CHAPTER 1

INTRODUCTION

1)   Body iron content and distribution

The human body contains about 50 mg Fe/kg body weight in males and 40 mg Fe/kg body weight in females. This iron is distributed between various compartments in the human body (1). The erythroid marrow (developing red blood cells) and circulating red blood cells contain about 32 mg Fe/kg body weight in males and 28 mg Fe/kg body weight in females as functional iron in haemoglobin. The remainder of functional iron is found as myoglobin mainly in muscle, 5 mg Fe/kg body weight in males and 4 mg Fe/kg body weight in females, and as iron-containing and iron-dependent enzymes throughout cells in the body, 1-2 mg Fe/kg body weight. Storage iron is contained in hepatocytes and in macrophages of the monocyte-macrophage system found in the liver, bone marrow, spleen and muscle. This comprises about 10-12 mg Fe/kg body weight in males and 5-6 mg Fe/kg body weight in females.

About 1 mg of iron is absorbed daily in the gastrointestinal tract. Gastrointestinal iron absorption from the gut lumen into the circulation occurs in three phases: 1) uptake of iron across the apical membrane of enterocytes, 2) intracellular translocation of iron and 3) transfer of iron across the basolateral membrane into the circulation (2). Uptake across the apical membrane (membrane facing the lumen) involves binding of inorganic iron (non-haem iron) or haem-iron to specific proteins (transporters) on the membrane. The major transporter involved in cellular inorganic iron uptake is the divalent metal

transporter – DMT1. DMT1 exclusively transports divalent metals (Fe^{2+}) and when inorganic iron in the diet is present as the Fe^{3+}-form, it necessitates the luminal conversion of Fe^{3+} to Fe^{2+} (2, 3, 4). This conversion of Fe^{3+} to Fe^{2+} in the lumen is achieved by an intestinal iron reductase – duodenal cytochrome B – Dcytb. Fe^{2+} is taken up by DMT1 into the cytoplasm of the enterocyte. Absorption of haem-iron occurs by means of a different transporter, an enterocyte haem importer, haem carrier protein 1 – HCP1. Once haem-iron has entered the enterocyte, it is likely cleaved by intracellular haem oxygenase 1 to release iron. Before the beginning of the third phase of iron absorption the absorbed and liberated Fe^{2+} joins the intracellular labile iron pool. From this intracellular labile iron pool, Fe^{2+} can either be stored in the iron storage protein ferritin or translocated to the basolateral membrane for transport into the circulation (2, 3). For transport into the circulation, Fe^{2+} binds to the basolateral membrane iron exporter protein – ferroportin. Upon reaching the extracellular face of the basolateral membrane of the enterocyte, Fe^{2+} is oxidized to Fe^{3+} by a membrane-bound multi-copper oxidase – hephaestin (3). The oxidation of Fe^{2+} to Fe^{3+} facilitates its transfer to the plasma iron transport protein transferrin (2, 3).

Iron circulates between the iron-containing compartments (intercellular iron shuttling) bound to the plasma iron transport protein transferrin. About 3 mg of iron is found in association with transferrin at any one time and the daily exchange of iron by plasma transferrin is about 30 mg. This transport iron amounts to about 0.2 mg Fe/kg body weight. Transferrin has two high-affinity binding sites for iron. Diferric transferrin, i.e., transferrin containing Fe^{3+} bound to both the iron binding sites, binds to a highly specific transferrin receptor on the cell membranes of cells allowing cellular uptake by receptor-mediated endocytosis. Iron is chelated by transferrin at the cell membrane of the cell releasing iron (such as the enterocyte) and the transferrin is subsequently bound by a transferrin receptor on the membrane of the cell accepting the iron (such as a developing

red blood cell). The receptors collect in clathrin-coated pits and facilitate transferrin internalisation into endocytic vesicles. The endosomes then become acidified. When the endosome reaches pH 5.5 protein conformation changes take place and thus result in the dissociation of Fe^{3+} from transferrin. Fe^{3+} is reduced to Fe^{2+} most likely by the endosomal reductase – Steap3. The formed Fe^{2+} is then transported from the endosome to the cytoplasm by the transporter DMT1. The transferrin-uptake cycle is completed when the endosome returns to, and fuses with the plasma membrane, returning apotransferrin (transferrin containing no iron) to the circulation and transferrin receptor to the plasma membrane. This allows both molecules to participate in another transferrin-uptake cycle (2, 3).

Cells contain a pool of chelatable or transit iron (Fe^{2+}) known as the labile iron pool bound to low molecular weight intracellular iron transport compounds, and it is thought that the endosomal iron released from transferrin transits this pool of iron. From this labile iron pool, Fe^{2+} can enter functional compartments or can be stored in ferritin (3). Since the labile pool of iron contains the metabolically and catalytically reactive iron, the magnitude of the labile pool of iron is maintained by sophisticated control mechanisms that regulate cellular iron uptake and storage in a coordinated manner. Storage of iron in association with ferritin is important since this protects cells against the deleterious effects of iron. In the labile iron pool, iron exists in the highly toxic Fe^{2+}-form. In this form iron can catalyse the production of harmful oxygen radicals in the Haber-Weiss reaction resulting in peroxidative damage to cellular structures. Whereas, in association with ferritin, iron is present as a soluble, nontoxic ferrihydrite mineral within the confines of the ferritin molecule. In order to ensure that the labile iron pool is kept within physiological ranges, the expression of the proteins involved in iron homeostasis such as transferrin, transferrin receptor and ferritin, is coordinately regulated. An increase in the intracellular free iron will, for instance, result in up-regulation of ferritin (increase in

efficiency of iron storage) and down-regulation of transferrin and transferrin receptor expression (decrease in iron uptake), whereas a decrease in intracellular free iron will result in the opposite. This coordinated, but divergent regulation of the expression of these proteins is governed by a single protein namely the iron-responsive protein (IRP), which can bind to iron-responsive elements (IREs) on the mRNAs of the iron homeostasis proteins. This protein is sensitive to intracellular iron levels and responds to these levels by regulation, at a posttranscriptional level, of the expression of the mRNA of the proteins responsible for iron uptake and storage. The presence of IREs in DMT1 and ferroportin mRNA suggests that their expression may also be controlled, at least in part, by the cellular iron content (5).

Red blood cell production and destruction is fairly dominant in iron metabolism. About two thirds of the iron in the human body is contained as functional iron in the haemoglobin of the red blood cells. Haemoglobin is responsible for binding oxygen and delivering it to the tissues and consists of a haem molecule, i.e., a protoporphyrin ring containing Fe^{2+}, as well as four globin protein chains, i.e., two α-chains and two β-chains. Haemoglobin production in developing red blood cells is a complex process requiring meticulous coordination of iron acquisition, protoporphyrin biosynthesis, and globin protein production (1). Iron is acquired by developing red blood cells through binding of transferrin to the transferrin receptor. Two to three million red blood cells are produced every second, therefore sufficient amounts of iron are required to enable the developing red blood cells to synthesise haemoglobin. In order for the bone marrow to supply this amount of new red blood cells, 30-40 mg of iron is needed on a daily basis by the erythroid (red blood cell forming) marrow. Since only 1 mg of iron is absorbed daily from the intestinal tract, iron from dying red blood cells is recycled (6).

The life-span of a circulating red blood cell is about 120 days. At this time these dying red blood cells will be taken up by macrophages of the monocyte-macrophage system present in the spleen, bone marrow and liver for recovery of their iron. The recovery of iron from these dying red blood cells provides most of the iron utilized by developing red blood cells for haemoglobin synthesis. After the red blood cell has been internalized into an acidic phagosome and most of the cellular constituents broken down, an enzyme, haem oxygenase, liberates iron from haem. The iron is transferred out of the phagosome by DMT1 into the cytoplasm where it will join the intracellular labile iron pool. From this intracellular iron pool, the iron will be either incorporated into ferritin for cellular storage or returned to the plasma. The movement of iron from the cell into the plasma is mediated by the plasma membrane transport protein – ferroportin. Iron will traverse the plasma membrane as Fe^{2+}. Upon reaching the extracellular side of the plasma membrane Fe^{2+} is oxidized to Fe^{3+} by caeruloplasmin. Caeruloplasmin is a serum multicopper oxidase, which facilitates movement of iron out of tissue stores and into transferrin by this oxidation process, similar to the protein hephaestin at the basolateral membrane of the enterocyte (3).

Various changes in body iron needs can bring about the modulation of iron homeostatic mechanisms in order to change plasma iron concentrations. Factors that can result in an increase in plasma iron concentrations include an increase in red blood cell production, hypoxia and iron deficiency. In contrast with these factors, diminished red blood cell production, iron overload and inflammation can bring about a decrease in the concentration of plasma iron. The molecule most likely responsible for bringing about these changes in iron homeostatic processes, is the liver-produced hepcidin. Hepcidin levels are increased in response to increased plasma iron, iron overload and inflammation and diminished in response to increased red blood cell production, hypoxia and iron deficiency (7, 8). Hepcidin binds to ferroportin and brings about the internalization and

degradation of ferroportin. This will result in less ferroportin available at the basolateral membrane of the enterocyte and the plasma membrane of the hepatocyte and macrophage for iron export into the circulation. In the enterocyte, this hepcidin-dependent internalization and degradation of ferroportin will reduce the dietary iron absorption, whereas in the macrophage hepcidin activity will attenuate cellular iron release. A decrease in iron transport into the circulation from the enterocyte will result in the accumulation of iron as ferritin in the enterocyte. Since enterocytes are viable for only one to two days, iron that accumulates within them is lost from the body when these senescent enterocytes are shed into the gut lumen (2, 3).

Ferritin is the major protein responsible for the sequestration, storage and release of intracellular iron. Ferritin can exist as different isoferritins. Various factors seem to play a role in the precise composition of a ferritin molecule and thus in its functioning as an iron storage protein.

2)   Ferritin and ferritin isoforms:

Structure-function relationships, synthesis, degradation and secretion

Ferritin is the major intracellular protein involved in the storage and release of intracellular iron and since iron is needed in various cellular functions it does not come as a surprise that ferritin is expressed in every cell type thus far studied (9). Iron is essential for cellular functions such as oxygen transport, electron transfer, nitrogen fixation, DNA synthesis and the production of haemoproteins like haemoglobin and myoglobin (10). However, not all cell types harness iron for the same purposes or to the same extent and therefore the role of ferritin in the management of intracellular iron differs between the various types of cells. Ferritin can play a role in specialized functions, e.g. recycling of iron in macrophages and short- and long-term storage of iron as in hepatocytes, as well as in intracellular housekeeping functions where it provides a reserve for cytochromes,

nitrogenases, ribonucleotide reductases, haemoglobin and myoglobin (11). Perhaps equally important to its function in the storage and release of iron is the role that ferritin plays in the protection of cells against the deleterious effects of iron. Iron exists in two readily interconvertible redox states and, at physiological pH and oxygen tension, Fe^{2+} is readily oxidized to Fe^{3+}, followed by the hydrolysis of the Fe^{3+}-containing compounds and the formation of insoluble ferric hydroxide and oxyhydroxide polymers (10, 12). In addition, Fe^{2+} can catalyse the production of harmful oxygen radicals in the Haber-Weiss reaction resulting in peroxidative damage to cellular structures (10, 13). Within cells, iron probably exists in a low molecular weight, redox-active form only for short periods – mainly within the lysosomes. Lysosomes are therefore particularly vulnerable to oxidative stress and may burst due to intralysosomal Fenton-type chemistry with ensuing peroxidative destabilization of lysosomal membranes. This could result in leaky lysosomes and the induction of cellular damage, or even apoptotic or necrotic death due to the release of a range of powerful hydrolytic enzymes into the cytosol (14). By sequestering large amounts of iron as a soluble, non-toxic ferrihydrite mineral within the confines of the ferritin molecule, ferritin protects the cell against insoluble ferric oxide and oxyhydroxide formation, as well as against the production of oxygen radicals (15, 16).

Ferritin is widely distributed in cells and is found in the cytosol, nucleus, mitochondria and lysosomes and appears in plasma as a result of cellular secretion. This specific distribution enables ferritin to supply the particular enzymes and other proteins with appropriate amounts of iron, and equally important, places ferritin in close proximity to sites where large amounts of iron are metabolized. Ferritin is actively transported into the nucleus to provide, amongst others, ribonucleotide reductase with iron and to protect DNA from oxidative damage as a result of inappropriate oxygen radical production due to surplus amounts of iron (17). A specific type of ferritin is also found in mitochondria.

Mitochondria are confronted with large amounts of metabolically active iron and although most cell types contain only very small amounts of a mitochondrial ferritin, in certain conditions, a specific ferritin is translocated to the mitochondrium to sequester unwanted amounts of iron (18). The regulation of ferritin distribution in different parts of the cytosol and in cellular organelles is controlled, in part, by the various mechanisms involved in the uptake of ferritin by the cellular organelles. Furthermore, ferritin binds to microtubules and this interaction can not only contribute to transport of ferritin to specific sites and organelles within the cell, but also to secretion of ferritin from the cell. Depolymerization of microtubules increase ferritin secretion and support the probable role of microtubules in regulating the intracellular concentration, distribution and release of ferritin under different physiological circumstances (19).

2.1) Structure of ferritin

Ferritin, like some of the other proteins involved in the regulation of iron homeostasis in the body, including transferrin and lactoferrin, can serve either as an iron donor or iron acceptor (20). However, unlike transferrin and lactoferrin, ferritin is capable of accepting, storing and donating vast amounts of iron. These abilities of ferritin are all a function of the structure of ferritin – especially that of the ferritin protein shell. Ferritin is a protein with a molecular weight of 450 000 daltons (11) and consists of an outer three-dimensional protein shell enclosing an 80 Å diameter inner cavity (21). In this inner cavity ferritin is capable of sequestering variable amounts of Fe^{3+}-atoms as a ferrihydrite mineral. When fully saturated, ferritin can store up to 4500 Fe^{3+}-atoms, but the usual amount is closer to 2000 Fe^{3+}-atoms (15, 21).

2.1.1) Structure of the ferritin protein shell

The outer three-dimensional protein shell contains a total of 24 protein subunits arranged symmetrically (15, 21). Two types of protein subunits exist, the H-subunit and

the L-subunit. The H-subunit (21 kDa) contains 178 amino acids while the L-subunit (19 kDa) contains 174 amino acids (9, 10). Each subunit is folded into 4 long α-helices (A, B, C, D), with a long loop between C and D, and a fifth short helix (E) at the C-terminal (21). Each subunit is roughly cylindrical (5.5 nm long and 2.7 nm wide) (9). For the L-subunit the amino acid arrangement into the various α-helices is as follows: long α-helix A (amino acid residues 10-39), long α-helix B (amino acid residues 45-72), long α-helix C (amino acid residues 92-120), long α-helix D (amino acid residues 124-155) and short α-helix E (amino acid residues 160-169) (15). The 4 long α-helices are aligned parallel to one another and are tightly packed into a cylindrical subunit bundle (9, 15), thus forming the main subunit axis. Between the 4 long α-helices the inter-helical contact region extends over a length of 35 Å (15), while the longest helix, i.e., the α-helix D, protrudes beyond this main inter-helical contact region and folds sharply back so that the α-helix E lies at an angle roughly 60 degrees to the main axis of the subunit (15, 21). The other feature of the subunit, that is, the long loop L (amino acid residues 73-91), joins the C-terminus of α-helix B to the N-terminus of α-helix C (15). Due to the inter-helical contacts and the arrangement of the subunit in the protein shell, the α-helices B and D have one face towards the inside of the ferritin protein shell, the α-helices A and C have one face each towards the outside of the shell, and the loop L is displayed on the outermost surface of the ferritin protein shell (15). This arrangement determines the types of amino acids situated on the inner face and the outer face of the subunit and therefore the subsequent intra-subunit and inter-subunit interactions. The secondary structure of the H-subunit is very similar to that of the L-subunit, despite the fact that they share only 55% amino acid sequence homology (9).

