, aplastic anaemia and myelodysplasia. Body iron can be measured by several parameters including serum ferritin concentration and transferrin saturation. The normal range of serum ferritin is 18–300 ng/ml. A decreased value of serum ferritin is associated with iron deficiency, while an increased value may indicate an increase of total body iron. However, it is also elevated in liver diseases, inflammatory conditions and malignant neoplasm. Another simple measure but insufficiently indicating total body iron (Beaton *et al.* 1989; Cook *et al.* 1976) is transferrin iron saturation, which is calculated as the concentration of serum iron divided by TIBC. Estimation of total body iron using this measure is less conclusive owing to high individual variation and strong influence of inflammation. The normal range of transferrin saturation is 15–55 per cent and the value increases in haemochromatosis. Furthermore, when moderate to severe iron overload is suspected, liver biopsy is necessary to be performed.

Magnetic resonance imaging (MRI) potentially provides the best available technique for examining the three-dimensional distribution of excess iron in the body; however, measurements and techniques must be calibrated for each individual machine. Biomagnetic susceptometry such as superconducting quantum interference device (SQUID) susceptometry (Brittenham *et al.* 1982) or, potentially, magnetic resonance susceptometry (Brittenham *et al.* 2001) provides the only noninvasive method to measure tissue iron stores that has been calibrated, validated and used in clinical studies, but the complexity, cost and technical demands of the liquid-helium-cooled superconducting instruments required have restricted clinical access to the method.

As previously mentioned, iron in the circulation is normally attached to transferrin. However in the case of iron-overload, NTBI is present (de Valk *et al.* 2000). Some species of NTBI may be safely bound to endogenous chelators; other species, however, may be catalytically active and capable of generating oxygen radicals, which is the major source of iron toxicity. This active NTBI is termed labile plasma iron (LPI) (Esposito *et al.* 2003). This iron species can also accumulate inside the cell and is termed labile iron pool (LIP). LIP may become catalytically active and is crucial for regulating the expression of many iron-related proteins. The findings mentioned above stress the need to identify the potentially toxic species of iron in both plasma and cells. Different means for small- and large-scale estimation of NTBI, LPI and LIP should be available with reliable and inexpensive methods to detect subjects at risk. Providentially, one of them is being developed (Breuer & Cabantchik 2001).

6.6 Methods of preventing iron damage

Screening for iron overload with biochemical methods and genotyping in patients who are suspected of having iron overload, such as type 2 diabetes

mellitus, atypical cardiac failure, early onset impotence in men and amenorrhea in women, early arthritis, high concentrations of liver enzymes, irritability, depression, joint pain and fatigue, is a recommended medical practice. Early detection of haemochromatosis is essential to prevent the potentially serious complications such as progression to severe organ damage. Currently, screening is not commonly done as part of routine medical care or check-ups, and many cases go undetected. A relatively inexpensive screening test for iron overload can actually be done by measuring the ferritin concentration together with the transferrin saturation from a blood sample.

Phlebotomy is the best solution for preventing iron damage in classical haemochromatosis. However, this treatment is not suitable for anaemia-related iron overload, and several other distinct forms of iron-overload. For these patients, iron damage can be prevented by administering an iron chelator. Desferoxamine (reviewed by Giardina & Grady 2001) commercially named Desferral®, is the only fully registered iron chelator that has been. However, the drug needs to be administered intravenously, is expensive and occasionally has some toxic side effects such as pain and swelling at the injection site, and rarely, impairment of vision and hearing or a general allergic or anaphylactic reaction. Patients may dislike wearing the pump, and fail to carry out the treatment. An alternative, safe, and effective oral iron chelator is urgently needed. An oral iron chelator, 1,2-dimethyl-3-hydroxypyrid-4-one (also known as L1, CP20 or Deferiprone) (Huehns *et al.* 1988), which is less expensive and relatively safe, has been recently recommended in Europe to be used in patients suffering from iron overload who cannot receive (too expensive) or cannot tolerate Desferral® (Kontoghiorghes *et al.* 2000). Experience with this drug has also been gained in Canada and India. Extensive research to develop safer and more effective oral iron chelators is in progress.

