The transferrin receptor: the iron bridge between the cell and its environment

A diagnostic tool in daily practice?

Bij de omslag:-

Evenals de Van Brienenoordbrug is de transferrinereceptor een dimeer.

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De transferrinereceptor: de ijzer brug tussen de cel en de omgeving

Belang als diagnostische test in de dagelijkse praktijk?

Proefschrift

Ter verkrijging van de graad van doctor aan de
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Het onderzoek beschreven in dit proefschrift werd voornamelijk verricht in het klinisch chemisch laboratorium van het Sophia Ziekenhuis in Zwolle. Een onderdeel van hoofdstuk 6 werd uitgevoerd bij de Vakgroep Biochemie, afdeling Chemische Pathologie van de Erasmus Universiteit Rotterdam.

Naar gelang van zijn ontwikkeling kent de mens de waarheid;
hoe meer hij weet, hoe meer hij ontdekt dat er nog meer valt te weten

Uit: Reizigers op het innerlijke pad, Hazrat Inayat Khan

Voor Egbert

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Introduction

1.1 Iron metabolism

"The versatility of uses Nature has found for iron originates in the simple aqueous chemistry of this essential transition metal. Of the diverse chemical reactions of iron in solution the most important is the facile and reversible one-electron oxidation-reduction reaction that takes iron between its two common oxidation states, the ferrous and the ferric" (1). It is for this reason that iron is a key element in many biochemical processes and shortage of iron causes damage to cells and organs. On the other hand it is also this feature which makes iron one of the most harmful elements, because it is able to catalyse the formation of highly reactive oxygen and hydrogen radicals when present in the unbound state. These radicals can cause permanent damage to intracellular proteins and DNA.

Almost all organisms from micro organisms and plants up to the higher organisms like humans require iron, but at a neutral pH the solubility product of iron is extremely low ($Fe(OH)_3 = 4x10^{-38}$), which makes iron almost insoluble. For this reason proteins have developed to manage the storage and transport of iron between cells.

1.1.1 Iron uptake

The body's iron status is almost completely dependent on the dietary intake. The gastrointestinal tract plays a major role in maintaining iron balance, especially the mucosa cells of the upper intestine (2).

To study iron absorption a distinction has to be made between the absorption of haem iron and non-haem iron. The major source of haem iron is food derived from animal tissues with haemoglobin and myoglobin being the main precursors. Non-haem iron is mainly derived from foods of both plant and animal origin. The bioavailibility of iron from haem components is much higher than that obtained from non-haem components.

Absorption of iron from both haem and non-haem components is greatly influenced by the body iron stores and is increased in the case of iron deficiency. This relationship, however, is less pronounced for haem iron (2).

Haemoglobin is converted to haem in the digestive tract. The haem molecule is taken up by the mucosa cell by a specific receptor and after cleavage from the haem molecule iron enters the intracellular pool of iron. The absorption of non-haem iron is more complicated and depends on more factors. In the first place bioavailability is different in different foods. Secondly a low pH of the gastric juice is required to solubilise iron and probably mucosa factors are important. Apart from that, enhancers of iron uptake can influence the bioavailability. An example of such an enhancer is ascorbic acid which enhances the uptake by reducing the ferric to the ferrous state and

by its action as a chelator in the stomach and small bowel to keep iron in solution. In some foods inhibitors of iron uptake may interfere with the absorption process. These can be found in cereals and some vegetables so contributing to the development of iron deficiency in vegetarian diets.

The precise mechanism of iron uptake has not yet fully been understood but it is supposed to be a combination of non-specific binding and specific uptake by a receptor or proteins which act as a shuttle (2). The regulation of iron uptake by the mucosa cell is dependent on the iron status and on the rate of erythropoiesis.

After uptake by the mucosa cell iron will either be stored in the mucosa cell or transported to the basolateral site of the cell where iron enters the blood. The precise mechanisms have not yet been clarified (2). In the serum iron binds to transferrin, an 80 kDa glycoprotein which has as main function the transfer of iron between sites of absorption, utilisation and storage (3,4).

1.1.2 Iron distribution

As has been stated before, the iron status is regulated at the level of iron uptake instead of at the level of iron excretion. The average iron secretion and uptake is 1 mg per day. The body of an adult male contains about 4 grams of iron (table 1.1). Almost two thirds of the total amount of body iron is incorporated in haemoglobin and myoglobin (5,6,7). In females the need for iron is increased in the fertile period of life due to increased loss as a result of menstruation and pregnancy.

Table 1.1 - Average overall distribution of iron in the human body, male 75 kg (adapted from 7).

		mg iron/75 kg	mg iron/kg
functional iron	haemoglobin	2,300	31
	myoglobin	320	4
	haem enzymes	80	1
	non-haem enzymes	100	1
	transferrin	4	0.05
storage iron	ferritin	700	9
	haemosiderin	300	4
total		3,800	50

Another group of patients at risk for developing iron deficiency is the child. Especially in age groups where rapid growth and development are combined with a high susceptibility to infections iron present in the food often does not match the requirements. This effect is even more important when the intake of food is insufficient (8,9). Many children develop iron deficiency anaemia which can, in advanced cases lead to impaired growth and central nervous system dysfunction. Studies in school children reveal that iron deficiency anaemia is accompanied by poor IQ test score. Some studies indicate that in this age group correction of the iron deficiency anaemia is not accompanied by improved IQ performance (8,10,11,12). However, a recent study has found the opposite (13). More research is required but attention has to be paid to the study design and the analytical methods used (14).

1.1.3 Iron storage

The storage protein for iron is ferritin. Ferritin consists of an apoprotein shell, Mw 480 kDa, surrounding a core which can contain up to 4,500 iron atoms in the form of the mineral ferrichydrite. The apoprotein is built up of 24 subunits. Two structurally distinct subunits can be found: the H and the L subunit. Different isoferritins can be found built up of different proportions of the two subunits. Different isoferritins can be found in different tissues. Isoferritins with an increased percentage of H-subunits are mainly found in organs like the heart and red blood cells, which are not primarily concerned in iron storage. On the contrary, isoferritins with increased percentages of L-subunits are predominantly found in tissues involved in iron storage such as the liver and the spleen (15).

The process of iron uptake into the ferritin shell has not been clarified yet. The hollow shell displays 14 channels through which iron can enter the core (7,16,17). Iron is generally taken up in its ferrous form, which is oxidised to the ferric form at the outer surface by a ferroxidase centre located on the ferritin H-chains (7,15,18,19). Ferric iron is stored in the core of the apoferritin in complex with oxygen and phosphorous.

Iron release in vivo is thought to occur in lysosomes or endosomes with an acid pH (15). The precise mechanism, however, is still unknown.

It has been postulated that a difference in subunit composition could lead to a difference in function between the isoforms of ferritin (7,16,17,20,21). This would be caused by the location of ferroxidase, which is exclusively located on the H-chain. As a result of this, isoferritins which are rich in H-subunits can take up "free" iron more rapidly. In the acute phase response the production of H-rich ferritin is stimulated.

The serum concentration of ferritin is used as a diagnostic tool in the assessment of iron status (22). In iron deficiency the serum ferritin concentration has been shown to correlate very well with iron stores. However, in those cases in which iron deficiency

is complicated by an acute phase reaction, the ferritin concentration can be elevated as a result of this despite the presence of depleted iron stores (22,23,24). Additionally, the serum ferritin concentration is a good indicator of iron stores but gives little information as to the severity of iron deficiency (22).

Recently, a method has been described to determine the iron concentration of ferritin. It has been postulated that this parameter should be a better indicator of iron stores than the serum ferritin concentration (25).

1.1.4 Iron transport

Iron is transported from sites of absorption to sites of utilisation or storage complexed to transferrin. Transferrin is a glycoprotein (Mw 80 kDa). It consists of a single polypeptide chain of 679 amino acid residues. The transferrin molecule can be divided in two homologous domains, an N-terminal domain and the C-terminal domain. Each domain contains an iron binding site which makes the transferrin molecule capable of binding two iron (FeIII) ions. The two iron binding sites act independently of each other (3,7,26). To bind iron to one of the binding sites, a synergistic anion is required to stabilise the complex.