2.1.1.1) Intra-subunit and inter-subunit amino acid side-chain interactions of the ferritin protein shell

Highly conserved amino acid residues are involved in intra-subunit and inter-subunit interactions (9, 11, 22). The different types of intra-subunit and inter-subunit amino acid side-chain interactions that are important for ferritin folding and stability include hydrogen bonds, salt-bridges and hydrophobic interactions (11). The contributions of these types of interactions to the folding and stability of the H- and L-subunits differ significantly between the two types of subunits. The intra-subunit hydrogen bonds are about 50% more abundant in the H-subunit than in the L-subunit, whereas the salt bridges are more important in the stabilization of the L-subunit and accounts for about 30% of the stabilization energy of the L-subunit (23). Due to the large number of intra- and inter-subunit salt bridges the ferritin molecule is highly stable to thermal and chemical denaturation (21). In addition, the differences in intra-subunit interactions between the H-subunit and L-subunit result in a linear increase in the resistance to denaturation, with L-subunit homopolymers and heteropolymers containing a high L-subunit proportion significantly more resistant than H-subunit homopolymers (23, 24). One important difference in intra-chain interactions that contribute to the L-subunits being more stable than the H-subunits is the salt-bridge lysine 62 – glutamic acid 107 in the L-subunit which replaces the ferroxidase center of the H-subunit (24). However, these differences in intra-chain interactions between the H-subunit and L-subunit appear to be largely masked by the presence of strong inter-subunit contacts in assembled molecules consisting of a combination of H- and L-subunits (23). The interactions between subunits responsible for ferritin assembly involve about 50% of the subunit surface and most of the inter-subunit contacts are conserved in H- and L-subunits. The first inter-subunit interaction to take place in the assemblage of the ferritin protein shell is the formation of interactions along the main subunit axis between two subunits creating a dimer pair (11). One face of the subunit contains hydrophobic residues from

α-helix A (valine 20, leucine 24, tyrosine 28, leucine 31) and loop L (phenylalanine 78, leucine 81, proline 84). By interaction with an equivalent region of a second subunit this hydrophobic patch of some 22 Å in length is buried from solvent (15). Another hydrophobic region on the subunit surface is that comprising one face of α-helix E (leucine 154, leucine 161, tyrosine 164, leucine 165, leucine 169) that will also be buried from solvent upon subunit interactions. There are a large number of inter-subunit interactions that form regions of marked hydrophobicity and other regions where polar interactions predominate (15). If these hydrophobic residues are to be partially buried from solvent then further assembly of dimers must occur. Since the assembly of ferritin consisting of 24 subunits results in the complete concealment of all hydrophobic patches (15), the formation of such a molecule is very favourable.

2.1.1.2) Channels present in the ferritin protein shell

For either mineralization or demineralization of the iron core to occur it is important that substances such as Fe^{2+}/Fe^{3+}, oxidants, reductants and chelators can gain access to the interior of the ferritin molecule. In order for these molecules to gain entrance to the interior of the protein shell the ferritin protein shell contains two main types of channels with strikingly different physical properties. The first type of channel comprises six hydrophobic channels with fourfold symmetry (12 Å long and 3-4 Å wide), which are lined by 12 leucine side-chains in the L-subunit and 8 leucine plus 4 histidine side-chains in the H-subunit belonging to the α-helix D. The second type of channel includes 8 hydrophilic channels with threefold symmetry (3-4 Å wide), each lined by 6 carboxyl groups, 3 aspartate residues (on the cavity side of the shell) and 3 glutamate residues (towards the outside of the molecule) belonging to the α-helix E (15, 21, 25, 26). The carboxylate groups of the hydrophilic threefold channels are essential for rapid iron transport across the protein shell (27). The hydrophobic character of the 6 hydrophobic channels argues against a possible role for these channels in the transport of Fe^{2+}/Fe^{3+}-

ions into the interior of the ferritin molecule. These fourfold hydrophobic channels are found to be impermeable to all cations with the possible exception of protons. It is suggested that these fourfold channels facilitate proton transfer in and out of ferritin in order to maintain electroneutrality during iron deposition (28). However, the substitution of leucine for histidine in the H-subunit may confer iron transfer properties to the hydrophobic channel of the H-subunit since histidine has a strong affinity for iron (22). The hydrophilic channels are the most likely routes of iron entry into the protein shell and are probably functional in both the H-subunit and the L-subunit. This is indicated by a high degree of conservation of the three glutamates and three aspartates in both subunits (29). Furthermore, alteration of residues of the hydrophobic channels has little effect on the rate and specificity of the reaction whereas modifications of the carboxyl groups lining the hydrophilic channels reduces the rate of iron uptake by about 2-fold (30). The hydrophilic channel is funnel-shaped, broadening out towards the outside surface to give a wide hydrophilic region (15, 30). The hydrophilic regions at the outside surface of the hydrophilic channels contain negative charges surrounded by patches of positive charges creating electrostatic fields in order to direct Fe^{2+}-ions toward the channel entrance (29). Once iron has been directed to the opening of the hydrophilic channel, it is bound to hydrophilic residues located at the outer and inner openings of the channels (31). These residues include cysteine 130 and histidine 118, both of which face the outer opening of the hydrophilic channel (31), and aspartic acid 131 and glutamic acid 134 in the narrowest part of the channel (11, 30). Aside from the hydrophobic and hydrophilic channels present in the ferritin protein shell, a H-subunit specific channel exists that connects the ferroxidase site in the center of the H-subunit to the outer protein surface (22). Although, transfer of iron through this channel is in general less efficient, the transfer of Fe^{3+}-ions to the outside of ferritin may become important in certain conditions.

2.1.1.3) The ferroxidase catalytic center of the H-subunit of the ferritin protein shell

The first step in the iron sequestration process by ferritin involves the oxidation of Fe^{2+} to Fe^{3+} by oxygen and is facilitated by the ferroxidase center contained in the H-subunit (31). This ferroxidase center comprises various amino acid side-chains as important iron ligands in the multi-step oxidation of Fe^{2+} to Fe^{3+} and includes a cluster of hydrophilic amino acid residues (glutamic acid 27, glutamic acid 61, glutamic acid 62, histidine 65 and glutamic acid 107) which are embedded within each of the 4 α-helix bundles comprising the subunit (24, 32, 33). This ferroxidase center is present in the H-subunit, but absent in the L-subunit (24). The L-subunit's potential ferroxidase activity is lost (32) due to amino acid changes including glutamic acid 62 to lysine and histidine 65 to glycine, formation of a salt-bridge between lysine 62 and glutamic acid 107 (34) and swinging of glutamic acid 61 into a position facing the cavity (31).

2.1.1.4) The nucleation site of the L-subunit on the inner iron/protein interface of the ferritin protein shell

The second step in the iron sequestration process by ferritin involves the formation of Fe^{3+}-nuclei and the subsequent growth of an iron-core. In order for ferritin to support Fe^{3+}-nuclei formation and the growth of the iron mineral, ferritin must contain the Fe^{3+}-atoms in the inner cavity of the protein shell and stabilize subsequently incoming Fe^{3+}-atoms on the growing iron-core. This is accomplished by amino acid side-chain ligands present on the inner surface of the ferritin protein shell. Binding of Fe^{3+} to these ligands supports Fe^{3+}-nuclei formation, as well as the subsequent growth of the iron-core. This results in the contact of the ferritin protein shell with the iron core at several points on the inner surface, forming an iron/protein interface. These iron/protein interfaces, which define the sites of core nucleation, probably excist where the protein subunit dimers interact (11). In contrast to the first step in iron sequestration, i.e., oxidation of

iron, which is accomplished mainly by the H-subunits, this second step, i.e., Fe^{3+}-nuclei formation and the growth of the iron-core is mainly a function of the L-subunits, which supply the appropriate amino acid side-chains. These amino acid side-chains act as ligands for the initially formed Fe^{3+} or as negatively charged domains that lower the activation energy of iron-core formation (24). The reason that the H-subunits are less efficient in nucleation and iron-core formation is that only 7 of the hydrophilic amino acid side-chains in L-subunits that line the inner surface, and which are not involved in the formation of salt bridges or hydrogen bonds, but thought to bind iron, are conserved in H-subunits. Amino acids important in nuclei formation and growth of the iron-core include histidine 49, arginine 52, glutamic acid 53, glutamic acid 56, glutamic acid 57, arginine 59, glutamic acid 60, glutamic acid 61, arginine 64 and lysine 67 on α-helix B, with glutamic acid 136, lysine 139 and lysine 142 on α-helix D, as well as 3 residues at the C-terminus, i.e., lysine 172, histidine 173 and aspartic acid 174. The differences in amino acid side-chains between H- and L-subunits result in the loss of negative charges in the H-subunit on the inner iron/protein interface so that H-subunits have a lower ability to nucleate iron (15, 22, 24). A cluster of three L-subunit glutamates has specifically been implicated in Fe^{3+}-nuclei formation. This cluster of glutamates, i.e., glutamic acid 57, glutamic acid 60, and glutamic acid 61, form a region of negativity for the binding of Fe^{3+}. In the H-subunit glutamic acid 57 and glutamic acid 60 are substituted for histidine and glutamic acid 61 is involved in the ferroxidase center of the H-subunit. It is suggested that glutamic acid 61 could also play a role in the nucleation of Fe^{3+} after oxidation of Fe^{2+}. However, this putative nucleation site, involving glutamic acid 61, glutamic acid 64 and glutamic acid 67, does not play a role in nucleation and growth of the iron-core (35). In the L-subunit glutamic acid 61 is swung into a position on the inner cavity surface in proximity to glutamic acid 57 and glutamic acid 60 to participate in this cluster of negative charges responsible for the greater efficiency of L-subunits in iron-core nucleation (32, 36).

2.1.2) The iron mineral

The iron mineral is enclosed in a cavity by a protein shell and contains different types of environments in which the Fe^{3+}-ion is located. These different types of environments include:

1)   iron atoms located at nucleation sites – these Fe^{3+}-ions are coordinated by amino acid side chain ligands from the inner protein surface and inorganic bonds from the mineral surface (13).

2)   iron atoms at the surface of the mineral – these Fe^{3+}-ions are connected to the bulk mineral, as well as to the Fe^{3+}-ions within the nucleation sites in the protein shell through inorganic oxide/hydroxide linkages and there may be additional linkages to the Fe^{3+}-ions of the shell through dinucleating amino acid side-chain ligands (13).

3)   iron atoms which are in environments corresponding to those of the bulk mineral connected through inorganic oxide/hydroxide linkages, where the Fe^{3+}-ions are located in the interstices between two hexagonally closely packed layers of oxygen (37, 38). The Fe^{3+}-ions appear to be in predominantly octahedral environments but up to one-third of the Fe^{3+}-ions are in tetrahedral sites, with an average of six oxygen atoms at a distance of approximately 2 Å. This, more or less, corresponds to the structure for ferrihydrite (13, 15, 29).

The iron core may differ between ferritin molecules, as well as within the same ferritin molecule, and can contain single or multiple crystallites and amorphous regions (29). One or more of such electron-dense crystallites are anchored to the inner surface of the protein shell (37). The average diameter of the iron cores range from 2.5-9 nm (29). The formation of a single crystallite approaching the inner diameter of the protein shell or the formation of various smaller crystallites depends on the availability of Fe^{2+}-ions/Fe^{3+}-ions during the later phases of mineral growth after nucleation has occurred (11). Striking features of the iron cores are their variable phosphate contents (21, 29).

Phosphate ions are found on the surface of the iron cores where they replace some of the surface hydroxyl groups (15). However, recent observations indicate that phosphate can also be found throughout the iron core, that cores can have ordered and disordered regions, and that the disorder increases when phosphate increases (11). *In vivo* the phosphate contents of ferritin iron cores appear to be in dynamic equilibrium with cell phosphate (15) and it has long been suggested that phosphate plays a role in iron homeostasis (13). This assumption has recently been supported by the fact that phosphate can stimulate the rate of iron uptake by providing binding sites on the mineral surface for incoming iron atoms and as such may play a role in the oxidation of Fe^{2+} on the mineral surface (39).

2.2) Mechanism of iron sequestration and release: The role of the ferritin protein shell in iron mineralization and demineralization

Ferritin concentrates iron in cells by directing the formation of a ferrihydrite mineral in the hollow cavity enclosed by the ferritin protein shell. This results in effective cellular iron concentrations of more than 10^{11} times the solubility of the Fe^{3+}-ion (40). In times of iron need ferritin releases iron by demineralization of the iron core. The exact steps involved in iron release are not completely known, but iron release involves the reduction of Fe^{3+} to Fe^{2+}. The rates of the processes involved in iron mineralization and demineralization are controlled by the protein shell (11) – this by influencing the local pH and redox potentials (13). The formation of the ferrihydrite mineral by ferritin is a multi-step process governed by the protein shell. Iron enters the ferritin cavity by passage through the channels situated in the protein shell facilitated by the presence of local iron binding sites (21). Upon entering the protein shell the iron is oxidized followed by hydrolysis, nucleation and iron core growth (15). Each of these steps contributes to the formation of a ferrihydrite mineral core from soluble Fe^{2+}-ions.

2.2.1) Oxidation of iron

2.2.1.1) Oxidation of Fe^{2+} by the ferroxidase center of the H-subunit

Oxidation of Fe^{2+} is an obligatory first step in order for an iron atom to finally be deposited in the cavity of ferritin. The H-subunit's ferroxidase center, formed by various amino acid side-chains, enzymatically oxidizes Fe^{2+} to Fe^{3+}. Enzymatic oxidation of Fe^{2+} by the ferroxidase center results in rates of iron oxidation several-fold faster than that which would occur during auto-oxidation of iron. This faster rate of iron oxidation results from the proper placement of Fe^{2+} atoms by the ligands of the ferroxidase center of the H-subunit for subsequent oxidation by O_2 (41). X-ray analysis has revealed three iron-binding sites per H-subunit. Sites A and B (3.8 Å apart) form a di-iron site and include a common bridging carboxylic acid residue. Ligands of site A also include one equivalent histidine and one glutamate glutamic acid 27, whereas site B has two carboxylate ligands in addition to the bridging carboxylate. These are glutamic acid 107 and glutamic acid 61. The third site, i.e., site C lies in the inner surface of the protein shell at a distance of 7 Å from the di-iron site. The first step in Fe^{2+} oxidation involves the binding of incoming Fe^{2+} atoms with each of sites A and B, followed by the formation of a μ-oxo-bridge (42). The affinity of site A for Fe^{2+} is higher than the affinity of site B, resulting in the occupation of site A by Fe^{2+} before site B. Binding of Fe^{2+} to site B follows O_2 binding and/or oxidation of the first Fe^{2+} (27). After about an hour of intermediate μ-oxo-bridged dimer formation, this complex splits into highly mobile Fe^{3+}-monomers that can move to the cavity for hydrolysis of the Fe^{3+}-compounds, nucleation and growth of the iron-core (33, 41). An unusually short distance of 2.53 Å between the two Fe^{2+} ions suggests the presence of a unique triply bridged structure requiring a small Fe-O-O angle. This geometry should favour decay of the peroxodiferric complex by the release of μ-oxo or μ-hydroxo diferric mineral precursors (43, 44). This would result in freeing of the ferroxidase sites for binding of additional Fe^{2+} and the start of another round of Fe^{2+} oxidation (31). A by-product

produced during the ferroxidase center oxidation of Fe^{2+} is H_2O_2, which can result in the subsequent production of Fenton chemistry-derived radicals (31).

2.2.1.2) Oxidation of Fe^{2+} on the surface of the growing iron core

During the initial stages of ferritin iron core mineralization, oxidation of Fe^{2+} takes place in the ferroxidase centers of the H-subunits of the ferritin protein shell. Once the mineral attains a certain critical size, oxidation of Fe^{2+} can additionally, and perhaps preferentially, occur on the surface of the growing iron core (13, 15, 31). Therefore, the main function of the ferroxidase center may be oxidation of sufficient iron from Fe^{2+} to Fe^{3+} for the initial nucleation events, and once these nuclei attained a sufficient size for oxidation to take place on the mineral surface, the H-subunit's role as a ferroxidase is superseded by oxidation on the mineral surface (34, 42).
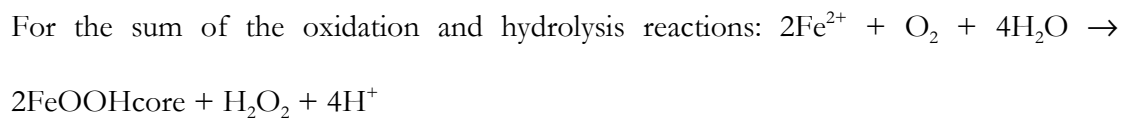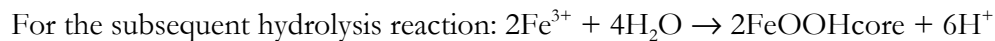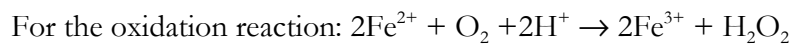
2.2.2) Hydrolysis and nucleation of the formed Fe^{3+}-compound

The subsequent hydrolysis and nucleation of the generated Fe^{3+}-compound is governed by the amino acid side-chains of the L-subunit (31). The highly mobile Fe^{3+}-monomers are directed to the inner cavity and properly placed on the protein shell/mineral interface by these ligands. The subsequent hydrolysis and nucleation involves the hydrolysis and aggregation of Fe^{3+}-ions to form nuclei containing perhaps as few as four or five constituent Fe^{3+}-ions resulting in the production of iron oxyhydroxides and oxides (13, 29). Hydrolysis and nucleation of the Fe^{3+}-compound is favoured since binding of Fe^{3+} to the ligands lowers the activation energy of nucleation (13, 21). Such nuclei can become the solid phase once some critical nucleus size has been reached and will sustain iron mineral growth from these sites (13). These initial sites of nuclei formation on the inner surface of the protein shell, involving specific amino acid side-chains, will maintain contact with the iron core (13). This interaction with the iron core seems to be a key factor in the stabilization of the crystal by providing a neutral container, or more likely,
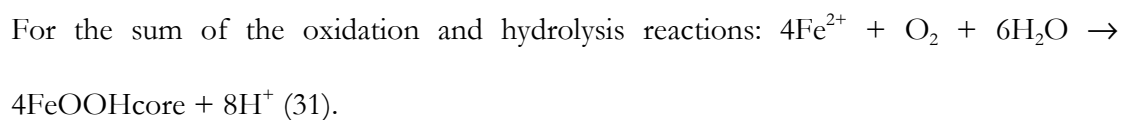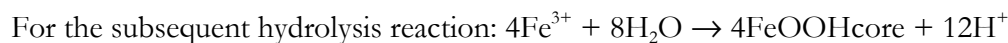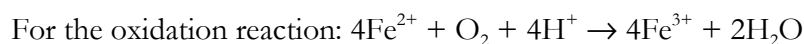
the interactions with negatively charged and neutral amino acid side-chains such as carboxylates, alkoxides, phenolates and imidazoles provide the necessary charge compensation (13).