Furthermore, non-absorbable non-toxic oral iron chelators may also be beneficial to reduce iron absorption by the enterocytes of not only *HH* patients but also *HH* carriers. Additionally, since iron toxicity has been generally associated with a condition of free radical-mediated cell and tissue damage, the use of dietary antioxidants may help to protect against toxic effects of iron.

6.7 Conclusion and future trends

In summary, there is growing evidence for the role of iron in the development of atherosclerosis and related cardiovascular diseases. With all the possible confounding factors being taken into account, the epidemiological studies suggest a positive correlation between body iron level and this vascular disease. *In vitro* studies show several possible mechanisms in which iron may play a role in atherosclerosis. *In vivo* studies confirm the mechanisms of iron action in atherosclerotic plaque formation. The animal studies further validate the involvement of iron in this disease. Furthermore, elevated body iron level and NTBI have been observed in the carriers of hereditary haematochromatosis (de

Valk *et al.* 2000). The affected *HFE* gene has a prevalence of 10 per cent in Caucasian population. Together with the tendency of having iron overload in the modern world, many more people may thereby suffer from the risk of developing early cardiovascular disease.

Iron-deficiency anaemia remains a prevalent and debilitating illness in developing countries as well as in specific groups of the Western population. The disease mostly affects pregnant women and young children at levels of 79 per cent in South East Asia and 44 per cent in Sub-Saharan Africa, based on UN figures from the mid-1990s. In children, the illness may cause a permanent cognitive impairment. Identifying and caring for iron deficiency cases is certainly crucial for people especially from these regions.

As a consequence, iron supplementation is a common practice in many countries. WHO programmes also endorse this practice. Companies advertise their products, mentioning the benefits of iron fortification. However, these beneficial measures can have deleterious effects, especially in people with hereditary haemochromatosis and anaemias associated with iron overload.

There are many markers for iron deficiency, with serum ferritin and hypochromic red cell percentage currently the best markers available in clinical practice. Iron fortification is necessary in this easily diagnosed condition. Oral iron supplementation is inexpensive and safe, but poor patient compliance and reduced intestinal absorption may limit its efficacy. Intravenous iron, on the other hand, is effective, but it may have the potential of inducing iron overload.

The side effects of iron overload, such as infections, malignancies and vascular diseases, are well recognised. However, no guidelines exist for safe practice. A well-balanced diet containing a sufficient amount of iron is always necessary. In the Western world, however, the case of iron overload is increasing. Some dietary restriction would be useful to avoid iron accumulation in the body, for example by limiting the consumption of iron-rich foods such as liver, red meat and iron-fortified cereals, in subjects who are not prone to iron deficiency.

In the case of haemochromatosis, early screening has become cost effective, particularly for certain groups of people. Relatives, especially siblings, of patients with haemochromatosis should be tested for genes that indicate predisposition to the disease, allowing early treatment and prevention of the disease and tissue damage.

6.8 Sources of further information and advice

The sources mentioned below provide further relevant information. Two books provide more and up-to-date information on the process of atherosclerosis, the risk factors including nutrient iron, and some prevention advice.

1. *Immune mechanisms of atherogenesis*, Ming K. Heng and Madalene Heng (eds), Landes Bioscience; 1st edition (June 2003), ISBN: 1–58706–037–X,

2. *Atlas of atherosclerosis: risk factors and treatment*, Peter Wilson (ed.), Current Medicine; 3rd edition (June 2003), ISBN: 1573401870.

Another book explains more details of both primary and secondary haemochromatosis, the symptoms, screening strategies, available treatments and furthermore the complications including vascular problems.

3. *Hemochromatosis: genetics, pathophysiology, diagnosis and treatment*, James C. Barton and Corwin Q. Edwards (eds), University Press; 1st edition (March 2000), ISBN: 0521593808.

The last book provides more recent information on available iron chelators, especially for medical use.

4. *Iron chelators: new development strategies*, David G. Badman, Raymond J. Bergeron, Gary M. Brittenham (eds), Saratoga Publishing Group, Incorporated; (May 2000), ISBN: 1879894203.

Furthermore, we would like to introduce a website of a joined scientific project supported by the European Commission: http://www.nutrientirontoxicity.nl, which especially examines the deleterious effects of this essential iron nutrient, in different target organs.

6.9 Acknowledgement

Financial support is received from the European Commission, key action 'Food, Nutrition and Health', project QLRT-2001–0044.

6.10 References

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