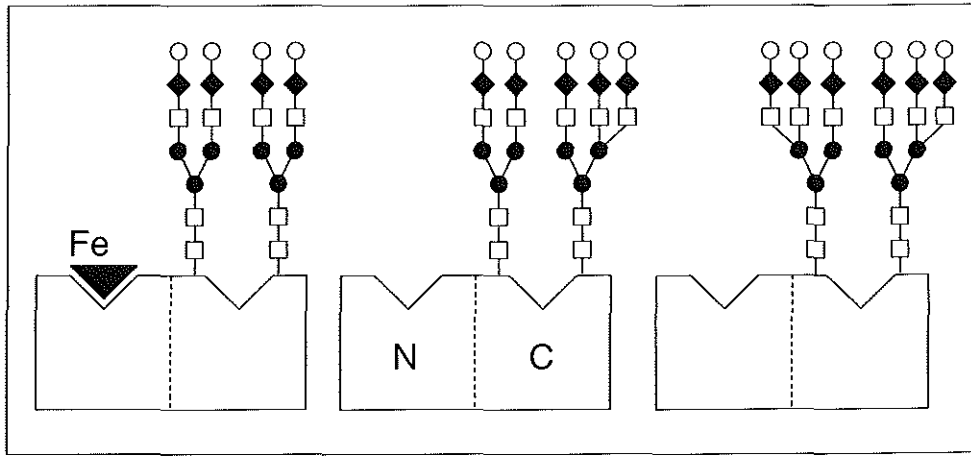
At the C-terminal domain two N-linked glycan chains are attached. These glycan chains may vary in carbohydrate content and in degree of branching, sialylation and galactosylation (3,26). This variation is called the microheterogeneity of transferrin. In different body fluids different microheterogeneity patterns have been described (27). For example in cerebrospinal fluid a raised percentage of asialotransferrin can be seen. This has been used as a diagnostic tool in the diagnosis of cerebrospinal fluid leakage in case of rhinorrhea or otorrhea. Also in certain clinical conditions different microheterogeneity patterns have been observed (28). One example is the appearance of an increased fraction of carbohydrate deficient transferrin (CDT) in case of high alcohol intake (29,30,31). A diagnostic test based on this principle has been developed (32).

Synthesis of transferrin mainly occurs in the hepatocyte in the liver. Transferrin synthesis starts with the association of mRNA and ribosomes in the cytoplasm. Initially a polypeptide chain is formed which is 19 amino acids longer than the final molecule. After translocation to the rough endoplasmic reticulum, the N-glycosylation of the protein takes place at the two possible glycosylation sites (26). At first a uniform glycan is positioned at the specific sites. The processing and subsequent elongation of the oligosaccharide chains occurs when the protein passes through the different compartments of the Golgi system (26).

1.1.5 Transferrin microheterogeneity

As has been described before, two branched glycans are attached to asparagine residues in the C-terminal domain of transferrin (figure1.1). These oligosaccharide chains vary in the degree of branching and sialylation. In normal serum bi-, tri- tetra- and even penta antennary chains have been identified. Usually a sialic acid residue is attached to the terminal sugar of the different branches.

Figure 1.1 - Schematic representation of the human Tf molecule.



The glycans of transferrin are structurally variable. They can differ in the degree of branching or the terminal sialic acid molecule can be lacking. Apart from this they can differ in the terminal sugar residue.

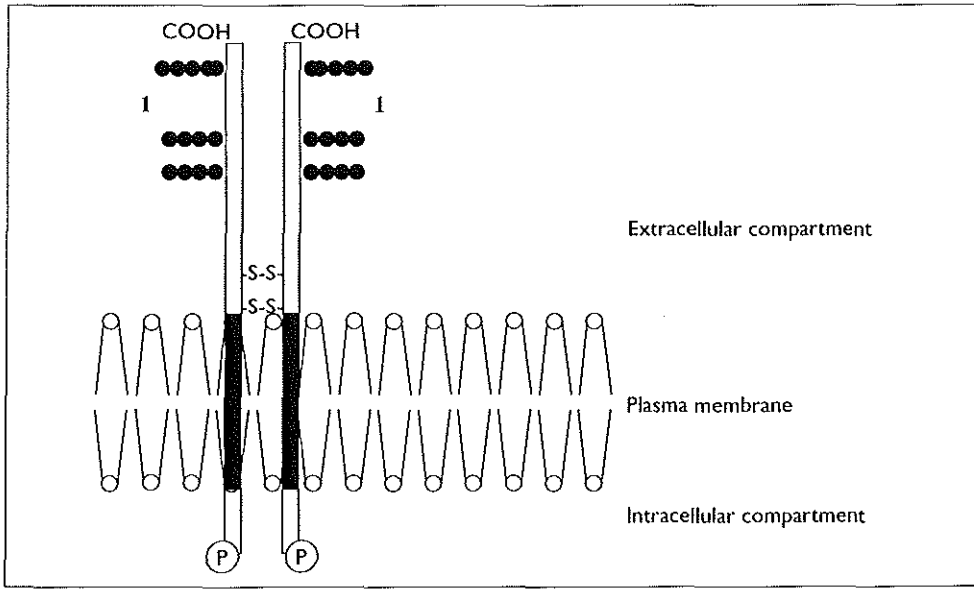
1.1.6 The transferrin receptor

In 1963 the concept that cellular iron uptake was mediated by the transferrin receptor was proposed by Jandl and Katz (34,35). Since then much research has been done on cellular iron uptake and in the last decade many articles have been written on the structure and the function of the transferrin receptor.

The transferrin receptor was found to be a dimeric structure, composed of two subunits linked by two disulphide bridges (figure1.2). This transmembrane protein has a molecular weight of approximately180 kDa. Each monomer can be divided in an extracellular region, an intermediate trans-membrane region and an intracellular part. The intracellular part is necessary for normal endocytosis of the receptor (37,38). The transmembrane part functions mainly as an anchor for the protein and as a signal peptide for translocation across the rough endoplasmic reticulum during biosynthesis

(35,39,40). The extracellular part of the transferrin receptor is a large protein which contains three N-linked glycosylation sites. Oligosaccharide chains are attached to these sites (41,42). In addition, in some cell types O-linked glycans have been identified as well (43). The function of the glycan hasn't been identified yet but they obviously play a role in the binding of transferrin to the receptor (35,44). The affinity of the transferrin receptor for transferrin depends on two parameters: pH and transferrin iron saturation (7). At pH 7.4, the pH of the extracellular space, the affinity is highest for fully saturated transferrin, i.e. diferric transferrin. However when the pH decreases to the pH of the endosomes, pH 5.5, the transferrin receptor affinity for apo transferrin increases, whereas the affinity for diferric transferrin decreases (45-48).

Figure 1.2 - The transferrin receptor is a dimer consisting of two identical 95 kDa polypeptide structures. Each monomer can be divided in (i) a large extracellular region (empty box) with a number of sugar residues (1) attached to the C-terminal end, (ii) a small and hydrophobic intermediate segment (black box) that spans the plasma membrane and (iii) a short Cytoplasmatic "tail" harbouring a phosphorylation site (P) (7).



The transferrin receptor is present on most cells but since erythroid cells have a special requirement for iron due to the synthesis of haemoglobin most receptors can be found on erythroid tissue. In vitro studies have shown that in cell culture soluble transferrin receptors are released from erythroid cells mainly to the serum at the middle to late phases of maturation, mostly from polychromatic erythroblasts (49). In serum soluble transferrin receptors can be found as truncated fragments of the intact cellular

receptors. These fragments appear in serum due to shedding or cleavage (50-52). The level of soluble transferrin receptors has been found to be directly proportional to the total amount of tissue receptors (50). Since the majority of tissue transferrin receptors can be found on erythroid cells, this is directly proportional to the erythroid mass. The concentration of soluble transferrin receptors in serum shows an individual variation around its homeostatic set point. This annual rhythm appeared to be synchronised with those in transferrin concentration and number of erythrocytes, while it was negatively related to the annual rhythms in serum iron, MCV and MCH. The intra-individual variation has been suggested to reflect changes in nutritional and immune status (53).

Recently, many clinical studies have been performed regarding the concentration of soluble transferrin receptors in different conditions (52-62). According to some authors the increased concentration of soluble transferrin receptors in iron deficiency is a useful parameter to differentiate it from anaemia of chronic disease (24).

Recently commercial Elisa tests have been introduced to the market for the detection of soluble transferrin receptors in serum using specific monoclonal antibodies (63,64). This test is expected to serve as a diagnostic tool in the clinical diagnosis of different types of anaemia.