2.2.3) Different iron oxidation kinetics and the formation of different reaction products by the ferroxidase center oxidation of iron and oxidation of iron on the mineral surface

Oxidation of Fe^{2+} by the ferroxidase center and oxidation of Fe^{2+} on the mineral surface take place under different conditions. These processes have different iron oxidation kinetics and result in the formation of different reaction products (36). At low Fe^{2+} concentrations (<50 Fe^{2+}-atoms/ferritin molecule) oxidation of Fe^{2+} is catalysed by the ferroxidase center and the reaction stoichiometry is as follows:

For the oxidation reaction: $2Fe^{2+} + O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O_2$

For the subsequent hydrolysis reaction: $2Fe^{3+} + 4H_2O \rightarrow 2FeOOHcore + 6H^+$

For the sum of the oxidation and hydrolysis reactions: $2Fe^{2+} + O_2 + 4H_2O \rightarrow 2FeOOHcore + H_2O_2 + 4H^+$

In the presence of higher Fe^{2+} concentrations (>250 Fe^{2+}-atoms/ferritin molecule) the H-subunit ferroxidase site becomes kinetically saturated and the size of the growing mineral reaches a size sufficient to sustain oxidation of Fe^{2+} on the mineral surface. This results in the changing of the dominant mechanism of iron oxidation from the ferroxidase center to the mineral surface catalysed mechanism (31). The reaction stoichiometry changes to the following:

For the oxidation reaction: $4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O$

For the subsequent hydrolysis reaction: $4Fe^{3+} + 8H_2O \rightarrow 4FeOOHcore + 12H^+$

For the sum of the oxidation and hydrolysis reactions: $4Fe^{2+} + O_2 + 6H_2O \rightarrow 4FeOOHcore + 8H^+$ (31).

Such differences in iron oxidation kinetics by ferritin are displayed in cells under varying conditions. Under cellular conditions where low Fe^{2+} concentrations relative to the amount of apoferritin may be expected, the ferroxidase activity of H-subunits is probably essential to initiate the formation of iron-nuclei and for iron mineralization to proceed at a significant rate (34). Autocatalytic Fe^{2+} oxidation on the surface of the growing iron mineral can only be significant once an initial iron-core has been established (34). However, it has certain advantages above the process that occurs in the ferroxidase center of the H-subunit. The first biologic advantage is that the potentially toxic production of hydrogen peroxide by the oxidation reaction, that takes place in the ferroxidase center, is replaced by the harmless reduction of di-oxygen to water via the oxidation reaction on the mineral surface (31, 42). As ferritin lacks catalase activity, the H_2O_2 produced during the ferroxidase center catalysed oxidation of iron results in some degradation of the ferritin protein shell. In effect, the protein itself acts as an anti-oxidant (31). Another advantage of such a change in iron kinetics is that it enhances the ability of ferritin to increase the rate of iron oxidation when challenged with a large amount of Fe^{2+}-ions. The ferroxidase center can be reutilized for another round of Fe^{2+} oxidation only once the generated Fe^{3+}-ion moves from the ferroxidase center into the iron storage cavity for subsequent nucleation. However, this rate of regeneration may be too slow to process a large number of Fe^{2+}-ions added at once, and under such conditions, oxidation on the mineral surface may occur even during earlier stages. These different oxidation abilities of ferritin to mineralize iron result in the formation of crystallites of different sizes. Under conditions favouring fast iron accumulation the 'ferrihydrite' produced has a relatively large crystallite size and a nearly all-or-none distribution within ferritin molecules, since the mineralization process will be favoured by oxidation of Fe^{2+} on the mineral surface (15) and the rate of iron deposition will be governed by the surface area of the iron mineral (38). However, under conditions of low

Fe^{2+} concentrations oxidation of Fe^{2+} will take place on different ferroxidase centers resulting in the formation of many small crystallites (15).

2.2.4) Migration of iron between ferritin molecules

Iron can migrate between ferritin molecules (24), either as Fe^{2+} or as Fe^{3+}. Migration of iron between ferritin molecules occurs if the movement of Fe^{2+}/Fe^{3+} to the next ferritin molecule results in favouring of the oxidation or hydrolysis/nucleation processes. Oxidation of Fe^{2+} results in the generation of highly mobile Fe^{3+}-ions, which subsequently have to be incorporated into a growing mineral crystal by a hydrolysis/nucleation process. Since L-subunits promote hydrolysis and nucleation of Fe^{3+} by binding of Fe^{3+} to specific L-subunit ligands, insufficient quantities of the L-subunit present in the ferritin molecule can result in the migration of Fe^{3+} to a ferritin molecule with sufficient quantities of L-subunit (32). The generated Fe^{3+} would move to the outer surface of the ferritin protein shell via a H-subunit specific channel linking the ferroxidase centers with the outer surface of the protein shell (30). This results in competition between ferritin molecules for iron and a tendency towards all-or-none distribution (15, 21, 24).

2.2.5) Non-specific Fe^{3+}-compound hydrolysis on the outer surface of the ferritin protein shell

When insufficient quantities of L-subunits exist for the entrapment of Fe^{3+} in the inner cavity Fe^{3+} can move to the outer surface of the protein shell where non-specific hydrolysis of Fe^{3+}-compounds can take place (36). Non-specific iron hydrolysis on the outer surface of the ferritin molecule can result in protein aggregation and precipitation (36). Spontaneous aerobic iron hydrolysis such as that which occurs on the outer surface of the ferritin protein shell appears to be caused by the formation of transient mono-

nuclear hydrated Fe^{3+}-compounds which have a strong tendency to aggregate and coagulate (36).

2.2.6) The cooperative roles of the H-subunit and L-subunit of the ferritin protein shell in iron mineralization

Since the H-subunit and the L-subunit of the protein shell have separate roles in the mineralization process of iron by ferritin and the specific properties of the two subunits are complementary and act synergistically, cooperation between these subunits is paramount for the efficient mineralization of iron by ferritin. The ratio of H-subunits to L-subunits is therefore important. While oxidation of Fe^{2+} by the ferroxidase center of the H-subunit accelerates the supply of Fe^{3+}, the subsequent hydrolysis and nucleation processes are driven by the L-subunit (29, 32). If insufficient quantities of the L-subunit are assembled into the protein shell non-specific hydrolysis of Fe^{3+}-compounds can take place on the outside of the protein shell. This may lead to aggregation and precipitation of ferritin molecules (36). Although very few H-subunits are necessary to initiate the process of iron oxidation, a deficit in H-subunits can result in poor iron sequestration abilities. Furthermore, H- and L-subunits can act cooperatively when in separate molecules (32).

The cooperative roles of the subunits of ferritin in the mineralization process of iron have been demonstrated by the formation of recombinant H-subunit or L-subunit homopolymers and recombinant H-subunit/L-subunit heteropolymers. *In vitro* experiments involving recombinant ferritin molecules showed that:

a)   Both the H-subunit and L-subunit homopolymers have the capacity to incorporate iron, however, since the recombinant L-subunit homopolymer relies on auto-oxidation of iron for the initial generation of Fe^{3+} the recombinant H-subunit

homopolymer exhibits iron uptake and ferroxidase kinetics several-fold faster than the recombinant L-subunit homopolymer (9, 24, 36).

b)   The rate of iron uptake increases with an increase in the H-subunit proportion from 0-35% of the ferritin molecule. A plateau is however reached with further increase in the H-subunit content (24).

c)   Ferritin H-subunit homopolymers have a low ability to nucleate the generated Fe^{3+}, and therefore takes up and release iron faster since the generated Fe^{3+} is not tightly bound in an iron mineral (22).

d)   Ferritin L-subunit homopolymers, when supplied with Fe^{3+} are more efficient in promoting iron mineralization than the corresponding H-subunit homopolymers (36).

e)   H-subunit/L-subunit heteropolymers are more efficient in taking up iron than the parent homopolymers (36). A high synergism between the two subunits occurs in ferritins with low H-subunit content (10-30%) and high L-subunit content (70-90%), a few ferroxidase centers are sufficient to promote fast iron oxidation, and many L-subunits are needed to reduce non-specific iron hydrolysis and facilitate iron mineralization (36).

f)   L-subunit homopolymers can incorporate some of the iron oxidized by H-subunit homopolymers (36).

g)   The amount of soluble ferritin molecules increases sharply in heteropolymers with an L-subunit content higher than 70-80%, thus preventing the non-specific iron hydrolysis on the outside of the protein shell and the subsequent protein aggregation/precipitation (36).

h)   H-subunit/L-subunit heteropolymers with low H-subunit content (18-30%) incorporate 3-4 times more iron than H-subunit homopolymers (36).

i)   The most efficient heteropolymers for *in vitro* iron incorporation are structurally similar to the ferritins found in the tissues which accumulate iron, such as liver and spleen, which typically contain 80-95% L-subunit and 5-20% H-subunit. Other tissues

with lower needs for iron storage may prefer ferritins with higher H-subunit content which, having a higher ferroxidase activity, are probably more efficient for iron detoxification (32, 36).

j)   L-subunit homopolymers containing nuclei will develop at the expense of L-subunit homopolymers without nuclei by autocatalytic growth processes on the surface of the iron mineral (24).

The respective and synergistic roles of the H-subunit and L-subunit of ferritin are also indicated *in vivo*, since *in vivo* H-subunit homopolymers are much less efficient than the H-subunit/L-subunit heteropolymers in taking up iron (41).

2.2.7) The release of iron from ferritin

Two mechanisms have been proposed for the release of iron from ferritin. Iron can be either released from the intact ferritin molecule or released upon the degradation of the ferritin molecule (45, 46). However, the relative importance of iron release *in vivo* by the one or the other of these mechanisms is not known. Two processes are chemically feasible for removing iron from the intact ferritin molecule – the first process the reduction of Fe^{3+} to Fe^{2+} followed by chelation of Fe^{2+} and the second the direct chelation of Fe^{3+} (21, 47, 48). The release of iron from ferritin by these two processes is accomplished with the aid of reductants and iron chelators that can cross the protein shell. Reductants and chelators gain access to the interior of the ferritin molecule through the threefold channels of the protein shell. It is suggested that the channels of the ferritin protein shell are dynamic and control the access of reductants and chelators, since reductants and chelators too large to pass through the channels can under certain conditions gain access to the interior of the ferritin molecule (47). Chaotropes can increase the access of reductants and chelators to the interior of ferritin by influencing the gating of the channel (47, 48). Various reductants and chelators, including

physiological and toxicological substances, can release iron from ferritin (48, 49, 50, 51). With the first of the two processes that remove iron from intact ferritin, i.e., the process involving the reduction of Fe^{3+} to Fe^{2+}, followed by chelation of Fe^{2+}, the reductant has to gain access to the interior of the protein shell to reduce the Fe^{3+} to Fe^{2+}. However, the formed Fe^{2+} will only leave the ferritin protein shell in the presence of a chelator (52). With the second of the two processes, i.e., the direct chelation of Fe^{3+} where the Fe^{3+} is not reduced, Fe^{3+} leaves the ferritin protein shell as an Fe^{3+}-complex. The hydrous ferric oxide cores can be reduced by one electron per iron atom accompanied by an uptake of two protons per electron from the surrounding medium (53). This is then followed by the chelation of Fe^{2+} and the transport to sites where Fe^{2+} is needed (53, 54). Effective reducing agents for the release of iron from ferritin include flavins, cysteine, glutathione, ascorbic acid and superoxide (10).

The initial rate of iron release shows a dependence on iron content, with maximum rate of release for relatively iron-poor molecules, which are one-third to one-half saturated with iron. It also depends on the surface area of the crystallite. This dependence resembles that for iron uptake and suggests a direct interaction between the surface of the iron core and the reducing agent (9, 11, 25). Furthermore, as iron atoms at the surface of the iron core could be expected to be more accessible to reducing agents than those in the interior, a last-in-first-out principle is obeyed (9). It has also been suggested that the release of iron from the intact ferritin molecule is sensitive to changes in conserved amino acids near the outside of the ferritin channels which are likely to be involved in regulating the localised unfolding of the protein shell in order to open the channels and release the reduced iron (55).

2.3) Isoferritins

2.3.1) Different H-subunit/L-subunit compositions of the ferritin protein shell

The multiple forms of ferritin have their molecular basis in the ratio of the two subunit types present, i.e., the H-subunit and the L-subunit (25). The ferritin protein shell exists as heteropolymers of various combinations of these two types of subunits (56) – a phenomenon that gives rise to the existence of isoferritins. As the roles of the H-subunit and L-subunit differ in the mineralization process, the subunit composition of ferritin will influence the metabolic properties of the assembled ferritin molecules (21, 36). H-subunit rich ferritins have been shown to accumulate and release iron faster than do L-subunit rich ferritins (9, 25, 57, 58) and it is suggested that the H-subunit rich ferritins permit more dynamic intracellular traffic of iron (25, 59). L-subunit rich ferritins apparently contain more iron than those ferritins rich in H-subunits (25, 60) and there are indications that the L-subunit rich ferritins predominate in cell types that play a role in the storage of iron (22, 25, 61, 62). However, increases in ferritins rich in the H-subunit have been shown to provide cells with increased resistance to H_2O_2 toxicity (41). It would further appear that a specific subunit composition may cater for iron storage, and that iron loading would increase the expression of the L-subunit whereupon these L-subunit rich isoferritins will sequester the bulk of the surplus iron (60, 63). In general L-subunit rich ferritins contain 1500 iron atoms or more whereas H-subunit rich ferritins contain less than a 1000 iron atoms (21). In situations of iron overload it may be advantageous to the cell to synthesize L-subunit rich ferritins, since these ferritins are not only able to store more iron but can also retain iron more firmly and turn over iron more slowly than H-subunit rich ferritins (64). The assumed role that the L-subunit rich ferritins play in the sequestration of the surplus iron during iron overload is underlined by the fact that their concentration in liver, serum and cultured cells is related to iron levels, whereas the H-subunit rich ferritins appear either to be non-affected (in liver) or negatively affected (in serum) by increases in iron concentrations (65). Furthermore,

upon iron supplementation of patients with functional iron deficiency in the presence of tissue iron overload there would appear to be a proportionately greater change in L-subunit rich ferritins than in H-subunit rich ferritins (65). Due to the H-subunit rich ferritin's more dynamic ability of iron uptake and release it would appear to be largely found in cells having high iron requirements for metabolic activities and a non-existent role in iron storage (66). Cells with a high content of H-subunit rich ferritins include erythroid cells, heart cells, pancreatic cells, kidney cells, lymphocytes and monocytes (38, 67), whereas the L-subunit rich ferritins are found predominantly in liver and spleen – organs associated with long-term iron storage (38, 62). The H- to L-subunit ratio of a specific type of cell does, however, not remain constant and the proportion of the H- and L-subunits present in the ferritin shell changes during differentiation and in various pathological states (10, 11, 22, 68).

The variations in the type of isoferritins present in erythroid cells are well studied. The presence of different isoferritins with different metabolic properties reflects the changing iron needs of the erythroid cell. The erythroid cells contain mainly H-subunit rich ferritins, which play a major role in the intracellular transport and donation of iron for the active synthesis of haem (61), particularly in immature erythroid cell precursors such as proerythroblasts and basophilic erythroblasts (63). However, when iron accumulates in erythroid tissue due either to an increase in the cellular uptake of iron or a decrease in iron usage for haem synthesis, the L-subunit rich ferritins seem to increase and to be closely related to the iron status of the cells (63, 66). The H-subunit/L-subunit ferritin composition is reported to decrease with erythroblast maturation (66), with H-subunit rich ferritin content higher in the early erythroblast fractions and decreasing with maturation. The content of L-subunit rich ferritin, apparently does not show such consistent changes with maturation (69).