1.1.7 The cellular uptake of iron

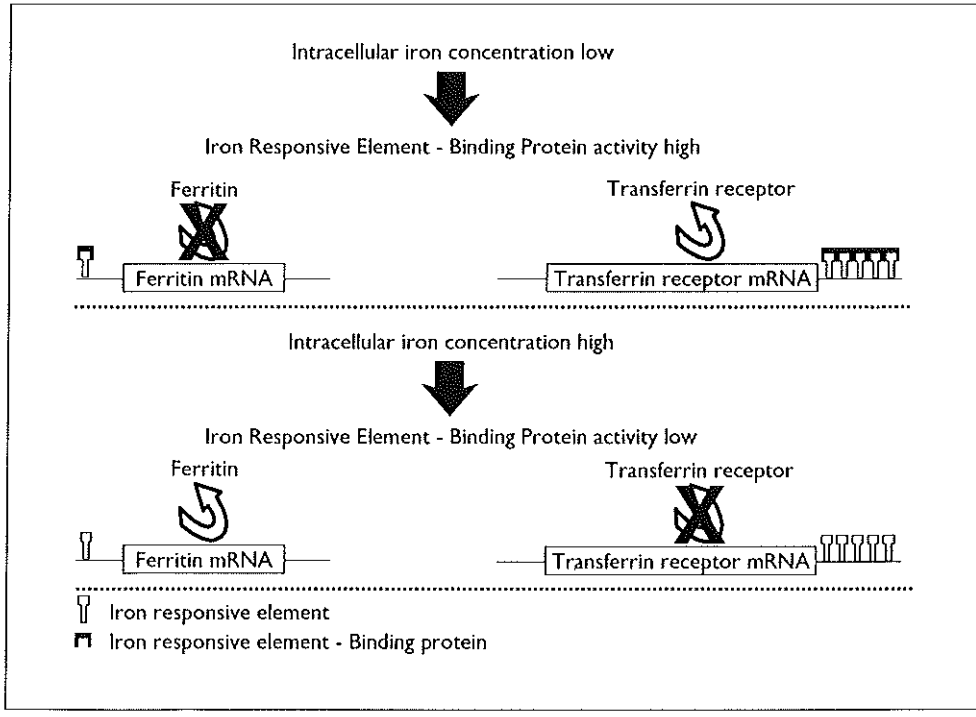
Most iron is taken up by the cell by means of a process called "receptor mediated endocytosis". After the transferrin iron complex has bound to the transferrin receptor, the transferrin-iron-receptor complex is internalised into the cell. All cells which have transferrin receptors on the cell membrane are capable of endocytosing transferrin (35). Several steps in this process can be identified. Firstly, the receptors on the cell surface bind transferrin and cluster into coated pits. This is followed by endocytosis, uncoating of the vesicles and acidification of their contents (35,26). The acidification of the endosome results in the release of iron from transferrin. The iron is subsequently released into the cytosol and apotransferrin as well as the receptor are recycled. Apotransferrin, which has a low affinity for the receptor at pH 7.4, is released into the circulation whereas the receptor is recycled on the cell membrane ready to bind iron-transferrin again (35,26).

1.1.8 Intracellular iron homeostasis

The expression of transferrin receptors on the cell membrane and the production of intracellular ferritin have been shown to be linked and both depend on the intracellular iron content (figure1.3). An interesting fine tuning system has been described which regulates the iron uptake at the molecular level, the "iron responsive element" (65,66,67). The iron responsive elements (IRE's) are short sequences in the mRNA of ferritin and erythroid 5-aminolevulinate synthase as well as in the mRNA of the

transferrin receptor. These hairpin shaped structures are located either at the 5' untranslated region of ferritin mRNA or at the 3' untranslated region of transferrin receptor mRNA. Although an RNA secondary structure within the 5' untranslated region of an mRNA can directly influence translation, the predicted stability of the ferritin IRE stem loop is insufficient to be likely to impede translation on its own (67,68,69). In fact, evidence has been gained which suggests that the IRE alone is a positive effector of ferritin translation (66,70). In human cell lysates a cellular factor was identified which was capable of interacting specifically with an RNA transcript containing an IRE (71). This factor was identified as the IRE-binding proteins (IRE-BP's). The IRE's are the target structures of the IRE-BP's. The IRE-BP has a structure similar to aconitase, an enzyme in the citric acid cycle (72,73).

Figure 1.3 - Schematic presentation of the interaction between the iron responsive element (IRE) and the iron binding protein (IRE-BP) and the effects of this interaction. Depending on the intracellular iron concentration either ferritin or the transferrin receptor is produced (7).



The IRE/IRE-BP system functions as a fine tuning system regulating the production of both ferritin and the transferrin receptor depending on the intracellular iron concentration. In case of a high intracellular iron concentration, the IRE-BP will

contain several molecules of iron. This makes the enzyme unable to bind to an IRE, so enhancing the synthesis of ferritin. In the case of a reduced intracellular iron concentration, the IRE-BP is activated and will bind to the IRE's. The binding of the IRE-BP to the IRE on the 5' untranslated region on the ferritin mRNA inhibits the production of ferritin. At the same time the IRE-BP will bind to the 3' untranslated region of the transferrin receptor, so stimulating the production of the transferrin receptor by blocking rapid degeneration of this transferrin receptor mRNA (7,66,74).

1.1.9 Iron metabolism in pregnancy

In pregnancy many physiological changes occur, as a result of the great demands of the foetus on the maternal metabolism. Despite the amenorrhea, pregnancy places a significant demand on iron homeostasis (75).

The foetus requires large amounts of iron, having the maternal iron stores as the only donor. In humans 23% of the maternal iron stores is transported to the foetus, mainly in the third trimester (76,77,78).

Much research has been done on transplacental iron transport. The placenta has been shown to be autonomous in iron uptake, with iron transport acting as a one way process towards the foetus (78,79,80). This process is active, operating against a concentration gradient. The amount of iron transported to the foetus increases towards the end of pregnancy. In the second trimester approximately 0.4 mg iron per day is transported across the placenta rising to 5 mg iron per day at term (76,78,80).

The human placenta is composed of a cellular trophoblast layer consisting of an inner layer of cytotrophoblast cells and an outer layer of multinucleated syncytiotrophoblast. The syncytiotrophoblast layer develops by fusion of numerous cells resulting in a multinucleated layer without cellular membrane. The syncytiotrophoblast layer has an apical side facing the maternal blood and a basal side resting on the cytotrophoblast cells and a basement membrane. As pregnancy progresses, the number of cytotrophoblast cells decreases. At term only 20% of these cells are still present. The human placenta has a villous structure. The foetal trophoblast villi project into lacunas filled with maternal blood. The distance between the maternal and the foetal circulation decreases as pregnancy progresses by fusion of the cytotrophoblast and the syncytiotrophoblast cells. This facilitates diffusion (77,81-84).

Transferrin receptors are present abundantly on both the apical and the basal cell membrane of the syncytiotrophoblast cells (77,86-92) and in very low concentrations on freshly isolated cytotrophoblasts (77). It has been shown that the expression of transferrin receptors on cytotrophoblast cells is part of the biochemical differentiation towards the syncytiotrophoblast (93,94). In culture the number of transferrin receptors depends on the iron concentration in the culture medium (95-97). In vivo the

expression of transferrin receptors on the syncytiotrophoblast layer depends on the foetal need for iron. Maternal transferrin can bind to the placental transferrin receptor and iron uptake can occur by receptor mediated endocytosis as has been described for other cell types.

As has been described by De Jong (26) the microheterogeneity pattern of transferrin is redirected towards the synthesis of highly sialylated transferrins as pregnancy progresses. The effect becomes statistically significant in the second trimester and this trend appeared to be continued up to the last week of pregnancy. After delivery the pattern rapidly changes to normal again within a period of 5 weeks (26). The physiological significance has not been identified yet, but an increased mobilisation of iron from the liver due to an increased concentration of the highly sialylated fractions has been proposed (26).

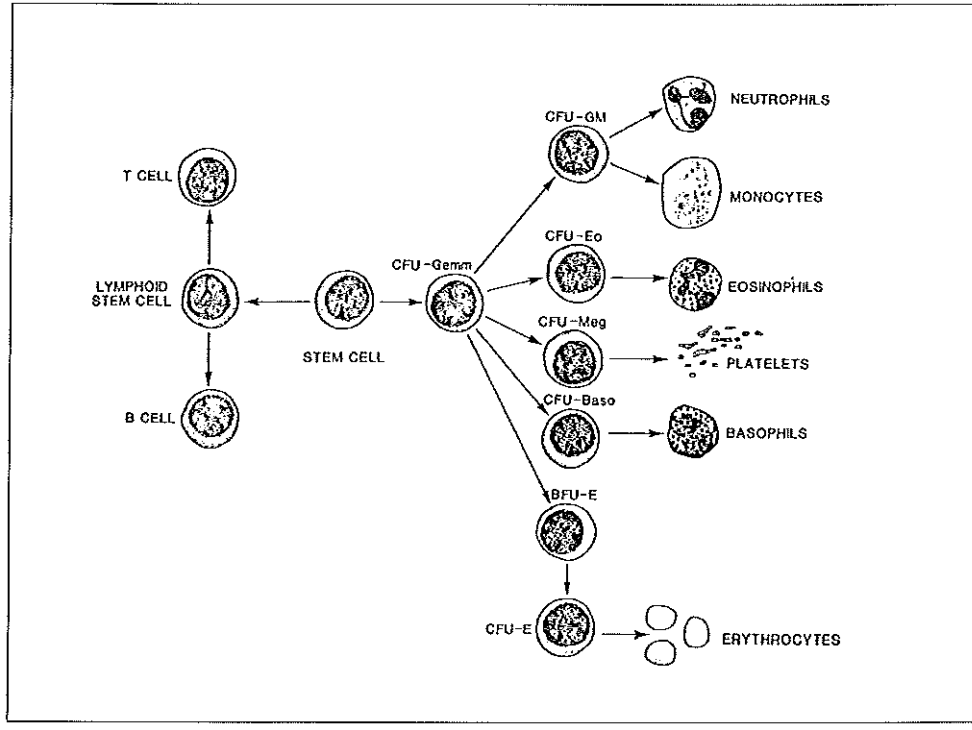
1.1.10 Foetal development of erythropoiesis and iron metabolism

During foetal development the first sign of haematopoiesis is the appearance of "blood islands" in the mesenchyma, lateral to the primitive streak at 2 to 3 weeks after fertilisation. At 16 days of gestation pluripotent stem cells arise in the yolk sac. These cells give rise to different cell lines and erythroid cells. Primitive forms of haemoglobin can be detected early after gestation. As pregnancy progresses the erythropoiesis moves from the yolk sac and intravascular erythropoiesis to the liver and the bone marrow. The liver becomes the main place of erythropoiesis by approximately 8 weeks of gestational life, whereas the bone marrow acquires this function at about 28 weeks of gestational life (98,99).