The ferritin present in reticuloendothelial cells (63, 70) and other macrophage-like cells are predominantly L-subunit rich (71, 72). An exception is the ferritin in human peripheral blood monocytes, which seems to be rich in H-subunits (67). However, *in vitro* macrophages, which originate from monocytes, would appear to develop a L-subunit predominance. This phenomenon is mainly associated with the loss of H-subunits (67). At present indications are that the addition of iron can cause an increase in both H- and L-subunits despite the persistence of a very low H-subunit/L-subunit ratio. The presence of mainly L-subunit rich ferritin is in keeping with the role of the reticuloendothelial cell/other macrophage-like cells in the scavenging of effete cells and the sequestration and storage of large amounts of iron. An interesting phenomenon is the fact that the same type of process may also occur in the brain. Microglia, a macrophage-like cell present in the brain (71, 72), is responsible for the phagocytosis of cellular debris during axon remodeling and naturally occurring cell death in the developing brain (71). These cells contain predominantly L-subunit rich ferritins consistent with its role in long-term iron storage (72).

2.4)   The synthesis of ferritin

The apoferritin molecule, consisting of 24 H- and L-subunits, is assembled from a cytosolic pool of available H- and L-subunits. This cytosolic pool of free H- and L-subunits is maintained by the supply of H- and L-subunits upon translation of H- and L-subunit mRNA by free polyribosomes and the proper folding of the polypeptide chains. While the composition of H- and L-subunits in the ferritin molecule is determined by the H- and L-subunits available in this pool, the quantity and composition of the H- and L-subunits in this pool of available subunits is regulated at both the transcriptional and translational levels of expression of the ferritin H- and L-subunit genes.

2.4.1) Assembly of ferritin from the pool of available H- and L-subunits

The twenty four-subunit ferritin protein shell is assembled in the cytosol from a pool of free, unassembled, or only partly assembled H- and L-subunits (21). These H- and L-subunits are synthesized by free polyribosomes and a basal concentration of free H- and L-subunits is maintained in this cytosolic pool of subunits. When this concentration rises as a consequence of the synthesis of new H- and L-subunits by free polyribosomes, subunits will be assembled into apoferritin in the cytosol near the polyribosomes. Since H- and L-subunits have the same conformation and many identical or similar amino acids are involved in the inter-subunit contact regions between H-subunit/H-subunit, H-subunit/L-subunit or L-subunit/L-subunit interactions, a complete range of subunit compositions of homopolymers and heteropolymers is possible (11, 21). However, homopolymers of ferritin consisting of either only H-subunits or only L-subunits are poorly represented in cells, suggesting the existence of preferential interactions between H- and L-subunits. This is in agreement with cross-linking experiments showing a preferential formation of H-subunit/L-subunit dimers (73).

Iron is incorporated into apoferritin or iron-poor ferritin molecules only once the ferritin shell is completely assembled from the available H- and L-subunits (21). This fraction of H- and L-subunits synthesized by free polyribosomes is destined for the intracellular sequestration of iron while a much smaller fraction of H- and L-subunits are synthesized by membrane-bound polyribosomes and once assembled the ferritin is secreted by the cell to the extracellular fluid (38).

2.4.2) Regulation of the expression of the H-subunit and L-subunit genes of ferritin

The H- and L-subunit genes of ferritin are expressed in most cells but the concentration of the assembled ferritin can vary 1000-fold among different cell types (11).

Furthermore, the composition of the H- and L-subunits in the ferritin molecule differs between different cell types, resulting in the cellular-dependent variation of isoferritin populations (21). This is achieved by the regulation of the expression of the H- and L-subunit genes of ferritin. Different mechanisms of regulation exist including transcriptional, modulation of transcript stability, translational depending on the metabolically available iron concentration and translational irrespective of the metabolically available iron concentration. The regulation of the transcription of the H- and L-subunit genes occurs mainly irrespective of the metabolically available iron concentration. However, there are indications that the transcription of the H- and L-subunit genes can be influenced by the metabolically available iron concentration in specific conditions (11, 64). The transcriptional regulatory mechanisms and stability of the mRNA determine the mRNA concentrations of the H- and L-subunits, whereas the translational regulatory mechanisms determine the magnitude of mRNA translation and the subsequent formation of the H- and L-subunits of ferritin. Therefore, the relative proportion of H- and L-subunits in the final ferritin molecules depends mostly on multiple transcriptional regulations (transcription or stability) that affect the respective proportion of H- and L-subunit mRNA in the total pool of translatable ferritin mRNA, whereas the total amount of ferritin produced in the cells depends on the magnitude of translation of the H- and L-subunit mRNA (10). Ferritin synthesis is stimulated during development, during cell differentiation, by pro-inflammatory cytokines, as well as by some hormones (74). Furthermore, a preferential increase in a specific subunit is elicited by the differential transcriptional regulation of the H- and L-subunit genes. The differentiation of various cells is associated with a consistent increase of ferritin mRNA and ferritin levels, and a preferential accumulation of the H-subunit as a consequence of a selective transcriptional regulation of the H-subunit gene needed to produce ferritin with a structure appropriate to a differentiated cell type (11, 21, 74). During inflammation, stimulation by the cytokine tumor necrosis factor (TNF), results in

transcriptional up-regulation of the H-subunit without a change in L-subunit expression. This gives rise to an increase in the H-subunit to L-subunit ratio of the produced ferritins (74). Up-regulation of the transcription of the H-subunit gene has also been found in cell lines overexpressing c-myc, in the pregnant uterus, in denervated skeletal muscle, in the atherosclerotic aorta, in response to other cytokines and after exposure to exogenous heme (10). Somewhat surprising, iron itself does not seem to affect the amount of H-subunit mRNA, although it has a stimulatory effect on the accumulation of L-subunit mRNA (10). L-subunit gene transcription and total cellular L-subunit mRNA appears to be dramatically increased by iron, whereas H-subunit transcription rates and H-subunit mRNA levels are only slightly increased (64).

2.4.3)   The gene sequences of the H-subunit and the L-subunit of ferritin

The genes for the H- and L-subunits are contained on different chromosomes – the gene for the H-subunit on chromosome 11 and the gene for the L-subunit on chromosome 19 (9). The genes for the H- and L-subunit contain 3 introns and 4 exons (16) and the gene sequences for these two subunits show extensive homology in their coding regions with several common stretches of 20-30 nucleotides. However, they differ markedly in their non-coding regions (22). These differences are extremely important for differential regulation of the expression of the genes for these two subunits. Functional analysis for the 5' non-coding region for the H-subunit gene but not for the L-subunit gene has been reported. The 5' non-coding region of the H-subunit gene contains three regulatory regions. The first of these regions, the B-box, –42 to –62 nucleotides upstream from the start codon and closest to the transcription initiation site is responsive to cAMP. This B-box regulatory region is sensitive to the initiation of transcription by hormones and second messengers and binds to a protein complex termed B-box binding factor (Bbf). The B-box binding factor comprises the transcription factor NFY, the co-activator p300 and the histone acetylase p300/CBP associated factor (PCAF) (16, 21). The second

regulatory region identified in the 5' non-coding region of the H-subunit gene includes a region called the A-box at position –109 to –132 upstream from the start codon (transcription initiation site), which contains a consensus sequence for binding the polymerase II transcription factor SP1 responsible for about 50% of the activation of gene expression in several cell lines (16, 21). The third regulatory region consists of a stretch of 10 G's which are termed "G-fer" between –272 and –291 upstream from the start codon. It is suggested that binding of inhibitory factor 1 to this sequence results in the inhibition of H-subunit gene transcription (16, 21).

2.4.4) Translational regulation of the H-subunit and L-subunit mRNA expression via metabolically available iron

Ferritin is the major intracellular protein involved in storage and detoxification of iron. It is therefore not surprising that the expression of ferritin is extremely sensitive to the amount of metabolically available iron. In order to accomplish a finely tuned system of ferritin expression as a function of the size of the metabolically available iron pool (the labile iron pool) it is important that the ferritin gene structure contains sequences that sense the size of the labile iron pool (75). The 5'-untranslated region (5'-UTR) of both the H- and L-subunit mRNA contains a highly conserved 28-base sequence known as the iron-responsive element (IRE) sensitive to the metabolically available active iron (9). The IREs are comprised of cis-acting nucleotide sequences. These nucleotide sequences form stem-loop structures that contain a six-membered loop with the sequence CAGUGN (10). These stem-loop structures are recognized by trans-acting cytosolic RNA-binding proteins required for the coordinated expression of the H- and L-subunits (11). These cytosolic RNA-binding proteins, IRP1 and IRP2, cause a decrease in H- and L-subunit mRNA translation by binding to the stem-loop structures of the 5'-UTR of the respective mRNAs. IRP1 and IRP2 mediate the translational efficiency by obscuring the subsequent binding of the 43S translation pre-initiation complex needed for the initiation

of translation (76). IRP1 and IRP2 both sense and homeostatically control the metabolically available iron. For IRP1 this is accomplished by the existence of two conformationally distinct forms. IRP1 is a 90 kD iron-sulfur cluster protein. When iron is abundant it exists as a cytosolic aconitase. When iron is scarce it assumes an open configuration associated with the loss of iron atoms from the iron-sulfur cluster and the subsequent binding to the IRE stem-loop structure, acting as a repressor of ferritin translation (16). In contrast, the 105 kD IRP2 protein is regulated by degradation: IRP2 protein is abundant in iron scarcity, but is degraded rapidly in iron excess through targeting of a unique 73 amino acid sequence and subsequent oxidation and ubiquitination (16, 77). This response of ferritin synthesis to the size of the metabolically available pool of iron endows the cell with an exceptionally rapid system for increasing ferritin synthesis upon iron influx. Iron influx increases the labile iron pool and, via binding to the IRP1 and IRP2, causes a rapid increase in ferritin translation. This rapid response is achieved by a shift of stored mRNA from the ribonucleoprotein (RNP) fraction to polysomes (translational shift) (64). The translation of existing ferritin mRNA is more rapid than additional ferritin gene transcription followed by translation. The ferritin response to iron influx can thus be viewed as a protective rapid response system, allowing immediate formation of additional ferritin in which to store the surplus iron (78). Both the H-subunit and L-subunit mRNA shift from the RNP fraction to polysomes to the same extent (64). Nevertheless, the transcription of the L-subunit gene is preferentially stimulated by an increase in metabolically available iron (9) and results in an increase in the ratio of L-subunit to H-subunit mRNA, which appears first in the RNP fraction and later in the polysomes (64). This increase in the L-subunit to H-subunit mRNA ratio in the polysomes accounts for the change in the ratio of L-subunit to H-subunit protein synthesis following iron administration (64). Therefore, coordinated translational control and differential transcriptional control exists between these two genes (64).

2.4.5) Translational regulation of H-subunit and L-subunit expression irrespective of metabolically available iron

Various factors other than iron may alter the translational efficiency of the H- and L-subunit mRNA. This may be accomplished by binding of regulatory factors to specific sequences in the 5'-UTR other than the IRE or by changing the efficiency of the interaction between the IREs and IRPs. One specific sequence responsible for translational control is the twenty-nucleotide sequence downstream from the IRE known as the acute box (79). This sequence responsible for the enhancement of translation operates after iron-dependent translational initiation and the formation of the 43S ferritin mRNA scanning complex (76). Factors that can influence the efficiency of the interaction between the IRE and IRP include cytokines (80), various hormones that changes the phosphorylation status of the iron-responsive proteins (16, 79), oxidative stress – reactive oxygen species (61), haemin (61), phosphatases, hypoxia and reoxygenation (79) and nitric oxide (NO) that causes the activation of both IRP1 and IRP2. Mechanisms hypothesized to underlie NO-mediated induction of IRP binding activity include cluster disassembly (IRP1), intracellular iron chelation (IRP1 and IRP2), or increased *de novo* synthesis (IRP2) (16).

2.5) The degradation of ferritin

Two different processes can result in the degradation of cytosolic ferritin. The first of these involves the 20S proteasome enzymatic system in the cytosol and the second degradation in the lysosome by proteolytic enzymes. Depending on the type of cell, the iron status and whether ferritin is degraded free in the cytosol or within a lysosome, different amounts of iron are made available for metabolic processes. Degradation of ferritin in the cytosol results in the complete release of iron from ferritin, whereas degradation of ferritin in the confinements of a lysosome can result in the entrapment of ferritin iron (37). Iron-containing ferritin can ultrastructurally be identified in the cytosol

as either randomly dispersed ferritin particles or as clusters of ferritin particles. The ferritin clusters in the cytosol are accumulations of ferritin in which ferritin particles can be individually resolved. The existence of ferritin either as randomly dispersed ferritin particles or as clusters of ferritin particles depends on the magnitude of iron handling of the different cell types. In cell types handling relatively low quantities of iron, iron-containing ferritin occur as rare, isolated particles whereas in cell types handling greater quantities of iron such as haemopoietic bone marrow cells and cells of the reticuloendothelial system, iron-containing ferritin occurs more frequently as clusters (81). In most clusters the particles are of the iron-rich variety and thus appear larger and more electron dense than the dispersed cytosolic ferritin (81). Cluster formation prevents access of the proteins involved in the cytosolic degradation of ferritin and in this way protects the ferritin molecule against degradation. This may be a regulatory step in the pathway of ferritin degradation and iron release. However, as long as the ferritin cluster is not enclosed by a membrane, degradation of these ferritin clusters can result in the release of iron in times of iron shortage. Various studies indicated that the formation of large iron-rich ferritin particles, as a result of an increase in intracellular iron, results in the protection of ferritin molecules against degradation (38, 78, 82) and that iron-depleted ferritin is easily degraded (37, 83). The 20S proteasome enzymatic system is responsible for the degradation of damaged intracellular proteins and can recognize specifically, and degrade, oxidized proteins (84, 85). The ferritin protein shell is confronted by a multitude of possible oxidative stressors. The oxidation of Fe^{2+} by the ferroxidase center of the H-subunit results in the production of H_2O_2 which could oxidize the protein shell, and the surrounding Fe^{2+} can promote oxygen radical production by Fenton type chemistry. Oxidation of ferritin results in the loss of ferritin function and targeting of ferritin to the proteasome degradation system of the cell (84, 85). Oxidation of ferritin can also result in aggregation of ferritin molecules. Sulfhydryl groups, particularly, are oxidized followed by aggregation of ferritin as a result of the

formation of disulfide bridges between ferritin molecules (86). The H-subunit contains a cysteine at position 90 located on the BC-loop facing the exterior which is extremely susceptible to oxidation (87).

Ferritin cluster formation, however, might also stimulate the uptake of ferritin into lysosomes whereupon less iron will be released during ferritin degradation. The reason for this is that the release of iron during the degradation process relies on the accessibility of the iron core to the reducing system of the cell since dissolution of the iron core is generally determined by the reduction of Fe^{3+} to Fe^{2+}. Therefore, degradation of the ferritin protein shell in the cytosol gives $FMNH_2$ (the reducing system of the cell) easy access to the Fe^{3+}-ions and results in the complete dissolution of the iron core. The generated Fe^{2+}-ions are reutilized in metabolic processes or incorporated into new ferritin molecules. If however, ferritin is degraded within a secondary lysosome (charged with proteolytic enzymes) the iron can no longer readily be made available because it has been cut off from the $FMNH_2$ reducing system (37). Instead, digestion by lysosomal enzymes would proceed and the resulting aggregates of iron oxyhydroxide (ferritin cores) would no longer be provided with a mechanism for mobilizing and recycling their iron. This will result in aggregation of the ferritin cores and the formation of haemosiderin (37). Cytosolic degradation may therefore be the major iron turnover mechanism providing the cell with easily accessible iron for shunting into metabolic pathways, while degradation within membrane-encapsulated secondary lysosomes, with subsequent haemosiderin formation, may prevent the uncontrolled release of iron and may become prominent when there is iron overload (21, 37). Nevertheless, degradation of ferritin in lysosomes can also produce soluble iron, although these larger masses of ferritin/haemosiderin may require more time for the release of their iron contents. The iron so released would then be translocated back to the cytosol for reutilization in metabolic processes or sequestration by ferritin (10, 46, 88). Thus it seems that the

release of iron from lysosomes depends on the magnitude of aggregate formation and the subsequent deposition of iron as haemosiderin.

2.5.1)   The formation of haemosiderin from ferritin

The absolute and relative amounts of iron stored in the form of the two iron reserves, ferritin and haemosiderin, vary with iron loading and cell type (15).   There is slightly more ferritin than haemosiderin in the liver and spleen when total tissue iron content is normal.   As total iron content increases a progressively higher percentage of iron occurs as haemosiderin (9, 15, 37, 89, 90).   With overloading syndromes such as primary and secondary haemochromatosis, the iron content of haemosiderin can increase up to a 100-fold, whereas that of ferritin only increases 5- to 10-fold (13).

There is enough evidence to believe that haemosiderin is derived from ferritin as a result of degradation of the ferritin protein shell in secondary lysosomes (12, 15, 91).   For instance, haemosiderin contains various amounts of degraded ferritin, as well as aggregated dense particles of irregular shape with diameters ranging from 10-75 Å, which ultrastructurally resemble iron cores (37, 90) and haemosiderin granules are recognised by anti-ferritin antibodies (21).   Ferritin is frequently situated in secondary lysosomes and autophagosomes of normal cells, such as hepatocytes and macrophages but its quantity in these organelles increases greatly after loading with iron (92) – demonstrating the protective function of haemosiderin formation against the toxicity of iron.   Ferritin finds its way into lysosomes by autophagocytosis and/or fusion of ferritin clusters with the lysosomal membrane.   Autophagocytosis is responsible for the turnover of cellular constituents including cellular proteins and involves the formation of autophagic vacuoles by invagination of intracytoplasmic membranes enclosing a relatively large volume of cytoplasm, together with various cellular constituents (37).   The autophagic vacuole receives digestive enzymes by fusion with a primary or secondary lysosome and

becomes an autophagosome (37). It is within this lysosomal organelle that the ferritin protein shell is degraded by the action of lysosomal proteases (93). It is suggested that the polymerization of ferritin (formation of oligomers of ferritin), which results in a change in solubility, heat stability and surface charge, may predispose ferritin to incorporation within lysosomes and transformation into haemosiderin (25, 90). Only once the ferritin protein shell has been modified, most probably by denaturation, resulting in the formation of insoluble ferritin molecules, does proteolytic decomposition of the ferritin protein shell by lysosomal enzymes take place (93). However, not all ferritin molecules in these lysosomal organelles are susceptible to the action of lysosomal proteases. Degradation of the ferritin protein shell results in the exposure of the iron oxyhydroxide mineral cores followed by aggregation of these oxyhydroxide particles and the formation of insoluble masses of iron oxyhydroxide (haemosiderin) (91, 92, 94). Although the main purpose of the formation of haemosiderin would appear to be protection against iron overload, these larger masses of ferritin/haemosiderin can, at a much slower rate, also release iron. This iron is then translocated back to the cytosol for reutilization in metabolic processes or sequestration by ferritin (10, 46, 88). Haemosiderin is, however, not necessarily the end product as massive quantities of iron oxyhydroxide (haemosiderin) from these secondary lysosomes, can accumulate to form cytoplasmic organelles known as siderosomes (92). The haemosiderin-containing siderosomes can thus be regarded as the end-product of secondary lysosome action in which the wall of the original secondary lysosome now encapsulates the digested ferritin iron cores (21, 37) – although clusters of electron-dense material without membranes or only partially enclosed membranes can also occur (21, 46, 82). Within siderosomes, ferritin can be identified as individual particles, in clusters, in paracrystalline hexagonal arrays, or forming circular arrangements (81). Siderosomes not only contain ferritin and haemosiderin, but occasionally also contain electron-dense amorphous or spicular iron-containing compounds which have as yet not been identified biochemically or

ultrastructurally. In cells with marked iron overload, solitary siderosomes seem to fuse and form larger bodies described as "compound siderosomes" (81).

2.5.2) The increased susceptibility of H-subunit rich ferritins to degradation

It is suggested that H-subunit rich ferritins are turning over more rapidly than L-subunit rich ferritins (22, 38, 78). Haemosiderin, which contains the degraded ferritin molecules as a result of the lysosomal breakdown of ferritin, shows the predominance of denatured subunits to be of the H-subunit type (89). It was shown that ferritin is more abundant in the iron overloaded liver than in the normal liver, and that it is richer in L-subunits displaying a L-subunit:H-subunit ratio from 5:1 to 12:1. In contrast, haemosiderin displayed a predominance of denatured H-subunits over denatured L-subunits (89). A mechanism may therefore exist for preferentially directing ferritins rich in the H-subunit into lysosomes resulting in the formation of haemosiderin containing a high proportion of denatured H-subunits. It was shown, *in vitro*, that a too great proportion of H-subunits in the ferritin protein shell result in ferritin aggregation. This may be due to the inadequacy of the ferritin protein shell to retain the formed Fe^{3+} resulting in the loss of Fe^{3+} and hydrolysis of Fe^{3+} on the outside of the ferritin molecule (21). This may be the signal for ferritin to be incorporated into lysosomes. Once inside the lysosome the presence of a large number of H-subunits in the ferritin protein shell increases the chances of degradation (60), since H-subunit rich ferritins, in the presence of denaturing conditions, are less stable than L-subunit rich ferritins (89, 95). The salt-bridge present in the L-subunit appears to be important for the differences in stabilities between H-subunit rich ferritins and L-subunit rich ferritins (57). Futhermore, H-subunit rich ferritins are more susceptible to proteolysis due to less ordered secondary structures (60). In particular, the loop L becomes more exposed and/or less immobilized when the proportion of H-subunits increases and therefore more accessible to lysosomal enzymes (25).

2.5.3) The reticuloendothelial cell and haemosiderin formation

The reticuloendothelial cell responsible for taking up and digesting effete red blood cells is confronted by tremendous amounts of iron as a result of the breakdown of the heme contained by the red blood cells. These surplus amounts of iron could result in iron-induced damage to the reticuloendothelial cell. In order to prevent possible iron-induced damage, the reticuloendothelial cell is capable of storing vast amounts of iron as haemosiderin. The reticuloendothelial cell takes up red blood cells and incorporates these red blood cells into phagocytic vacuoles, where digestion of the red blood cell and degradation of heme take place. The heme contained by the red blood cell is degraded in 5 hours. However remnants of the red blood cells could still be detected in the phagocytic vacuoles 24 hours after uptake of red blood cells by the reticuloendothelial cell. At this time, moderate numbers of free ferritin molecules are present in the cytosol. Forty-eight hours after red blood cell uptake the number of ferritin molecules in the cytosol is increased and continue to accumulate in the cytoplasm up to 96 hours after red blood cell uptake. However, from 48 hours onward ferritin is translocated into aggregates situated in autophagic vacuoles by a process of invagination of intracytoplasmic membranes followed by the formation of haemosiderin (37). The formation of haemosiderin in reticuolendothelial cells and other macrophage-like cells are influenced by inflammatory and infectious conditions. Macrophages subjected to increased oxidative stress also degrade ferritin faster (84). It is therefore suggested that during inflammatory and infectious conditions the proportion of poorly accessible (non-chelatable) iron associated with ferritin similarly increases, suggesting a pathway from non-ferritin iron to loosely associated ferritin iron to a well-sequestered non-chelatable form existing as haemosiderin (96). Cytokines such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) may be responsible for these effects during inflammatory and infectious conditions. These cytokines may increase lysosomal activity resulting in increased degradation of intracellular ferritin, leading to the formation of haemosiderin,

from which iron would be less easily liberated for subsequent extracellular release (97). *In vitro* incubation of cells with either TNF-α or IFN-γ increases the expression of ferritin H-subunit mRNA but not L-subunit mRNA (96). Such a differential regulation of ferritin subunit expression might result in increased amounts of haemosiderin formation since H-subunit rich ferritins are more susceptible to lysosomal degradation.

2.6) Ferritin in cellular organelles

2.6.1) Nuclear ferritin

H-subunit rich ferritins are transiently present in the nucleus where the presence of these H-subunit rich ferritins can be controlled by various factors such as abnormal increases in cellular iron levels, developmental status, pro-inflammatory cytokines and oxidative stress (17). A specific pathway has been shown for the translocation of cytoplasmic H-subunit rich ferritins, but not cytoplasmic L-subunit rich ferritins, into the nucleus (98). This pathway for the transportation of cytoplasmic H-subunit rich ferritins into the nucleus involves the nucleur pore complex (NPC) (17) and the translocation is an active process that requires energy in the form of ATP (17, 98). Although many proteins are translocated into the nucleus by binding of a nuclear localization signal to the nuclear pore complex, no such nuclear localization signal could be identified for H-subunit rich ferritins. However, since H-subunit rich ferritins, as well as the H-subunit rich ferritin mutant containing no ferroxidase center, are translocated to the nucleus but L-subunit rich ferritins are not, specific amino acids on the outside of the H-subunit rich ferritin molecule are implicated in this process (17). O-glycosylation is predicted as the cue for the specific translocation of H-subunit rich ferritins. Once the H-subunit rich ferritins are inside the nucleus these ferritins can form stable complexes with the DNA (99). This places the H-subunit rich ferritins in a strategic position to ward off possible oxidative onslaughts to DNA or, alternatively, to donate iron for enzyme activity and possibly the nicking of double stranded DNA that could result in relaxation of superhelical stress

(99). It was shown that not only does H-subunit rich ferritin form a stable complex with DNA (99), but that DNA also contains specific iron-binding sites (17).

Although the precise functions of H-subunit rich ferritins in the nucleus are still somewhat unclear indications are that H-subunit rich ferritin protects DNA and other nuclear constituents against oxidative damage (17), that it donates iron for iron-dependent enzyme or transcription activities (99) and that it may play a role in the regulation of the transcription of specific genes (100).

2.6.2) Mitochondrial ferritin

Mitochondrial ferritin is found in the matrix of the mitochondria under specific physiological conditions, but is low in most cell types. Mitochondrial ferritin is, in general, structurally and functionally analogous to cytosolic ferritin and its main function, similar to that of cytosolic ferritin, is to sequester surplus iron. It does, however, differ from cytosolic ferritin in various aspects (101). Where the ferritin present in the cytosol exists mainly as heteropolymers consisting of different combinations of the H- and the L-subunit, mitochondrial ferritin consists of only homopolymers of a subunit similar to the H-subunit of cytosolic ferritins (101). The mitochondrial ferritin subunit is encoded from an intronless gene located on chromosome 5q23.1, which is different from the H-subunit gene for the cytosolic ferritin. Nevertheless, a high degree of sequence homology exists between the cytosolic H-subunit and the mitochondrial ferritin subunit (101, 102). The mitochondrial ferritin subunit has about 80% sequence identity to cytosolic ferritin H-subunit in its coding region and 55% to that of the cytosolic ferritin L-subunit (103), with a structure very similar to H-subunit ferritin (18). More important, the mitochondrial ferritin subunit has complete conservation of the amino acids constituting the ferroxidase center (18). The mitochondrial ferritin gene is expressed in the cytosol as a 30 kDa polypeptide containing a N-terminal mitochondrial targeting

sequence of 60 amino acids. Once the 30 kDa polypeptide enters the matrix space of the mitochondria the targeting sequence is removed and the polypeptide processed into a subunit of 22 kDa (101). Typical hollow spherical ferritin shells containing 24 subunits are then assembled from these subunits.

As mitochondrial ferritin contains ferroxidase activity it can take up large amounts of iron. However, the ferroxidase activity is significantly lower than that of H-subunit rich cytosolic ferritin (102). This is partially due to the fact that only 12 of the 24 ferroxidase centers of mitochondrial ferritin would appear to be actively oxidizing Fe^{2+} to Fe^{3+}, and this at a reduced rate, and although a μ-peroxodiferric intermediate is formed, mitochondrial ferritin does not regenerate its ferroxidase center. The underlying reason that only some of the ferroxidase centers are involved in the oxidation process is that the side-chain of serine, in place of alanine at position 144, protrudes toward a channel that connects to the ferroxidase center – a configuration that may create steric hindrance to the movement of iron accounting for only 12 active ferroxidase centers and a stoichiometry of 24 Fe^{2+} oxidized per ferritin molecule. The oxidized Fe^{3+} is stabilized followed by nucleation. The negative patch of glutamic acid residues near the ferroxidase center, similar to that for the L-subunit of cytosolic ferritin, might constitute the nucleation site. Once nucleation has taken place autocatalytic mineral surface oxidation of iron occurs, resulting in slower oxidation of iron, but less H_2O_2 production early in the process of core formation (102).

Iron present in the cytosol transverses the double membrane of the mitochondria and is therefore readily taken up by mitochondrial ferritin (104). Mitochondrial ferritin, *in vitro*, takes up iron in a similar fashion as cytosolic ferritin, where Fe^{2+} is initially oxidized to Fe^{3+} by a ferroxidase center. This is then soon followed by iron-nuclei formation and autocatalytic Fe^{2+} oxidation on the mineral surface – since mitochondrial ferritin does

not regenerate its ferroxidase centers. This process was shown to occur at a slower rate than for cytosolic H-subunit rich ferritin. Despite the slower uptake, *in vivo*, mitochondrial ferritin displays a higher avidity for iron than cytosolic H-subunit rich ferritin. Although excess iron, even when processed by mitochondria, is said not to be retained in mitochondrial ferritin, but is sequestered into cytosolic ferritin (104), it would appear that over-expression of mitochondrial ferritin can lead to the depletion of cytosolic iron. In situations where increased amounts of mitochondrial ferritin are available, cytosolic ferritin and mitochondrial ferritin competes for cytosolic iron and since mitochondrial ferritin has a higher avidity for iron this can lead to the accumulation of iron in mitochondrial ferritin (104). This depletion of cytosolic iron dramatically increases IRP binding to IREs, followed by a decrease in cytosol ferritin levels and an increase in transferrin receptor expression (104, 105). In short it would at present appear that the high avidity of mitochondrial ferritin for iron, together with a low availability of this iron for chelation, could lower iron bioavailability in the cytosol (105). The functional implication of this is as yet not clear.

Under normal conditions most cell types contain only low amounts of mitochondrial ferritin and the stimuli for the expression of mitochondrial ferritin is still somewhat unclear. As mitochondrial ferritin does not contain an iron responsive element in its 5' untranslated region, as is the case for the H-subunit and L-subunit of cytosolic ferritin (101), one would presume that the expression of mitochondrial ferritin is not regulated by the labile iron pool as for cytosolic ferritin. However, when haem synthesis fails in patients with sideroblastic anaemia an increase in mitochondrial ferritin occurs together with the occurrence of ring sideroblasts (106).

Although details of the regulation of mitochondrial ferritin expression are not known the purpose of the presence of ferritin in the mitochondria is self-evident. The

mitochondrium is confronted with a great amount of iron since most of the metabolically active iron of the cell is processed in the mitochondria due to the synthesis of heam and iron-sulphur complexes (102, 105). Not only are mitochondria surrounded by iron, but mitochondria also produce great amounts of reactive oxygen species during oxidative respiration. This combination of iron and reactive oxygen species in the mitochondrium calls for the protection provided by mitochondrial ferritin in times of iron dyshomeostasis that may result in the oxidative damage of mitochondrial constituents (105).

2.7) Extracellular ferritin

Most of the synthesized ferritin remains within the cell where it sequesters and releases iron in order to maintain intracellular iron homeostasis. However, the content of ferritin varies between different cell types and maturation stages. The content of ferritin in peripheral white blood cells, for instance, is about 10^3 times higher than that of peripheral red blood cells, with monocytes showing the highest values (63). Aside from the presence of ferritin in the cytosol of the cell, various quantities of ferritin are found in the plasma. It is suggested that ferritin may enter the circulation either *via* secretion of ferritin by cells or through the release of ferritin from damaged cells (9). Both mechanisms probably contribute to plasma levels. Ferritin destined for intracellular iron homeostasis is synthesized on free polyribosomes whereas a small amount of ferritin may be synthesized on the rough endoplasmic reticulum for secretion into the plasma (107, 108). The range of plasma ferritin in the normal adult varies between 15-300 μg/l (9, 107) and consists mainly of glycosylated L-subunit rich ferritins containing insignificant amounts of iron, even in conditions of iron overload (10, 107, 108, 109). While the iron content of ferritin in the liver and spleen could be more than 0.2 μg Fe/μg protein in conditions of iron overload, the iron content of plasma ferritin can be as low as 0.02-0.07 μg Fe/μg protein (110).

The regulation and functions of secreted ferritins in the plasma remain an enigma. However, a quantitative relationship exists between the level of plasma ferritin and the amount of storage iron (111). In conditions of iron overload there is generally an increase in the expression of intracellular L-subunit rich ferritins, paralleled by an increase in these ferritins in the plasma (83). Although the specific cellular origin of plasma ferritin is not known (112), various experiments indicated a large contribution made by the reticuloendothelial cell. An increase in plasma ferritin levels is known to occur in parallel with the increase in reticuloendothelial cell ferritin after an increase in reticuloendothelial cell iron during phagocytosis of non-viable red blood cells (12, 109). However, elevated plasma ferritin levels are also seen in patients with parenchymal iron overload whose reticuloendothelial cells are virtually devoid of iron (12). Therefore, it would appear that the plasma ferritin reflects storage iron anywhere in the body, regardless of the type of cell in which it was stored (12). However, plasma ferritin concentration is affected by a number of factors other than the amount of storage iron including tissue necrosis, damage to ferritin-rich tissue, inflammation, infections, neoplastic disease and increased red blood cell turnover (38, 111, 112, 113). When any of these conditions are present the relationship between plasma ferritin concentration and amount of storage iron no longer holds. With tissue necrosis, as in hepatocellular injury, the increase in plasma ferritin is for instance due to the release of ferritin from the damaged cells, since the increase in ferritin is dependent on both the magnitude of cellular damage and liver iron stores (83). Furthermore, an increase in non-glycosylated, iron-rich ferritin has been reported upon tissue damage, which is indicative of the release of tissue ferritin from the damaged tissue and not as a result of active secretion (108).