Erythropoiesis is the result of a finely tuned system of stimulators and inhibitors. The factors influencing erythropoiesis are diverse and act on different stages of erythroid development. Metcalf has proposed a model in which all haematopoietic cells are organised in subsequent compartments. As a certain cell develops from one compartment to the next it loses the ability of self replication but every subsequent compartment is larger and more differentiated (100,101).

The pluripotent stem cells are usually in the resting G0 stage but are capable of differentiating into one of the lineage specific progenitor cells (figure1.4). From the pluripotent stem cell the colony forming unit arises that can differentiate into erythroid, myeloid, monocytic and meagakaryocytic cell lines. The several cell lines that develop from this CFU-GEMM proliferate and differentiate into circulating blood cells. This process of proliferation and differentiation is influenced by several hormonal and micro-environmental factors (102,103).

Figure 1.4 - Hierarchy of haematopoiesis (104).



As the erythroid progenitor cell develops through erythropoiesis the cell matures into the erythrocyte thereby developing various membrane antigens (figures1.5 and 1.6). From the earliest stage the transferrin receptor can be detected on the cell membrane. The transferrin receptor is lost from the membrane at the stage of the reticulocyte (105). From the stage of the erythroblast until the erythrocyte glycophorin appears on the cell membrane.

Figure 1.5 - Schematic representation of the various stages of erythroid differentiation (106).

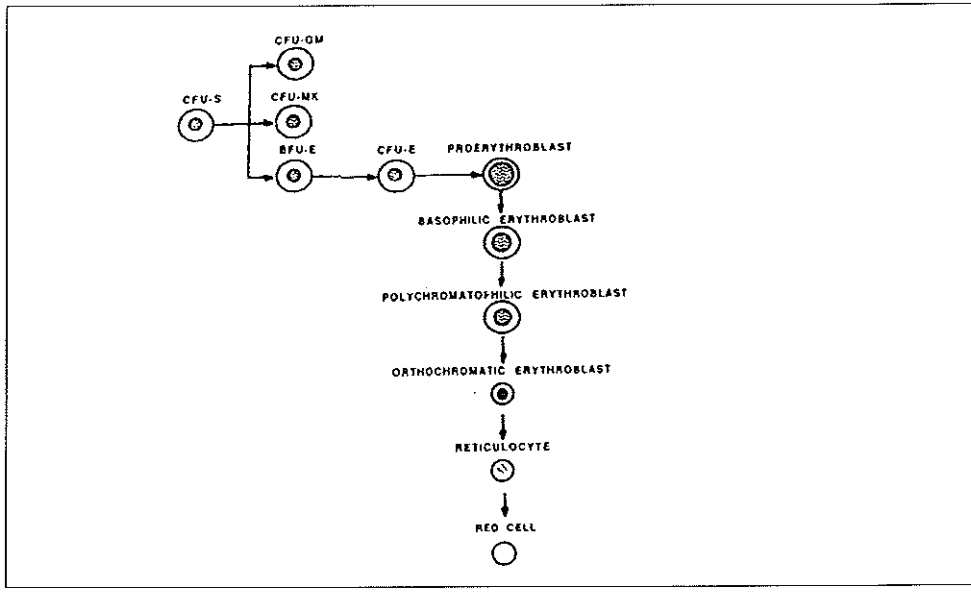
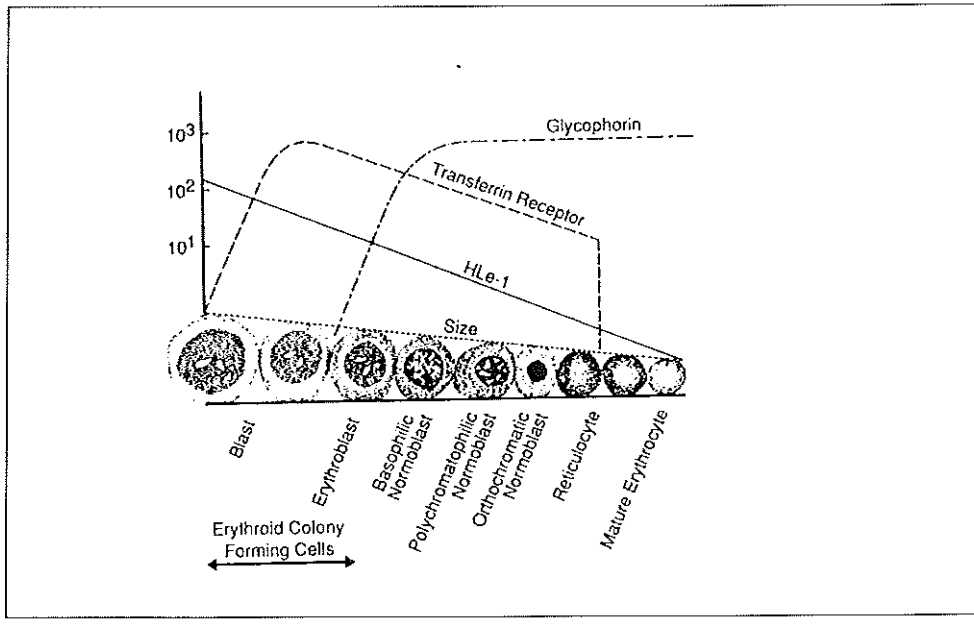


Figure 1.6 - Development of membrane antigens in erythroid development (105).



1.1.11 Iron metabolism of infancy and childhood

In the newborn infant polycythemia is usually found as a result of intra uterine hypoxia. After birth the oxygenation improves markedly leading to a depression of erythropoiesis. This can be shown by a cessation of erythropoietin production after the second day of life (107,108,109).

The two determinants of iron status at birth are birth weight and haemoglobin mass (table 1.2). The body iron content of the newborn is relatively constant over a wide range of maternal iron reserves (110).

Table 1.2 - Relationship of birth weight to total body iron at birth (adapted from 110).

birth weight (kg)	total body iron (mg)
1.5	120
2	160
2.5	200
3	240
3.5	280

The foetus seems to be an effective parasite for iron. Iron administered to the mother during pregnancy does not influence the iron status of the newborn and the haemoglobin concentration of blood from infants born to mothers with iron deficiency is normal (110-115). In a recent study the relation between maternal serum ferritin concentration and the foetal erythrocyte ferritin content was compared for different groups of patients (116). This study indicates that maternal erythropoiesis and the foetus compete for iron in the case of shortage for iron. During the first weeks of life iron shifts from erythropoiesis towards the storage pools as the haemoglobin concentration falls. As a result the infant is able to double its birth weight without exhausting the iron reserves. Thereafter iron absorption becomes the critical factor in maintaining a normal haemoglobin concentration (110,117,118). For term infants the iron reserve at birth is sufficient for the first 5 months of life. Preterm infants, however, only carry enough iron for up to the first two months. These changes are also reflected in the ferritin concentration. At birth the ferritin concentration is high and it increases during the first weeks of life as iron stores expand. As erythropoiesis increases again the ferritin levels fall. The same pattern is seen in premature infants, allthough the ferritin levels are lower (110,119-123). An even better indication of the foetal iron stores could be gained from the foetal erythrocyte ferritin concentration (116).

The iron status during infancy is important for the long term development in children. Iron stores at birth have been shown to correlate very well with iron stores at 6, 9 and

12 months of age (125). Both iron deficiency and iron overload have an adverse effect. Children who develop iron deficiency anaemia in infancy have a higher risk of long-lasting developmental disadvantages compared to their peers with better iron status (126). In contrast to adults detectable levels of non protein bound iron can be found in up to 25% of healthy newborns (127, 128). In a recent study the relationship has been studied between the severity of birth asphyxia, plasma concentration of non-proteinbound iron and TBARS (thiobarbituric-acid-reactive species). It was found that there appeared to be an association between an elevation of the non-proteinbound iron concentration and an adverse outcome after severe birth asphyxia. On the other hand all the severely asphyxiated children with no detectable non-proteinbound iron in their plasma had a normal neuro developmental outcome at 1 year of age (129).