It is suggested that the increase in plasma ferritin is related to an increased production of ferritin by the malignant cells in various neoplastic diseases. In leukemia the normal concentration of ferritin in circulating leukocytes is increased up to six-fold in acute

myeloblastic leukemia, more than twenty-fold in acute myelomonocytic leukemia and two- to three-fold in chronic granulocytic leukemia (109). In the presence of various solid tumours, including tumours of the breast, pancreas and liver, an increase of H-subunit rich ferritins was shown in the cells of the tumour, as well as an increase in plasma ferritin. In addition, the plasma ferritins reflected this increase in H-subunit rich ferritins of the tumour, therefore the tumours seem to produce and secrete these H-subunit rich ferritins (114, 115).

The concentration of ferritin in plasma is a function of the rate of secretion or release on the one hand, and the clearance by other tissues on the other (113). The major cell type responsible for the clearance of plasma ferritins is the hepatocyte. A specific receptor for both glycosylated and non-glycosylated ferritin has been demonstrated on the hepatocyte membrane (113). These receptors bind both the H-subunit and the L-subunit of ferritin (116). However, a significant difference is indicated between the rates of clearance for the non-glycosylated ferritins of tissues and the glycosylated plasma ferritins (113). These differences in clearance may result in a significantly longer half-life for the glycosylated, secreted ferritins in the circulation compared to that of the non-glycosylated tissue ferritins (9, 113, 116, 117).

2.7.1) The internalization of ferritin by cells

Iron delivery to cells in general, and to developing erythroid cells in particular, is largely attributed to diferric transferrin (118). However, developing erythroid cells possess on their surface, in addition to transferrin receptors, receptors that bind specifically, and internalize, H-subunit rich ferritin (118). This binding and internalization of H-subunit rich ferritins is accomplished by means of a specific saturable process and is highly regulated by the iron status of the cell (77). Since, extracellular ferritin, once internalized by the cell, is indistinguishable from intracellular ferritin, extracellular ferritin could

possibly also function as an iron donor (77). It is, for instance, known that internalized ferritin can increase the cellular labile iron pool and decrease the levels of the iron responsive protein, whereas apoferritin (containing no iron) has an opposite effect. This supports the notion that internalized ferritin is an iron donor and suggests that apoferritin behaves like an iron chelator (77).

Developing erythroid cells in the bone marrow are often found in close proximity to a central "mother" reticuloendothelial cell which "feeds" ferritin to these developing red blood cell precursors (46, 119). This process, known as rhopheocytosis, is a highly regulated pathway for iron assimilation by erythroid progenitor cells while cytosolic ferritin serves as an intermediate pool for iron for haem synthesis (118, 120, 121). Erythroid progenitor cells contain receptors for ferritin and ferritin finds its way into the erythroid precursors by receptor-mediated endocytosis (45, 118). Ferritin first binds to coated invaginations or pits before appearing in coated intracellular vesicles followed by joining of the cytosolic pool of ferritin (45).

Not only developing eryhroid precursors can take up ferritin but ferritin is also rapidly internalized by hepatocytes. This presumably also occurs via a receptor-mediated pathway. Other cells, capable of pinocytosis or more generally endocytosis, have also been shown to take up ferritin from extracellular fluid. Therefore, ferritin present intracellulary could have either been synthesized by the cell or could have been taken up (92). Internalized ferritin can subsequently be degraded, similar to intracellularly produced ferritin, within the cell (122, 123) and its iron contents released into the labile iron pool of the cell (124).

2.7.2) Other functions of ferritin

Ferritin seems to have functions beyond the control of iron bioavailability – amongst others the down-regulation of myelopoiesis and the suppression of certain immune responses. H-subunit rich ferritins are present in most biological fluids, but not, or only in low concentrations, in plasma (125). The ferritin present in plasma is mostly L-subunit rich. However, during certain disease states the concentration of H-subunit rich ferritin is increased. At present it would appear that the H-subunit rich ferritins are derived mostly from monocytes and macrophages as indicated by the secretion of H-subunit rich ferritins from many monocyte-macrophage cell lines, as well as by monocytes from blood and bone marrow (126). The release of H-subunit rich ferritins from monocytes is controlled by T-cell subsets. T-helper cells enhance release and T-suppressor cells suppress the release (9).

A number of effects have been attributed to these H-subunit rich plasma ferritins including the down-regulation of myelopoiesis and the suppression of various immune functions (112, 126). It has specifically been shown that H-subunit rich ferritins, but not L-subunit rich ferritins, down-regulate myelopoiesis (127), i.e., the growth and development of granulocytes, macrophages, erythrocytes and platelets (54, 128), both *in vitro* and *in vivo*. It has been suggested that H-subunit rich ferritins constitute part of a normal inhibitory feed-back mechanism for the proliferation of granulocyte-macrophage colony forming units (CFU-GM), multipotential colony forming units (CFU-GEMM) and erythroid burst forming units (BFU-E) (119, 128). H-subunit rich ferritin decreases the proliferation of cells during myelopoiesis by directly affecting these progenitor cells (128). Surface receptors specific for H-subunit rich ferritins have been shown on these progenitor cells (129). These effects of H-subunit rich ferritins are mediated via the ferroxidase activity of the H-subunits – most probably by inducing intracellular iron starvation (41, 125), since addition of iron completely counteracts the inhibitory effects

of the H-subunit rich ferritins (130). Not only does H-subunit rich ferritins down-regulate the production of cells involved in the immune system, but H-subunit rich ferritins also suppress various functions of immune cells. H-subunit rich ferritins can for instance exert inhibitory effects on E-rosette formation of T lymphocytes (CD2 is the surface molecule on T-lymphocytes which facilitates binding to sheep erythrocytes and the formation of so-called E-rosettes), suppress the *in vitro* responses of lymphocytes to various mitogens including PHA and con A, inhibit the mixed-lymphocyte reaction, inhibit delayed-type hypersensitivity responses, block the access to T-lymphocytes by various regulatory factors by sitting on the surface of the cells (9, 131) and decrease leukocyte migration (9). Receptors for H-subunit rich ferritins have also been found on various T-cell lines, CD4 and CD8 T-lymphocytes and on CD19 B-lymphocytes, and the expression of H-subunit rich ferritin binding sites on these cells appears to be closely and positively linked to their activation and proliferation status (118, 125). It would therefore appear that H-subunit rich ferritins may perhaps act as feedback inhibitors of activation of peripheral blood cells in a way similar to that suggested for the cells involved in myelopoiesis. Quiescent circulating lymphocytes, reticulocytes, erythrocytes and monocytes show little expression of the H-subunit rich ferritin receptor, but PHA-stimulated lymphocytes, Epo-induced BFU cells and differentiated macrophages have all been shown to express above average levels of the receptor (132) which may result in these cells being more susceptible to inhibition by H-subunit rich ferritins. Increased binding of H-subunit rich ferritins to peripheral lymphocytes have also been shown to occur in patients with malignant disorders and the magnitude of H-subunit rich ferritin binding to lymphocytes was shown to be related to the stage of the malignant process (133). It is postulated that two receptor systems exist for the binding and execution of H-subunit rich ferritin's effects. The first receptor system internalizes the bound ferritin. This system is similar to the receptor system operating in erythroid precursors. However, a regulatory effect on cell proliferation and maturation occurs, whereas in

erythroid precursors such a regulatory effect has not been observed (77, 118). The second receptor system, with a Kd three orders of magnitude lower, does not result in the internalization of the bound ferritin (77, 118). This suggests a mechanism for the regulation of cellular proliferation and maturation by ferritin not involving iron or the sequestration of iron.

2.8) In conclusion

Although we are still far from understanding the exact role of ferritin and its isoforms in health and disease new information on the functions of ferritin and the movement of iron within the protein shell are surfacing. Information on the recently identified mitochondrial ferritin (101, 103), a ferritin molecule with biochemical properties very similar to H-ferritin provides, for instance, new insight into the movement of iron within the ferritin protein shell rich in H-subunits (134).

There can be no doubt that the primary function of ferritin is to regulate the bioavailability of iron and that both the H-subunit, which contains the ferroxidase center for oxidation of Fe^{2+} to Fe^{3+}, and the L-subunit, which plays a paramount role in the subsequent nucleation of Fe^{3+} and growth of the iron-core, are required for optimal control of this bioavailability. However, even in the context of the regulation of iron bioavailability and the effects thereof many questions about the exact mechanisms and about the interplay between the H- and L-subunits remain. It would in general appear that an increase in expression of the H-subunit especially, during times of cellular stress such as occurs during inflammation, brings about rapid sequestration of iron. The H-subunit with its active ferroxidase center reduces the labile iron pool, which would result in increased IRP activity, decreased cellular proliferation and increased resistance to H_2O_2-induced oxidative stress – effects that are apparently down-regulated by prolonged iron overload or inhibition of the ferroxidase center (135, 136). Information on the

influence of the degree to which L-ferritin is expressed is still somewhat contradictory. While it is generally accepted that the L-subunit assists the H-subunit in enhancing the incorporation of iron into ferritin by providing the major nucleation sites the increased expression of the L-subunit or in the L-subunit/H-subunit ratio of ferritin is important in cells responsible for iron storage of vast amounts of iron. Although the results of a number of studies point to a role for L-ferritin in limiting the bioavailability of iron (64, 137, 138, 139), dramatic increases in the levels of L-ferritin as seen in hereditary hyperferritenemia cataract syndrome, constitutive down-regulation of L-ferritin due to a mutation in the L-subunit start codon (136) or modification of the levels of L-ferritin by transfection with siRNA and cDNA (136) do, in general, not seem to have a significant influence on iron availability or compartmentalization (136).

While the primary function of ferritin is generally considered that of an iron storage protein that regulates the bioavailability of iron, other functions, some related to iron bioavailability and others not, are emerging such as its role in cellular proliferation where L-ferritin seems to increase (136, 140, 141, 142) and H-ferritin to decrease proliferation (136), in erythropoiesis where the H-subunit/L-subunit ratio is important in supporting iron supply for haemoglobin synthesis or storage of excess iron (69) and its regulatory role in the immune system where H-subunit rich ferritins suppress certain immune responses and down-regulate myelopoiesis (112, 126). Of interest is the fact that the suggested opposing effects of H- and L-ferritin would appear to be mediated through different mechanisms with the suppressive function of H-ferritin brought about by its effect on bioavailable iron with that of L-ferritin being independent of bioavailable iron (136). In addition, H-ferritin expression would appear to have antiapoptotic effects – not related to its iron-binding function (143).

2.9)   Figure 1: Heuristic presentation of intracellular ferritin metabolism

Synthesis of ferritin: Transcription (I) of the H-subunit and L-subunit ferritin genes occurs in the nucleus of the cell. This is followed by (II) the translocation of the H-subunit and L-subunit mRNA to a pool of translatable ferritin mRNA. Translation of the H-subunit and L-subunit mRNA of ferritin from this pool of translatable ferritin mRNA is largely controlled by iron from the labile iron pool (III) that contains the metabolically and catalytically reactive iron. In this pool of translatable ferritin mRNA (II) translation of the H-subunit and L-subunit mRNA, respectively, is prevented by binding of the iron responsive protein (IRP) to the iron responsive element (IRE) on the 5' non-coding stretch of H-subunit and L-subunit mRNA (II.1). Displacement of the IRP takes place upon binding of iron to IRP followed by translation (II.2). Translation of the ferritin mRNA takes place on free polyribosomes in the cytosol (IV). This is followed by folding of the translated H-subunit and L-subunit polypeptides into the α-helix rich tertiary structures of the H-subunit and L-subunit. These subunits form a pool consisting of H-subunits and L-subunits (V). From this pool of H-subunits and L-subunits the protein shell of ferritin consisting of 24 subunits symmetrically arranged is assembled (VI). The completely assembled iron-free ferritin (apoferritin) forms a pool of apoferritin containing different combinations of H- and L-subunits (VII). Ferritin can also be secreted from the cell (VIII).

Sequestration of iron (IX): Sequestration of iron is shown to occur after the ferritin protein shell is fully assembled. (IX.1) Oxidation of Fe^{2+} is performed by the ferroxidase centre of the H-subunit. This is followed by nuclei formation and iron core growth facilitated by L-subunits. Once the iron core reaches a sufficient size oxidation of Fe^{2+} can take place on the surface of the iron core. (IX.2) Oxidation of Fe^{2+} is performed by the ferroxidase centre of the H-subunit. However, if ferritin contains insufficient quantities of L-subunit for nuclei formation the formed Fe^{3+} can leave the ferritin molecule and move to a ferritin molecule containing sufficient quantities of L-subunit or

an already developed iron core, or (IX.3) the formed Fe^{3+} can leave the ferritin molecule followed by hydrolysis of Fe^{3+}-compounds on the outer surface of the ferritin molecule and ferritin aggregation.

Release of iron from ferritin (X): The release of iron from ferritin is shown to occur either by (X.1) simultaneous entry of a reductant and a chelator to the interior of the ferritin protein shell whereupon Fe^{3+} is reduced to Fe^{2+} in the confinements of the ferritin protein shell by the reductant followed by the release of Fe^{2+} as a Fe^{2+}-chelator complex, or (X.2) entry of only a chelator to the interior of the ferritin protein shell in which case Fe^{3+} is not reduced and leaves as a Fe^{3+}-chelator complex.

Distribution of ferritin (XI): Ferritin occurs in the cytosol either as dispersed ferritin particles (XI.1) or as ferritin clusters (XI.2).

Degradation of ferritin (XII): Two different processes can result in the degradation of ferritin. (XII.1) Degradation by the 20S proteasome enzymatic system, which recognises and degrades oxidised ferritin. (XII.2) Degradation by lysosome enzymes in a secondary lysosome. Ferritin finds its way into the secondary lysosome by either autophagocytosis (XII.2.1) or by targeting of ferritin to the secondary lysosome (XII.2.2). The latter can lead to haemosiderin and eventually siderosome formation.

Nuclear ferritin (XIII): Ferritin is also found in the nucleus. The ferritin in the nucleus consists of cytosolic H-subunit rich ferritins that are translocated back to the nucleus from the pool of apoferritin (VII) where these ferritins can form stable complexes with the DNA.

Mitochondrial ferritin (XV): Mitochondrial ferritin contains 24 identical subunits transcribed from a different gene than that for the cytosolic H-subunit and L-subunit (XIV). Upon transcription and translation the mitochondrial ferritin subunit polypeptide is translocated into the matrix of the mitochondria (XV.1). This is followed by the cleavage of the signal sequence and folding of the mitochondrial ferritin subunit (XV.2). Typical hollow spherical ferritin shells containing 24 subunits are then assembled from

these subunits (XV.3). Cytosolic iron from the labile iron pool (III) transverses the double membrane of the mitochondria followed by sequestration by mitochondrial ferritin (XV.4).