1.2 Myelodysplastic syndromes

The myelodysplastic syndromes consist of a heterogeneous group of primary haematological disorders which are usually characterised by a discrepancy between a cytopenia in blood and cellular or hypercellular bone marrow aspirations. In 1982 the French American British (FAB) Co-operative Study group tried to classify these disorders which were earlier referred as preleukemia, smouldering leukaemia's or refractory anaemia (130,131). A new classification system was made and these bone marrow disorders were termed myelodysplastic syndromes (MDS).

1.2.1 Classification and morphological characteristics

In the group of MDS five subtypes can be found: Refractory Anaemia (RA), Refractory Anaemia with excess blasts (RAEB), Refractory Anaemia with excess blasts in transformation (RAEBt), Refractory Anaemia with ring sideroblasts (RARS) and Chronic Myelo Monocytic Leukaemia (CMML). These different forms can be distinguished on both morphological and numerical findings in blood and bone marrow (table 1.3). In all subtypes abnormalities can be found in the erythroid, myeloid and megakaryocytic lineages. MDS may either be apparently primary or may evolve in the course of other bone marrow diseases or be secondary to previous exposure to cytotoxic chemotherapy, irradiation or environmental toxins.

Table 1.3 - The FAB-classification of the myelodysplastic syndromes (adapted from 134).

category	blood	bone marrow
refractory anaemia (RA)	anaemia blasts 1%	blasts < 5% ring sideroblasts 15% of ANC
refractory anaemia with ring sideroblasts (RARS)	anaemia blasts 1%	blasts < 5% ring sideroblasts > 15% of ANC
refractory anaemia with excess of blasts (RAEB)	anaemia blasts 5%	blasts 5 · 20%
refractory anaemia with excess of blasts in transformation (RAEB-t)	blasts > 5% ± Auer rods	blasts 20 · 30%, or the presence of Auer rods
chronic myelomonocytic leukaemia (CMML)	blasts < 5% monocytes > 1x 10^9/l	blasts up to 20 % promonocytes often increased

The myelodysplastic syndromes have been found to be clonal in origin arising from the multipotent stem cell (131,132). Usually at least two maturation lineages are involved but the defect may be principally manifest in one lineage (133,134) (figures 1.7 and 1.8):-

(a) erythropoiesis: Dyserythropoiesis often leads to anaemia which can be normocytic, microcytic or macrocytic. In the blood film many abnormalities can be found like anisocytosis and poikilocytosis, circulating nucleated cells which may show dyserythropoietic or megaloblastic features or basophilic stippling. Erythropoiesis is often increased in usually hypercellular bone marrow aspirations showing megaloblastic erythropoiesis with dysplastic features like multinucleairity, nuclear lobulation and fragmentation, Howell-Jolly bodies, inter-nuclear bridges, increased pyknosis and gigantism. In some cases ring sideroblasts can be seen;

(b) granulopoiesis: In blood neutropenia is most frequently seen, although neutrophilia does not exclude the diagnosis MDS. Abnormalities in the nucleus or cytoplasm which could be indicative for the diagnosis are: acquired Pelger-Huet anomaly, bizarre shaped nuclei, increased chromatin clumping, hypersegmented nuclei, hypo- or agranular cytoplasm, cytoplasmic basophilia in mature cells and Döhle bodies. Apart from dysgranulopoietic features numerical and maturation defects are found in some subtypes: monocytosis, promonocytes and blast cells (sometimes with Auer rods). In bone marrow smears granulopoiesis can be either increased or diminished with hypogranular or hypergranular promyelocytes and hypogranular myelocytes. A maturation arrest can be seen as a lack of mature neutrophils. Granulopoiesis is usually morphologically abnormal showing the same characteristic as seen in blood. The number of blasts can be increased;

(c) thrombopoiesis: Apart from reduction or increase in the number of megakaryocytes different morphological abnormalities can be found including micro megakaryocytes, large mononuclear megakaryocytes or large hypersegmented forms with multiple small nuclei. In blood the presence of giant platelets or bizarre shaped platelets suggests a diagnosis of MDS.

Figure 1.7 - Bone marrow aspiration of a patient with MDS showing gigantism and nuclear fragmentation.

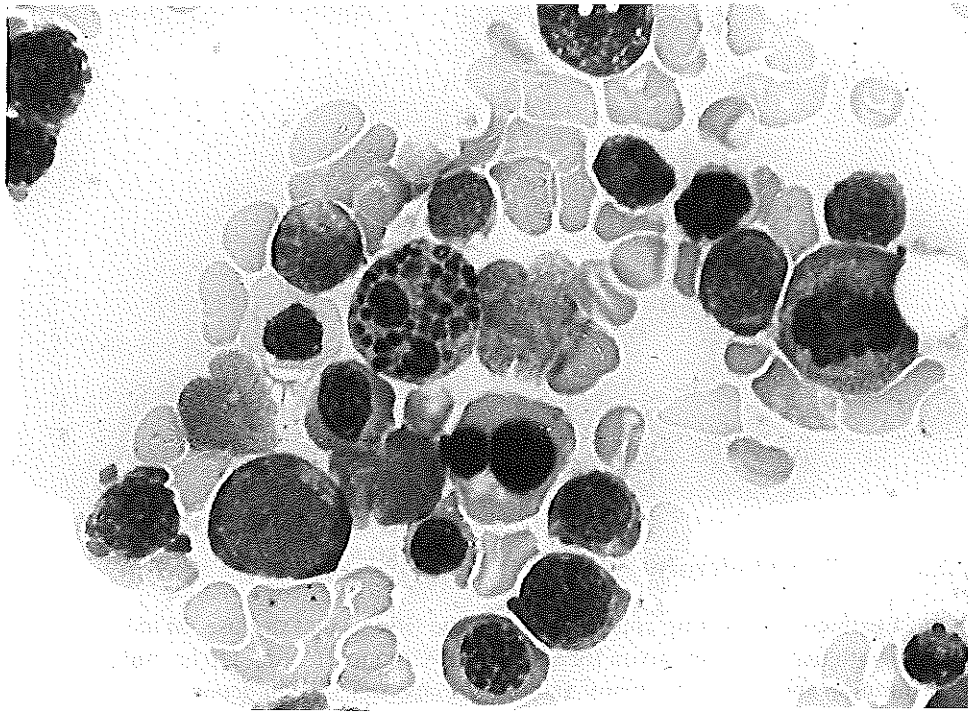
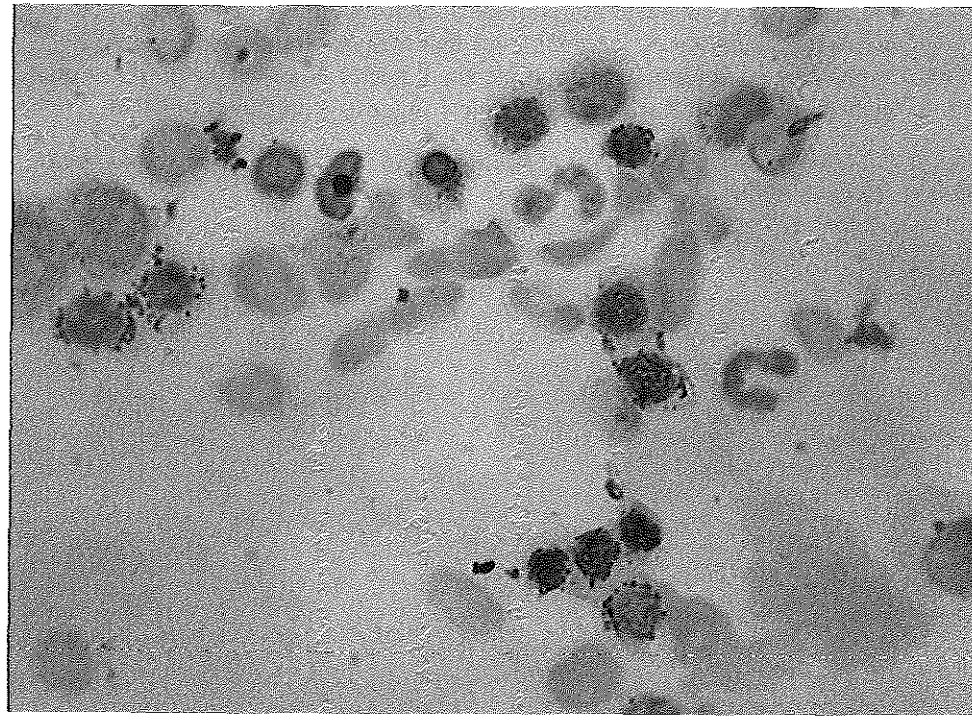


Figure 1.8 - Ring sideroblasts in a bone marrow aspiration of a patient with RARS.



1.2.2 Clinical picture

In most patients a persisting anaemia is often the first sign of disease. In many cases therapy with iron has been given for some time without affecting the haemoglobin concentration and eventually many patients become transfusion dependent. Most patients present at middle or older age but the diagnosis has to be considered in younger age groups as well. Even a childhood syndrome has been described, associated with monosomy 7 (136).

The long term prognosis is influenced by several factors such as patient characteristics and FAB classification. Patients with MDS may die as a result of bone marrow failure due to MDS or the MDS may transform to leukaemia (134). In some individuals evolution to another category of MDS happens, usually to a category with a worse prognosis. Also other factors may influence the prognosis of MDS (table 1.4). Several scoring systems have been developed to use estimate the prognosis in individual patients (134,137-143).

Table 1.4 - Factors which have been reported to be of prognostic significance in MDS (adapted from 134).

	factors indicating a good prognosis	factors indicating a poor prognosis
clinical features	younger age female de novo MDS	older age male secondary MDS splenomegaly (CMML)
FAB-category	RA or RARS	RAEB or RAEB-t
blood features		anaemia, neutropenia, thrombocytopenia, presence of blasts, neutrocytosis, monocytosis (CMML)
bone marrow aspirate		increased number of blasts, dysgranulopoiesis, dysmegakaryopoiesis, reduced megakaryocytes
bone marrow biopsy		abnormal localisation of immature precursors (ALIP)
ferrokinetics	near normal iron utilisation at 14 days	low iron utilisation at 14 days, increased ineffective erythropoiesis
karyotype	normal, both normal and abnormal metaphases, 5q-, +8 or 20q- as a sole abnormality	abnormalities of chromosome 7 or both 5 and 7; all metaphases abnormal; complex karyotype
bone marrow culture	normal numbers of CFU-GM colonies and clusters	reduced CFU-GM colonies or increased clusters
cell kinetics		low labeling index
biochemistry		elevated LDH

In the majority of cases non random cytogenetic abnormalities are commonly found (143). In primary MDS the most frequent abnormalities are deletions of chromosome 5 (-5/5q-), chromosome 7 (-7/7q-), chromosome 20 (20q-), or the gain of an additional chromosome 8. The presence of certain chromosomal abnormalities may correlate with survival and evolution into leukaemia (144-149). In a recent report on X inactivation patterns in purified sub populations of blood cells in elderly women, however, clonal granulocytes and polyclonal T-cells were observed in 23 % of normal women and in 63 % of patients with myeloproliferative and myelodysplastic

syndromes (150). This suggests an age related stem cell depletion or selection. This also implies that clonal blood cells may probably precede rather than follow mutations which subsequently give rise to myelodysplastic or myeloproliferative phenotypes. This implies that the demonstration of clonal granulocytes and polyclonal T-cells not a useful diagnostic marker in elderly women (150).

1.3 Aim of the thesis

The present thesis contains a study of the clinical significance of transferrin receptors in different clinical syndromes. In general practice many patients present with signs of anaemia and iron therapy is often started as a routine. This often leads to iron overload in patients with anaemia of chronic disease and in patients with myelodysplastic syndromes. In this thesis the clinical significance of the transferrin receptor as a predicting parameter for the efficiency of iron therapy was studied.

Another group of patients who are often treated with iron supplements are children. In (premature) infants transfusions can add to a state of iron overload, which has to be prevented. The duality of iron, being both an essential and a noxious agent, makes iron metabolism in the infant a fascinating topic. In this thesis the concentration of soluble transferrin receptors was studied in (premature) infants in the first days of life. To make interpretation of the results possible the microheterogeneity of transferrin was studied as well.

The following issues were studied:-

(a) Different commercially available tests for the evaluation of soluble transferrin receptors in serum were evaluated. The clinical and analytical implications were studied.

(b) In patients with different types of anaemia the membrane expression of transferrin receptors on erythroblasts in bone marrow was studied with a newly developed flow cytometric method.

(c) The relationship between the cellular expression of transferrin receptors on erythroblasts in the bone marrow and the concentration of soluble transferrin receptors in serum was compared.

(d) The serum concentration of transferrin receptors in a group of newborn infants was studied in relationship to other parameters of iron metabolism. In the same patients the microheterogeneity of transferrin was evaluated.

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Chapter 2

Materials and Methods

2.1 Introduction

In this chapter the techniques used in the experiments are described in detail. All reagents used were of analytical grade. Patient samples were taken with permission of the patients. Parents agreed that their babies participated in the study. The study design was approved by the medical ethics committee of the hospital after the methods had been optimised during a pilot study.

2.2 Flow cytometry

Cellular characteristics can be studied by immunological marker analysis. During differentiation the antigenic structure of the cellular membrane changes. By the expression of a certain combination of antigens different cell types can be distinguished and cells can also be classified according to a particular differentiation stage.

The antigenic structure of the cell membrane can be studied by the use of conventional antisera or monoclonal antibodies. In recent years monoclonal antibodies have been increasingly used. A monoclonal antibody raised against the antigen of interest is incubated with the cell. If the specific antigen is present on the cell membrane this antibody will bind to it. After a washing step to remove the excess of unbound antibody the bound antibody can be detected after addition of a fluorescent label. This detection can be performed both by microscopy and flow cytometry. A classification system has been developed for the increasing number of monoclonal antibodies available during the last decades. During international workshops monoclonal antibodies showing the same reaction pattern are grouped together in "clusters of differentiation or designation" (CD).

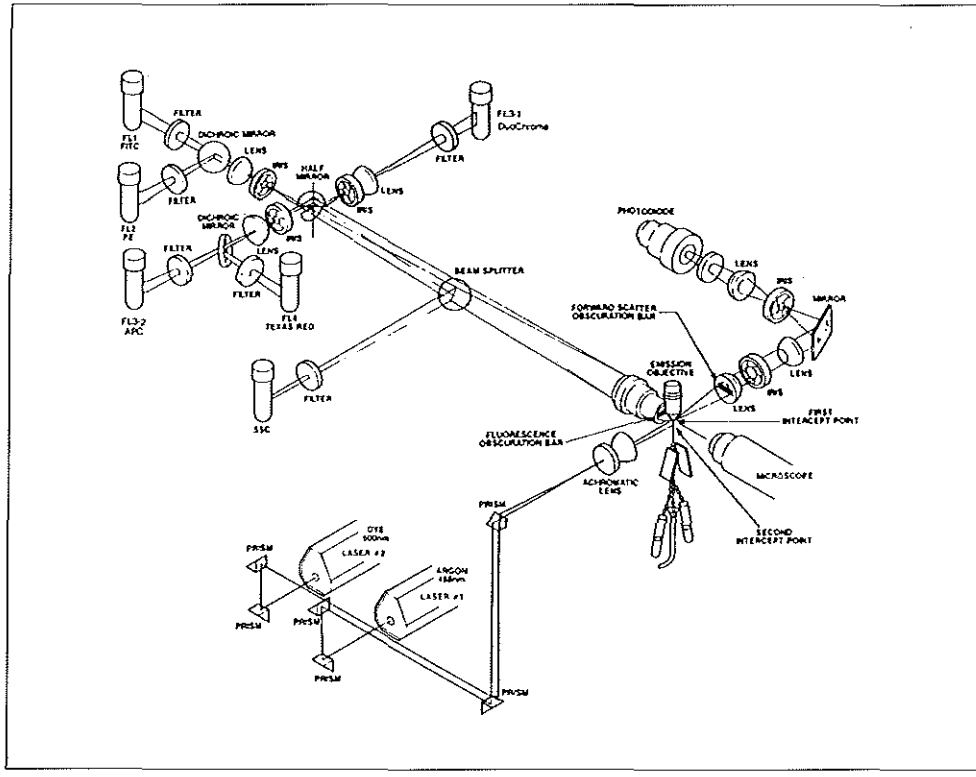
Many monoclonal antibodies are commercially available and attached directly to a fluorochrome. Different fluorochromes are available, fluorescein isothiocyanate (FITC) and phycoerythrin (PE) being the most frequently used in flow cytometry. Monoclonal antibodies that are not attached to a fluorochrome by the manufacturer (unconjugated antibodies) can be coupled to a fluorochrome after binding of the antibody to the cellular antigen. These unconjugated antibodies are mainly used for the detection of antigens which are weakly expressed.

Multiple immunological staining techniques make it possible to study the concurrent expression of two different antigens in one cell type. The easiest way is to combine two fluorochrome conjugated antibodies. These antibodies can be mixed and incubated with the cell suspension. Recent developments in flow cytometry and especially in the software have made the concurrent use of up to 5 fluorochromes in one experiment possible.