Processes involved in cellular iron acquisition (XVI-XVIII): (XVI) The transferrin receptor binds transferrin and is endocytosed. Upon acidification of the endosome iron is released into the labile iron pool (III). (XVII) The ferritin receptor binds ferritin and is endocytosed. Ferritin is degraded in a secondary lysosome and the released iron joins the labile iron pool (III). (XVIII) Red blood cells are phagocytosed followed by degradation and the release of heme iron by heme oxygenase. The released iron joins the labile iron pool (III).
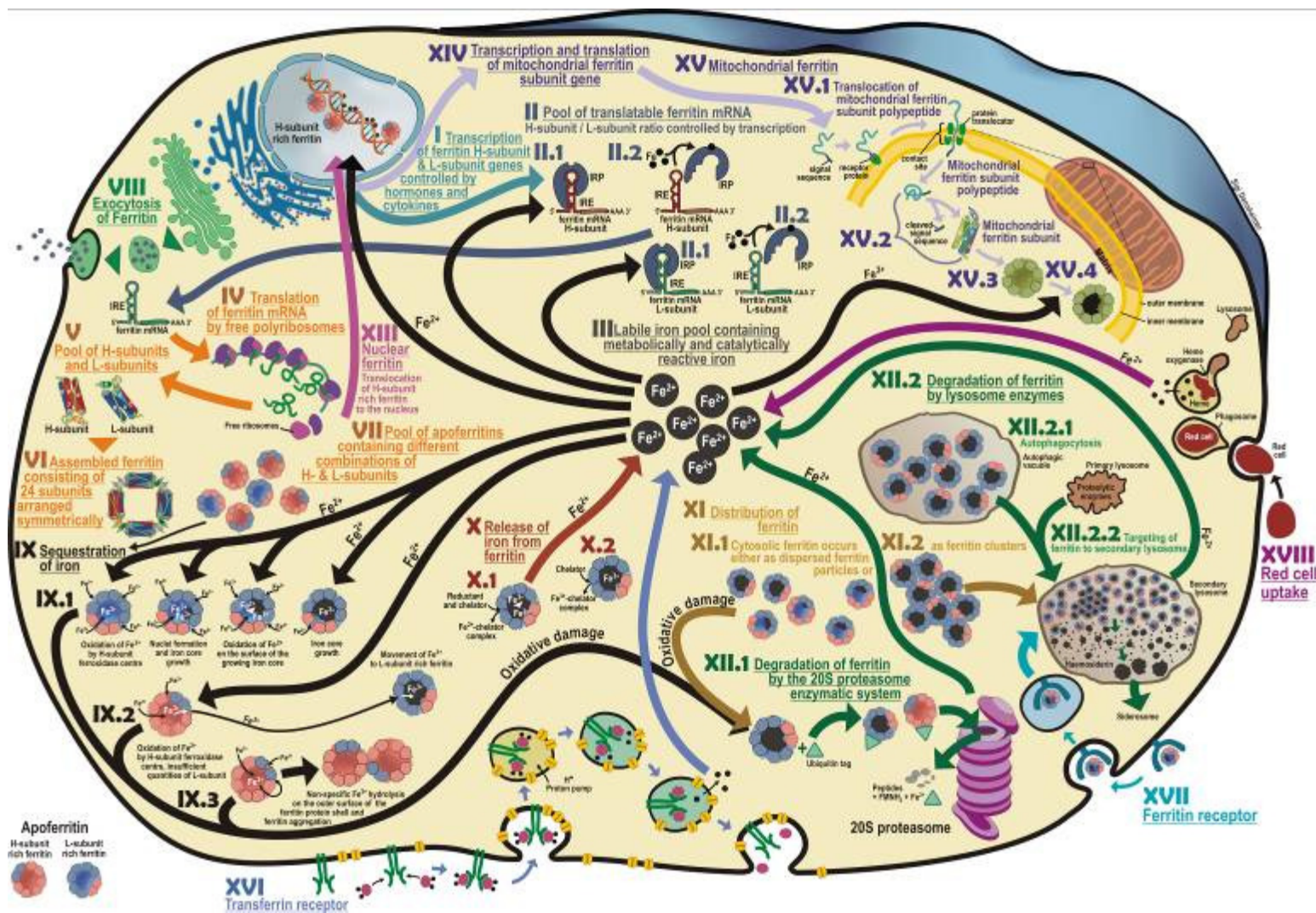
3) **Ferritin and ferritin isoforms:**

   Protection against uncontrolled cellular proliferation, oxidative damage and inflammatory processes

The regulation of iron availability is of paramount importance for the viability of cells. Iron is, on the one hand essential for the functioning of various enzymes, while on the other, supports the formation of reactive oxygen species (ROS). Ferritin is a major iron storage protein that controls iron availability in the body. Ferritin consists of a protein shell enclosing an inner cavity where variable amounts of iron are stored as a ferrihydrite mineral. The ferritin protein shell consists of 24 subunits of two different types, the H-subunit and the L-subunit. The H- and L-subunits have different roles in the sequestration of iron. In view of the role of ferritin in iron homeostasis and the functional differences between the ferritin subunits, it does not come as a surprise that the H- and L-subunits of ferritin are differentially expressed in cells with different functions and in different disease states. Present indications are that, depending on the cell type, the developmental status, and the presence of pathological conditions, ferritins from different cells form characteristic populations of heteropolymers or isoferritins (57, 83). These heteropolymers or isoferritins are functionally distinct and, depending on the ratio of H- and L-subunits present in the ferritin protein shell, iron is differently metabolized.

Different combinations of the two subunits of ferritin give rise to the existence of isoferritins with different metabolic properties. H-subunit rich ferritins have been shown to accumulate and release iron faster than L-subunit rich ferritins (9, 25, 57, 58) and it is suggested that the H-subunit rich ferritins permit a more dynamic intracellular traffic of iron than L-subunit rich ferritins (25, 59). H-subunit rich ferritins are also responsible for the rapid sequestration of iron in situations where iron can contribute to damage to the cell. The expression of the H-subunit and the L-subunit of ferritin is controlled by

both transcriptional and translational mechanisms. However, there are indications that the level of H-subunits and H-subunit rich ferritins is strictly controlled by an additional mechanism. Cells limit the accumulation of H-subunits by differentially secreting H-subunits in variance with L-subunits. Such a mechanism could play a significant role in regulating the amount of cytosolic H-subunit rich ferritin and might protect the cell against unwarranted rapid sequestration of iron by H-subunit rich ferritins (144). L-subunit rich ferritins apparently contain more iron than those ferritins rich in H-subunits (25, 60) and there are indications that the L-subunit rich ferritins predominate in cell types that play a role in the storage of iron (22, 25, 62).

The differential expression of the H- and L-subunits of ferritin, i.e., the distribution of isoferritins are documented for various disease states. However, no clear-cut picture has emerged, either of the homeostatic changes that control the differential expression of these two subunits or the functional importance of different isoferritins. It is suggested that the optimum differential expression of these two subunits for a specific type of cell offers protection against uncontrolled cellular proliferation, increased oxidative stress and inflammatory conditions.

3.1) Ferritin and the differential expression of the H- and L-subunits of ferritin during uncontrolled cellular proliferation

Plasma ferritin is elevated in various types of cancers, irrespective of the amount of total body iron (145). It has been suggested that plasma ferritin levels can be used as tumour markers for prognostic purposes and in monitoring the activity of certain types of cancer (146, 147). In patients with solid tumors, such as pancreatic carcinoma, lung cancer and hepatoma, there is a particularly high prevalence of elevated plasma ferritin, and in patients with breast cancer, with metastasis, ferritin plasma concentrations are commonly elevated (38). Squamous cell carcinoma of the head and neck is marked by increased

plasma ferritin concentrations, which show a tendency to increase and to remain high in patients with a poor prognosis in contrast to patients with a favourable prognosis (148). The plasma ferritin concentrations of patients with haematologic malignancies are well documented. Extremely high plasma ferritin levels are seen in acute myeloblastic leukemia whereas in complete remission ferritin plasma concentrations could be returned back to normal (145). In Hodgkin's disease plasma ferritin concentrations are related to the stage of the disease, increasing from stage 1 to stage 4 (38). In non-Hodgkin's lymphoma a remarkable correlation exists between plasma ferritin concentrations and tumour histology. The highest plasma ferritin concentrations are found in patients with active histiocytic lymphoma and the lowest plasma ferritin concentrations in patients with lymphocytic lymphoma whereas intermediate plasma ferritin concentrations are found in patients with mixed histology (38). Many factors are suggested to contribute to the hyperferritinaemia associated with cancer, including inflammation, hepatic necrosis due to metastasis and chemotherapy, blood transfusions and a decrease in hepatic clearance of ferritin (149). In addition, a modified and increased synthesis and secretion of ferritin by tumour cells occur (146, 149, 150, 151). In many instances the increased ferritin is shown to be H-subunit rich (149, 152, 153, 154, 155, 156) and it has been suggested that the measurement of H-subunit rich ferritin may be of value in the diagnosis of malignancy (154).

3.1.1) Cellular proliferation, ferritin subunits and cancer

The most common feature of cancer is the abnormal proliferation of cells, either contained in a specific location, or following metastasis at different sites involving various organs. Iron is a necessary element for cellular proliferation and it is generally accepted that rapidly dividing cells require more iron for their growth and metabolism than resting cells. It is also known that cells normally display an increase in cellular proliferation upon an increase in the labile iron pool (138). The reason for the high need

for iron is that iron is necessary for the functioning of different enzymes involved in cellular proliferation, including ribonucleotide reductase, which controls a rate-limiting step in DNA synthesis, and for various mitochondrial enzymes involved in the metabolism of the cell (151, 157). Not only do malignant cells require more iron for growth and metabolism than normal cells, but the cellular labile iron pool can in turn modulate the magnitude of induced cellular proliferation by the oncogene H-ras (158). One way in which to bring about an increase in the cellular labile iron pool is by suppression of ferritin synthesis. The transcription factor encoded by the proto-oncogene c-MYC, which is responsible for proliferation of normal cells, can during uncontrolled expression, result in cellular transformation and excessive cellular proliferation. c-MYC can, where appropriate, activate or repress target genes in order to bring about cellular proliferation (159). The expression of the H-subunit gene is shown to be down-regulated by c-MYC and to be essential for the control of cellular proliferation and transformation by c-MYC (159). This is in agreement with the fact that the H-subunit is responsible for controlling the labile iron pool and that down-regulation of H-subunit expression would result in an increase in the labile iron pool.

3.1.2) Cellular differentiation

Ferritin is also implicated in the differentiation of cells. Cellular differentiation entails the expression of specific proteins in order for the cell to establish a differentiated phenotype and to perform specialized functions. Cellular differentiation is a controlled event and can be activated by a vast number of stimuli depending on the type of cell. Oligodendrocytes are for instance responsible for the synthesis and maintenance of central nervous system myelin and the differentiated phenotype of the oligodendrocyte will therefore express all proteins necessary for the assembly of large quantities of membranes (160). The stimuli responsible for inducing differentiation of oligodendrocytes involve the adhesion of the oligodendrocyte to a substrate. It was

shown that the expression of the H-subunit of ferritin is up-regulated upon substrate-adhesion and the induction of oligodendrocyte differentiation (160). In various other cell types, including pre-adipocytes, erythroid cells, neuronal cells and monocyte to macrophage differentiation, the expression of the H-subunit of ferritin is similarly up-regulated upon differentiation (74, 161, 162). In various cancers cellular differentiation can bring about the suppression of tumourigenicity since cellular differentiation results in cell cycle arrest and inhibition of cellular proliferation. Differentiation of, for instance colon carcinoma cells is, as for normal cells, accompanied by an increased expression of the H-subunit of ferritin (163). Metastasis is characteristic of the uncontrollable progression of cancer whereas tumour cells displaying a more differentiated phenotype generally show less metastatic activity. H-subunit rich ferritins may perhaps be involved in curtailing the spread of cancer as it was shown that the over-expression of the H-subunit of ferritin in a rat transitional cell carcinoma progression model is associated with less tumour cell metastasis (164). It would thus appear that the increased expression of the H-subunit of ferritin might be involved in the initiation of cellular differentiation and therefore the prevention of uncontrolled cellular proliferation and metastasis.

3.1.3) Programmed cell death (apoptosis)

Programmed cell death or apoptosis, in which cells actively participate in their own self-destruction, is an important process in eliminating abnormally proliferating cells. Once again ferritin is implicated. Many tumour-suppressor genes can induce apoptosis upon DNA damage or other cellular distress signals. One such tumour-suppressor gene, the transcription factor p53, is up-regulated in many types of malignant cells in an attempt to kill-off rapidly proliferating cells by activating the programmed cell death pathway. A shift in the H-subunit/L-subunit ratio would appear to occur in such conditions. H-subunit ferritin mRNA is for instance differentially up-regulated in an immortal human breast epithelial cell line treated with chemical carcinogens and in various breast cancer

cell lines, in contrast to mortal or primary human breast epithelial cell lines (153). Increased H-subunit ferritin mRNA was also detected in breast cancer tissue samples and tissue with ductal hyperplasia had higher expression of H-subunit than normal adjacent mammary tissue (153). This up-regulation of the H-subunit of ferritin may be an attempt of the cell to induce apoptosis since a significant correlation was shown between H-subunit and p53 expression in primary hepatic carcinoma tissue compared to non-malignant liver and healthy tissue (165).

From the above discussion one can perhaps speculate that an increase in the H-subunit of ferritin may play a role in curtailing excessive cellular proliferation and, although conclusive evidence does not exist, it would appear that a) hyperferritinemia with an increase in the H-subunit/L-subunit ratio occurs in many types of cancers, b) the H-subunit may perhaps stimulate differentiation in an attempt to curtail uncontrolled proliferation and c) that H-subunit expression is up-regulated together with p53 expression as part of the process of apoptosis. Whether H-subunit rich ferritins in this context act through the modulation of iron or as a growth factor is unclear.

3.2) **The expression of the H- and L-subunits of ferritin in diseases and toxicities associated with an increase in reactive oxygen species (ROS) generation**

Oxygen free radicals are implicated in the pathogenic processes of various diseases and toxicities and ROS are known to damage critical cellular components including DNA, proteins and lipids (166). The two reactive oxygen species, superoxide and hydrogen peroxide, are virtually always present in cells due to basal cellular processes and could also be formed upon activation of polymorphonuclear leucocytes and macrophages. However, it is only in the presence of iron that these two reactive oxygen species become highly toxic. Superoxide and hydrogen peroxide can interact to form the very toxic

hydroxyl radical in the Haber-Weiss reaction. However, the reaction rate for this reaction is very low, but when iron is present and once reduced by superoxide, it can rapidly form hydroxyl radicals in a reaction with hydrogen peroxide (167, 168).

Ferritin plays a prominent role in the protection of cellular constituents against such possible oxygen free radical onslaughts by sequestering iron in a non-toxic form. This is due to modulation of the expression of ferritin by a variety of factors associated with oxidative stress that act either directly on gene expression, or indirectly via the modification of the activity of the iron regulatory proteins (139, 169, 170). In disease states associated with an increase in reactive oxygen species the H-subunit of ferritin is preferentially up-regulated. Ferritin containing a high proportion of H-subunits increases the resistance against oxidative challenges since H-subunit rich ferritins are capable of rapidly sequestering redox-active iron, thus suppressing the formation of reactive oxygen species (138, 171, 172, 173).

3.2.1) Oxidative stress and neurodegenerative diseases

The brain contains high levels of iron and an uneven cerebral distribution of iron exists with high levels in the basal ganglia (substantia nigra, putamen, caudate nucleus and globus pallidus), red nucleus and dentate nucleus (174). The brain accumulates iron with age due to the slow turnover rate of iron and a continuous up-take of iron throughout life (175). The amount of iron accumulating in each brain region varies (176). Such an accumulation of iron could result in increased oxidative damage if increases in anti-oxidative mechanisms do not match that of iron.

The expression of ferritin has been shown to increase in different brain regions with normal aging and this, as a result of ferritin's iron-sequestering capabilities, would provide the brain with additional protection against age-related increases in oxidative

stress (176, 177, 178). The two subunits of ferritin are expressed to different extents in different brain regions and it changes with normal aging. In the young brain the H-subunit of ferritin is generally more abundant than the L-subunit – except in the globus pallidus where the ratio of H-subunit to L-subunit is 1:1. With normal aging both the H- and the L-subunit of ferritin increases. The H-subunits of ferritin increase in most brain regions in a remarkably consistent pattern, i.e., approximately two-fold. However, in the globus pallidus the increase is eight to ten-fold greater than the increase in the other regions. The increase in L-subunit expression is more region-specific than that of the H-subunit. In addition, the age-related expression of the H- and L-subunit of ferritin shows an increase in the H-subunit/L-subunit ratio for all brain regions, except for the substantia nigra where this ratio decreases with aging (176).

It has been suggested that the pathology of various neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease, involves an excessive increase in iron in specific brain regions and that premature increases in iron may be an early risk factor for the onset of neurological disease (175). In both Parkinson's disease and Alzheimer's disease the normal age-associated increase in the expression of ferritin and the expected change in the H-subunit/L-subunit ratio fail to occur (176, 179, 180). An increase in iron content without the concomitant up-regulation of ferritin expression and the expected change in the H-subunit/L-subunit ratio would leave these brain regions vulnerable to oxidative stress. In Parkinson's disease, dopaminergic brainstem nuclei, particularly the substantia nigra pars compacta is destroyed. Clinical evidence that iron-induced oxidative damage may in fact be involved was provided by the observation of an increase in the total iron content without an increase in ferritin expression in the substantia nigra. Evidence of excessive oxidative damage was substantiated by the presence of increased basal levels of malondialdehyde and lipid hydroperoxides, markers of lipid peroxidation, as well as by a decrease in levels of reduced glutathione (179).