2.2.1 The flow cytometer

A flow cytometer is an instrument developed to analyse cell samples. The FacStar^{plus} (Becton Dickinson, USA) used in the experiments in this thesis is equipped with a 100 mW water cooled argon laser light source which provides monochromatic light at 488 nm and a low power helium-neon laser providing a monochromatic light beam at 633 nm. A schematic diagram of the instrument is given in figure 2.1. Cells in a suspension pass in a laminar non turbulent stream through the nozzle in an open air measurement region. The sample fluid is centred into the nozzle by a pure laminar flow. This is achieved by forcing the sample fluid into a sheath fluid flowing under pressure slightly lower than that of the sample fluid. The laser beam is focused perpendicularly to the cell stream. Cells sequentially pass the laser beam light and are detected individually.

Figure 2.1 - Diagrammatic representation of a dual laser flow cytometer.



As the cells pass the laser beam, the light is scattered in different directions. This scattered laser light is detected both at a low angle in the forward direction (forward

scatter signal, FSC) and at 90° (sideward scatter signal, SSC). The FSC signal depends primarily upon the volume of the cells, whereas the SSC signal is mainly determined by the internal organisation, cytoplasmic granularity, nuclear density and external cell structure (1,2). The FSC light and the SSC light are detected in detection devices such as a photodiode. Weak signals are first amplified in a photomultiplier tube. The light signal is then converted into an electric signal directly proportional to the light signal. The electric signal can be analysed using commercially available software. The FSC and SSC signals of a certain cell suspension can be plotted in a diagram. In this diagram different cell populations can be distinguished, which makes the analysis of a selected cell population possible.

As the cell suspension passes the laser light, fluorochromes attached to the cell will be excited by the laser light. These fluorochromes will emit fluorescence light. The fluorescence light will be detected in photomultiplier (PM) tubes in the same way as the scattered light. The magnitude of the electric signal is directly proportional to the number of fluorescent molecules which have bound to the cell. Different fluorochromes emit light with different wavelengths. The detection of these different wavelengths is performed by splitting the lightbeam by dichroic mirrors. The appropriate wavelength collected in each PM tube is selected by the filter system placed in front of the PM tube. This filter system makes it possible to easily apply new fluorescent labels. The measurement of the electric signal from a fluorescent label is performed with logarithmic amplification. The sensitivity of the light detection device depends on the voltage applied and on the amplifier gain setting. These settings have to be checked daily by the use of standard particles such as fluorescence beads and adjusted if necessary. These instrument settings can influence the results. For quantitative analysis the use of standard beads carrying well defined numbers of fluorochromes per bead is obligatory.

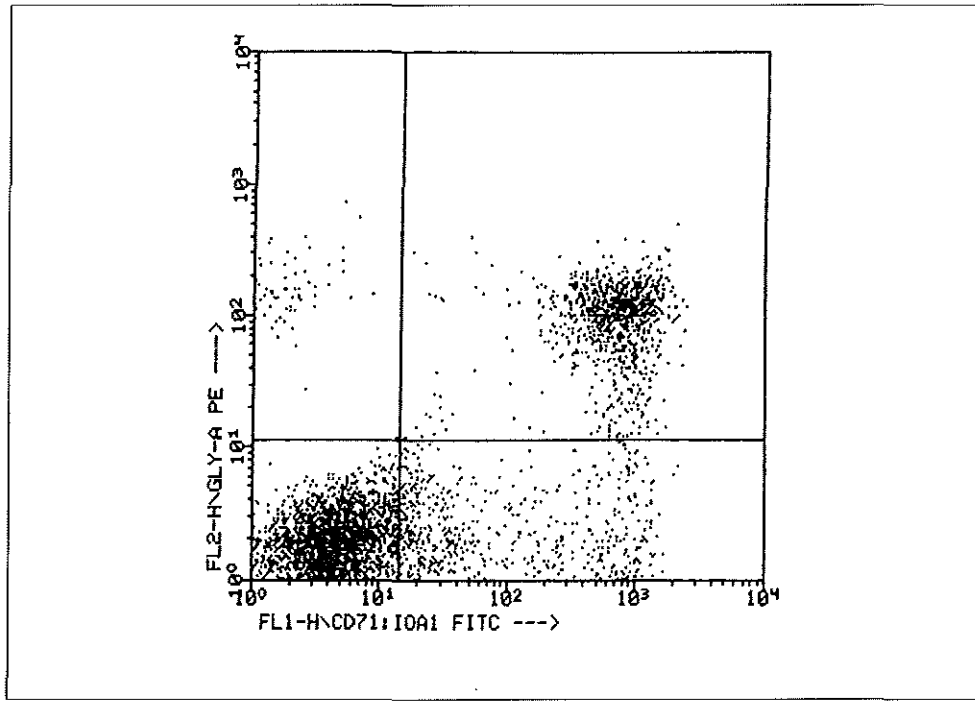
The light emitted from the fluorochromes can be plotted in a dual parameter dot plot as has been described for the FSC and SSC signal (figure 2.2). This can be done for all the cells or one population can be selected from the FSC/SSC diagram. The data gained from these diagrams can be analysed using the instrument software.

The FacStar^{plus} flow cytometer is equipped with a cell sorting unit. This makes it possible to isolate certain cells for individual analysis.

2.2.2 Preparation of the cell suspension and cytospin slides

Cells could be isolated for immunological marker analysis by density centrifugation using a commercially available density gradient (Lymphoprep, Nycomed Oslo). Bone marrow aspirations were obtained from the iliac crest or from the sternum. The bone marrow cells were drawn in citrate to prevent clotting: 1 ml of sodium citrate (3.8%, w/v) was added to 3 ml of bone marrow aspirate. The bone marrow aspirate was then diluted with Isoton Diluid Azid Free (Baker) and 6 ml of this mixture was carefully layered over 3 ml of lymphoprep. This mixture was centrifuged for 15 minutes at a force of 800 g for 20 minutes at room temperature in a swing out rotor. The mononuclear cells formed a distinct band at the sample/medium interface and could be removed using a Pasteur pipette. The cells were then washed twice with Isoton Diluid Azid Free containing 0.5% bovine serum albumin (w/v) and 0.05 % sodium azide (w/v) for 5 minutes at room temperature with a force of 400 g. For surface membrane analysis the cells were suspended in RPMI/Fetal Calf Serum (10%, v/v) to a final concentration of 10 x 10^6/l.

Figure 2.2 - Example of a two parameter dot plot.



Cells were suspended at a concentration of 10^4-10^5 cells per ml in a protein-containing suspension. Two hundred µl of this suspension were added to the sample chamber (Shandon,UK) which had been prespun with one drop of buffered saline. The sample chamber was placed in a cytospin centrifuge (Cytospin 3, Shandon,UK) and the cells were spun for 5 minutes at 200 g. The sample slides were removed from the centrifuge and allowed to air-dry.

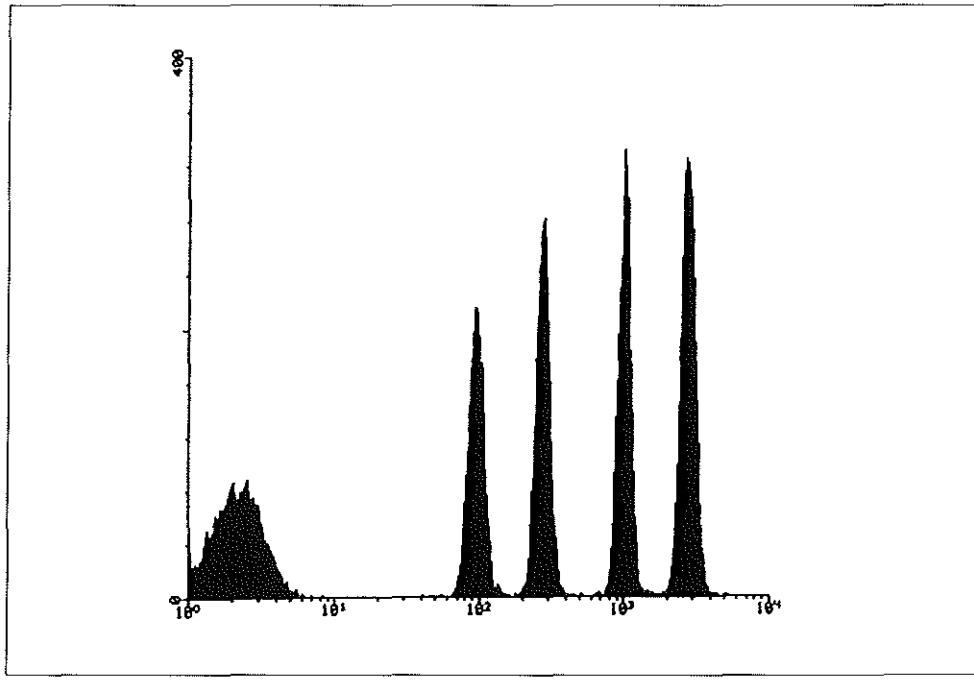
2.2.3 Detection of transferrin receptors on erythroblasts

To study the antigenic structure of the cells in the suspension the cells were incubated with sets of two monoclonal antibodies. These antibodies were conjugated with FITC and PE respectively for 30 minutes at 4 °C at a concentration recommended by the manufacturer. MoAbs used were LCA (CD 45), LeuM3 (CD 14), Leu4 (CD 3), Leu12 (CD 19), LeuM9 (CD 33) (Becton-Dickinson, San Jose, CA, USA); OKM5 (CD 36) (Ortho Diagnostic Systems, Raritan, NJ, USA); IOA71 (CD 71) and GLY-A (Immunotech, USA). Control mouse Ig's (IgG1 and IgG2a) were also purchased from Becton-Dickinson, USA.