3.2.2) Oxidative stress and vascular disorders

The endothelium plays an important role in the regulation of vascular function and integrity. It produces many mediators responsible for the regulation of vasodilatation and vasoconstriction, blood-clotting including pro-thrombotic, anti-thrombotic and fibrinolytic substances, as well as molecules promoting the adhesion of platelets, monocytes and leukocytes (181). The endothelium is highly susceptible to damage caused by reactive oxygen species. Reactive oxygen species, for instance, cause changes in the ionic permeability of the endothelium (182), modulation of the fibrinolytic response of the endothelium (183), changes in the endothelium's contribution to platelet aggregation (184) and abnormal functioning of endothelium-derived relaxing factor (185, 186, 187, 188). Iron is responsible for generation of the very toxic hydroxyl radical via Fenton-type chemistry and these reactive oxygen species are implicated in the pathogenesis of numerous endothelium-associated vascular disorders including atherosclerosis, microangiopathic haemolytic anaemia, vasculitis and reperfusion injury (189). Oxidant-mediated injury potentiated by iron includes direct damage to the endothelial cells by reactive oxygen species, as well as promotion of the conversion of low-density lipoprotein to cytotoxic oxidized products. This iron-catalyzed oxidant injury to endothelial cells can be attenuated by the addition of exogenous iron chelators such as the lazaroids and desferoxamine (190).

One abundant source of redox-active iron in the vasculature is haem and exposure of endothelial cells to haem greatly enhances cellular susceptibility to oxidant-mediated injury (189). The most important source of haem within the vascular endothelium is haemoglobin. Upon oxidation of haemoglobin the released haem, a hydrophobic iron chelate, can be transferred to the endothelium and is rapidly incorporated into the endothelial cells (189). As a mechanism of defense against such free haem-induced toxicity cells, including endothelial cells, up-regulate haem oxygenase-1, as well as ferritin.

The expression of haem oxygenase-1 can be induced by a number of stressful stimuli including its own substrate haem, various haem proteins, heavy metals, UVA radiation, hypoxia, hyperoxia, ischaemia-reperfusion and many others (191). Haem oxygenase-1 is a haem-degrading enzyme that opens the porphyrin ring, producing biliverdin, carbon monoxide and free redox-active iron (192). Ferritin, which is simultaneously up-regulated, effectively sequesters the released iron and prevents the iron from taking part in oxidant-mediated cellular damage (189, 193). Aspirin has been shown to increase endothelial resistance to oxidative damage in bovine pulmonary artery endothelial cells by inducing the synthesis of ferritin, up to five-fold above basal levels, in a time and concentration-dependent fashion (194). In addition to the endothelial defense strategies against haem iron, local macrophages also play a role. Vascular associated macrophages can endocytose senescent erythrocytes or free haemoglobin as a haptoglobin-haemoglobin complex (195). After the breakdown of the haemoglobin by the macrophage the haem iron is released followed by sequestration of the iron by macrophage ferritin. Haem iron leads to increased synthesis and secretion of ferritin by macrophages (196). Erythrophagocytosis and haemoglobin catabolism by macrophages occur in microvessel-rich regions and contribute to the formation of iron deposits and ferritin induction in atheroma (195). However, the accumulation of iron in atherosclerotic lesions in the macrophage is known to exacerbate iron-induced damage to the vasculature by promoting, amongst others, LDL oxidation by reactive oxygen radical production (196).

The effect of haem on ferritin and ferritin subunit expression is experimentally confirmed. In haemin (ferriprotoporphyrin IX – the oxidized prosthetic group of haemoglobin) treated cells the iron uptake capacity of cells was seen to be greatly enhanced and to contain approximately three times more iron per ferritin molecule. The expression of the H-subunit of ferritin was elevated 12- to 15-fold whereas the L-subunit

was essentially unchanged. These results are consistent with the idea that H-subunit rich ferritins sequester redox active iron rapidly and copiously, thereby enhancing cellular resistance to oxidative damage (172). The same shift towards H-subunit rich ferritin can occur during post-ischaemic reoxygenation where iron could catalyse the production of reactive oxygen species. Over-expression of the H-subunit gene has been reported to provide protection against such ischaemia-reperfusion injury and to prevent cellular damage upon organ transplantation (197). In addition, the H-subunit may in these circumstances prevent cells from undergoing apoptosis induced by reactive oxygen species (193). The up-regulation of the H- to L-subunit ratio in similar conditions is, however, not uniformly supported. A significant induction of both the H- and L-subunit mRNA of ferritin was reported by Chi *et al* as a result of ischaemic events and it was shown that the H- and L-subunit mRNA can remain elevated for up to 336 hours after the onset of reperfusion. This increase in H- and L-subunit of ferritin was associated with iron deposits in areas where cell death and tissue necrosis was noted (198).

More evidence for the up-regulation of ferritin expression by ischaemic injury was seen in the myocardium, cerebral arteries and spinal cord. Ischaemia was seen to result in an enhanced ferritin content relative to the degree of ischaemia in the myocardium (199). Ferritin expression is also up-regulated during bleeding episodes, since haemolysis will substantially contribute to deleterious free redox active iron. In adventitial fibroblasts of cerebral arteries and cells in the subarachnoid space the increase in ferritin levels are known to persist for prolonged periods of time after subarachnoid haemorrhage (200). With traumatic spinal cord injury with haemorrhage, ferritin expression in microglia and astrocytes was seen to be significantly increased and to correlate with the severity of the injury (201).

3.2.3) UV-induced oxidative damage

Ultraviolet irradiation results in increased oxidative stress in various cell types exposed to sunlight, including the corneal epithelial cell and epidermal and dermal fibroblasts. Free iron, by supporting the Fenton reaction is known to exacerbate UV-induced oxidative damage to cellular constituents. As for other cell types challenged by increased reactive oxygen species generation, corneal epithelial cells and epidermal and dermal fibroblasts also display an increase in ferritin expression upon UV irradiation (202, 203). It has even been shown that corneal epithelial cells that contain increased amounts of nuclear ferritin show less DNA breakage (203). Although over-expression of the L-subunit of ferritin does not seem to have a major effect on cellular iron distribution or to protect lens epithelial cells against UV irradiation, over-expression of the H-subunit of ferritin appears to result in increased storage of iron in ferritin as well as protection of cells from UV (photo-oxidative stress) damage (204).

One can summarize by saying that ferritin synthesis in general would appear to be up-regulated in the presence of an oxidative onslaught. Indications are that, an increase in H-subunit rich ferritins could be the mediating factor in the protection against oxidative damage. The H-subunit rich ferritins accomplish this through the rapid sequestration of iron, thus preventing the generation of highly toxic oxygen radicals.

3.3) The expression of ferritin and the differential expression of the H- and L-subunits of ferritin in inflammatory conditions

The inflammatory response can be triggered by mechanical injury, chemical toxins, invasion by microorganisms and hypersensitivity reactions (205) and may be described as the body's attempt to invade the area of tissue damage, contain the response to an isolated area, destroy the initial injurious agent, break down damaged tissue and finally repair/regenerate the destroyed tissue. As part of the inflammatory response neutrophils

and macrophages are recruited to the inflammatory site. Neutrophils and macrophages are responsible for phagocytosis and degradation of microorganisms, immune complexes and necrotic tissue. These phagocytes engulf the substance and encapsulate it in a membrane-bound phagocytic vacuole (phagosome). Many different active substances including enzymes and oxygen radicals are delivered to the phagosome to destroy and degrade the phagocytosed material (206).

Oxygen radicals, i.e., molecules containing unpaired electrons are generated in large amounts during infectious conditions and inflammatory responses (207). They react with proteins, lipids and nucleic acids, resulting in degradation of the phagocytosed material in the confinements of the phagosome in the neutrophil and macrophage. However, large amounts of these toxic metabolites leak to the fluids and tissues in the area of the inflammatory reaction and by reacting with cellular constituents can result in substantial damage (206, 208). Iron, due to its role in Fenton-type chemistry, can result in exacerbation of oxygen radical production. Such an increase in unwanted oxygen radical production due to toxic amounts of iron can be seen in chronic inflammatory conditions such as rheumatoid arthritis. An increase in the iron content in the synovium is, for instance, present in rheumatoid arthritis (209) and a significant correlation exists between thiobarbituric acid-reactive material (lipid peroxidation product), the amount of iron in the synovial fluid and the inflammatory activity of the disease (210). Furthermore, when anaemic rheumatoid arthritis patients receive iron supplementation, lipid peroxidation is stimulated resulting in worsening of the synovial inflammation (211).

In general, a reduction in the bioavailability of iron will offer protection against cell injury by hydroxyl radicals that are generated from neutrophil- and macrophage-derived superoxides (212). Iron sequestration by cells in the zone of inflammation may therefore provide protection against the free radical assault (76). This role of host cell protection

against an increase in the free radical onslaught is consistent with observations that a reduction in ferritin sensitizes cells to pro-oxidant cytotoxicity, and that overexpression of ferritin reduces reactive oxidant species in cells challenged by oxidants and by implication reduces the oxidative toxicity (112). Macrophages, although contributing to the production of ROS, can also provide protection against it by reducing the available iron.

3.3.1) The macrophage, iron metabolism and ferritin in inflammatory conditions

The macrophage and other macrophage-like cells are responsible for handling large amounts of iron in the body. In inflammatory conditions the magnitude of iron released by the macrophage is strongly influenced. Inflammation produces a shift in iron handling by the macrophage in favour of iron storage, in time leading to haemosiderosis of the macrophage (9, 12, 213) and a corresponding hypoferraemia (9, 96, 97). It has been proposed that this abnormal retention of iron by the macrophage is caused by an increase in ferritin synthesis as a non-specific acute-phase reactant of inflammation (9, 214). This increase in ferritin synthesis occurs prior to the reduction of serum iron levels and is considered to result in a diversion of iron from the intracellular labile iron pool to ferritin and subsequently to haemosiderin (9, 215, 216, 217, 218). The increase in ferritin expression by the macrophage mostly influences the early phase of iron release. In normal conditions two-thirds of the iron entering the macrophage/reticuloendothelial system (RES) is released during this phase, but an increase in ferritin expression can result in a decrease in the release of iron during this phase to only 10% of the iron entering the macrophage/RES (218, 219). However, the slow release phase of iron from the macrophage is also influenced and can result in a situation where 33% of the iron is still present in storage form in the macrophage/RES after 60 days (220). Furthermore, once the macrophage and other macrophage-like cells have been activated, as occurs during inflammation, these cells express increased levels of transferrin receptors (96) and

are therefore able to acquire increased amounts of iron by endocytosis of the iron-transferrin-transferrin receptor complex. The increase in iron uptake via this route will contribute to the high magnitude of iron sequestration and to the ensuing haemosiderosis that develops in macrophages and other macrophage-like cells. These iron-withholding mechanisms are implemented as a defense strategy in order to deplete biologically active iron in the zone of inflammation or, once the inflammatory response cannot be contained, systemically (221). This is important because elevated levels of available iron can promote both the growth of infectious microorganisms and oxidative stress. It has also been shown that up-regulation of H-subunits results in the inhibition of pro-inflammatory cytokine induced apoptosis by suppressing reactive oxygen species generation (222). This probably occurs as a negative-feedback anti-inflammatory response.

3.3.2) Increased ferritin expression as a result of cytokine activation

Cell-to-cell communication molecules known as cytokines play an important role in mediating the process of inflammation (205). Inflammation progresses as a result of the action of pro-inflammatory cytokines, including Il-1, TNF, IFN-γ, Il-12, Il-18 and the granulocyte-macrophage colony-stimulating factor and is resolved by anti-inflammatory cytokines such as Il-4, Il-10, Il-13, IFN-α and TGF (223). Ferritin is considered to be one of the acute phase proteins and its expression has been shown to be increased by various cytokines (80, 214). Cytokine-induced increases in intracellular ferritin expression during the acute phase response can result in the sequestration of metabolically available iron in the macrophage/RES and the subsequent decrease in plasma iron. It has in fact been shown that activation of macrophages by cytokines such as TNF-α and Il-1 could result in the slower release of iron compared to non-stimulated macrophages, thus supporting the proposed role of cytokines in ferritin-mediated iron sequestration by macrophages (215, 224). The concept that an increase in intracellular

ferritin, as well as secretion of ferritin, can be induced by pro-inflammatory cytokines as part of the acute phase response is further supported by the fact that the secretion of ferritin was shown to be stimulated by cytokines in a primary human hepatocyte culture where IL-1α and Il-6 induced a transient secretion of ferritin at 24 hours, followed by a decline to baseline, and TNF treatment resulted in a sustained increase in ferritin secretion (16).

Cytokines reported to have the ability to induce ferritin expression include Il-1α, Il-1β, Il-2, Il-6, TNF-α, and IFN-γ – all pro-inflammatory cytokines (Table 1). These cytokines modulate ferritin expression by both transcriptional and translational mechanisms (80), but largely by an increase in the rate of transcription of the ferritin gene (16, 225). The expression of the ferritin subunits is differentially regulated by cytokines and it is mostly the H-subunit of ferritin that is increased by cytokine induction at variance with the L-subunit (225). *In vitro* experiments with various cell types showed an increase in H-subunit expression relative to L-subunit expression upon cytokine activation (Table 1).

The role of ferritin in inflammatory conditions can be summarized by saying that pro-inflammatory cytokines increase the production of ferritin early in the inflammatory response and that H-subunit rich ferritins are preferentially up-regulated at variance with L-subunit rich ferritins. This process does not only protect the body against reactive oxygen species generation, but in addition reduces the bioavailability of iron needed by pathogenic microorganisms.

3.4) Table 1: The effects of cytokines on the expression of H-subunits and L-subunits of ferritin

Cytokine	Cell type	Effect/H-subunit	Effect/L-subunit
Il-1β	Human Hepatoma Cells (212)	Not determined ↑ translation	↔ mRNA ↑ translation
Il-1β	Human Hepatoma Cells (226)	↔ mRNA ↑ translation 15-fold	↔ mRNA ↑ translation 6-fold
Il-6	Human Hepatoma Cells (226)	↔ mRNA ↑ translation 3-fold	↔ mRNA ↑ translation 4-fold
Il-1β	Primary Human Umbilical Vein Endothelial Cells (226)	↑ expression 4.8-fold ↔ mRNA	↑ expression 2.4-fold ↑ mRNA 30%
TNF-α	Mesenchymal, Myo-blasts, Myocytes, Adipocytes, Fibro-blasts (16)	↑ mRNA	↔ mRNA
Il-1α	Mesenchymal, Myo-blasts, Myocytes, Adipocytes, Fibro-blasts (16)	↑ mRNA	↔ mRNA

TNF-α	U937 Macrophage (16)	↑ mRNA	↔ mRNA
INF-γ	U937 Macrophage (16)	↑ mRNA	↔ mRNA
Il-1β	U937 Macrophage (16)	↔ mRNA	↔ mRNA
TNF-α	A549 Type-2 Alveolar Pneumocyte (16)	↑ mRNA	↔ mRNA
Il-1β	A549 Type-2 Alveolar Pneumocyte (16)	↑ mRNA	↔ mRNA
Il-1β	THP-1 Monocyte (227)	↔ mRNA	↔ mRNA
TNF-α	THP-1 Monocyte (227)	↑ mRNA	↑ mRNA
TGF	THP-1 Monocyte (227)	↔ mRNA	↔ mRNA
PDGF	THP-1 Monocyte (227)	↔ mRNA	↔ mRNA
Il-1	Aortic Smooth Muscle Cells (227)	↑ mRNA	↔ mRNA
TNF-α	Aortic Smooth Muscle Cells (227)	↑ mRNA	↔ mRNA

TGF	Aortic Smooth Muscle Cells (227)	↔ mRNA	↔ mRNA
PDGF	Aortic Smooth Muscle Cells (227)	↔ mRNA	↔ mRNA
TNF-α	Alveolar Epithelial Cells (228)	↑ subunit	↑ subunit but less
TNF-α	Primary Human Myoblasts (225)	↑ mRNA	↔ mRNA

3.5) In conclusion

Ferritin is a major iron storage protein that regulates the bioavailability of iron in the body. Although H- as well as L-subunits of ferritin are both necessary for sequestration and release of iron into and from ferritin, different isoferritins seem to have preferential functions. While, under basal conditions, the L-subunit rich isoferritins would appear to be the major storage depots and predominate in tissues with iron storage functions, H-subunit rich ferritins predominate in metabolically more active tissues and are up-regulated in conditions of stress. H-subunits seem to be the up-regulated subunits with, preferentially, regulatory functions. H-subunits are up-regulated when the need for rapid change of iron availability arises – very often to suppress unwarranted iron-stimulated events, such as the formation of toxic radicals, uncontrolled proliferation of cells or the growth of pathogenic microorganisms.

4) Aim of the study

The primary aim of the present study is to quantitatively measure the expression of the H-subunit and L-subunit of ferritin in the bone marrow macrophage and cells of the erythron in patients with chronic immune stimulation. A second aim is to investigate the possible role that the expression of the H-subunit and L-subunit of ferritin may have in the establishment and maintenance of an iron transfer block in patients with chronic immune stimulation.

Due to the amount of data it was necessary to divide the thesis into two volumes for binding purposes. Volume 1 contains the thesis proper. Volume 2 contains raw data, photographs and micrographs and experimental evaluation of the technique for ultrastructural immunolocalisation of the H-subunit and L-subunit of ferritin.

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