The cells were analysed on a FacStar^{plus} flow cytometer equipped with Lysis II software (Beckton-Dickinson, USA). The nucleated population was gated on a two-parameter forward light scatter and 90° light scatter cytogram.

The percentage of erythroblasts expressing the surface antigen of interest (CD 71) was determined from a dual parameter dot plot (GLY-A-FITC/CD 71-PE). To quantify the mean fluorescence intensity Quantum 25 fluorescence beads (kindly provided by Becton-Dickinson, USA) were used as a standard. Beads with 0 - 19.0 x 10^5 molecules of equivalent soluble fluorochrome (MESF) per bead were analysed on the flow cytometer. Each bead had its own fluorescence intensity which could be seen in a histogram (figure 2.3). By plotting the mean channel of fluorescence intensity for every bead, a standard curve could be drawn. The mean fluorescence intensity of different antibodies could be read as MESF from the standard curve by use of the mean channel of fluorescence intensity in the histogram.

Figure 2.3 - Example of standard beads run on a flow cytometer. By plotting the mean fluorescence channel number against the given MESF a standard curve can be drawn.



Determining the MESF of CD 71 on GLY-A positive cells gave information on the number of transferrin receptors on erythroblasts of different patients.

2.3 Preparation of bone marrow films

Bone marrow aspirations were taken from the sternum or the iliac crest. The cells were drawn in citrate to prevent clotting and to make immunological marker analysis possible.

The preparation of bone marrow films was only satisfactory when both free marrow cells and marrow particles could be identified in a stained film. Preparations of marrow cells were made by isolating the marrow particles. Films were made by smearing out the particles between two slides under slight pressure. Care had to be taken since excessive pressure could cause disruption and distortion of the cells.

2.4 Staining methods for bone marrow films

Bone marrow films were air dried before staining. From all patients three slides were stained with May Grünwald-Giemsa and one slide was stained with the Prussian blue stain for iron. All slides were viewed by a panel of trained morphologists.

2.4.1 May Grünwald-Giemsa

Films should be stained as soon as they are dried in the air. Bone marrow films were fixed in methanol (Merck, Germany) for 5 minutes. The films were transferred to a staining jar containing diluted May Grünwald's stain: 10.0 ml of buffer A, containing 9.078 g KH_2PO_4 in 1000 ml water was mixed with 10.4 ml of buffer B containing 11.876 g $Na_2HPO_4.2H_2O$ in 1000 ml water and water was added to 500 ml. Twenty five ml of May Grünwald stain (Merck, Germany) was diluted with 25 ml of this buffer. The pH was measured and if necessary adjusted between 6.9 and 7.1.

After the films had been allowed to stain for 3 minutes they were transferred to a staining jar containing Giemsa's stain (Merck, Germany) freshly diluted with 7 volumes of distilled water. The films were left for 15 minutes and then washed with distilled water. Finally the films were dried and covered by a coverslip using Entallan (Merck, Germany) as a mounting medium.

2.4.2 Prussian blue stain for iron

Air dried films of bone marrow aspirates were fixed in methanol (Merck, Germany) for 5 minutes. The bone marrow slides were put in a staining jar containing potassium ferrocyanide in HCl. This was made by mixing equal volumes of a solution containing 50 g $K_4\{Fe(CN)_6\}.3H_2O$ in 1000 ml water and a solution containing 16.5 ml HCl, 37%, in 1000 ml water. The slides were left in this staining solution for 5 minutes at 37 °C in a microwave processor or 30 minutes at room temperature.

The slides were then rinsed in water for 30 seconds and transferred to a staining jar containing a filtered Kernechtrot staining solution made by mixing 200 mg Kernechtrot (Merck, Germany) and 10 g aluminiumsulfate-18-hydrate in 200 ml water. After the slides had been allowed to stain for 3 minutes at 37 °C in a microwave processor or for 15 minutes at room temperature they were rinsed with water, dried and covered by a coverslip using Entallan (Merck, Germany) as a mounting medium.

2.4.3 Staining for erythroid cells

Air dried cytospin slides or bone marrow films were fixed in absolute methanol (Merck, Germany) for 5 minutes. The slides were placed in a staining jar containing O-dianisidine stain made by mixing five volumes of an 0.2% solution of O-dianisidine (Koch-Light,) in absolute methanol, one volume of a 30% hydrogen peroxide solution (BDH Chemicals, UK) and one volume of a 1% sodium nitroferricyanide solution. The slides are incubated in this solution in the dark for ten minutes and rinsed in water. The air dried slides were then counterstained with May Grünwald-Giemsa.

2.5 Soluble transferrin receptors in serum

Soluble transferrin receptors could be identified by the use of an enzyme immunoassay based upon the double antibody sandwich method (16). For many years the purified transferrin receptor was not available, making the standardisation of the enzyme immunoassay complicated. Recently three test kits have appeared on the market for the determination of soluble transferrin receptors in serum or plasma. In chapter 4 these test kits have been compared and a small clinical study has been described.

As in every enzyme immunoassay the duration of the incubation steps was critical and accuracy of pipetting was essential for the reproducibility of the results.

2.5.1 Principle of the test

The reaction was performed in microwells that were precoated with a polyclonal antibody against the transferrin receptor. Plasma or serum samples were diluted and pipetted into the wells. Murine monoclonal antibodies with specific reactivity against the human transferrin receptor were added to the wells. This murine monoclonal antibody was conjugated to horseradish peroxidase (HRP) to make the reaction visible. During the incubation period the transferrin receptor in the sample bound to the polyclonal antibodies absorbed on the wells and the HRP-conjugated antibodies bound to the captured soluble transferrin receptors. The microwell plates were thereafter washed to remove any unbound transferrin receptor and excess of HRP conjugate. An enzyme substrate for the HRP was then added to the wells (chromogen TMB). The action of HRP formed a blue product during a second incubation period. To stop the enzyme-substrate reaction an acid solution was added to the wells. The blue product was then converted into a yellow colour. The intensity of the yellow colour was directly related to the amount of bound transferrin receptor in the well, which was directly proportional to the concentration of soluble transferrin receptors in the sample.

2.5.2 Test procedure

Three commercial tests were available on the market: the RAMCO assay distributed by DPC, Apeldoorn, The Netherlands, the Eurogenetics assay distributed by Eurogenetics, Tessenderlo, Belgium and the Orion test kit distributed by Imphos, Amersfoort, The Netherlands. In the following section the differences between the assay procedures are indicated. Once the assay has been started, all steps should be completed without interruption.

2.5.2.1 Sample preparation

One of the major differences between the three assays is the sample preparation. The Orion assay is the only assay which does not require predilution of the serum samples. In the RAMCO assay samples have to be prediluted 1:100 in sample diluent whereas in the Eurogenetics assay a dilution of 1:10 is required. In the RAMCO test kit the standards are provided at the required concentrations. For the Eurogenetics assay the standards have to be prediluted 1:10 as well. In the Orion test kit sTfR standards require reconstitution in distilled water.

2.5.2.2 The analysis

The prepared samples and standards were pipetted in individual wells in duplicate (100 µl in Eurogenetics, 50 µl in RAMCO and 20 µl in Orion). Two hundred µl of buffer was added to the wells in the Orion assay. In the RAMCO assay 150 µl of HRP-conjugate was added immediately. In the Eurogenetics and Orion assays the HRP-conjugate was added after incubation of the sample in the wells in a moist atmosphere at room temperature: for 30 minutes (Eurogenetics) or 1 hour (Orion). After this incubation the plates were washed 4-5 times and the HRP-conjugate was added to the wells (100 µl in Eurogenetics and 200 µl in Orion). After the addition of the HRP-conjugate the microtiter strips were incubated at room temperature in a moist atmosphere (2 hours in RAMCO, one hour in Orion and 30 minutes in Eurogenetics). After the incubation the strips were washed and freshly prepared substrate solution was added. Finally the strips were incubated for 30 (RAMCO and Orion) or 20 (Eurogenetics) minutes at room temperature. The colour reaction was stopped by the addition of 50 µl stopping solution. The absorbency of each well was read at 450 nm in a microplate reader which was blanked with 200 µl of substrate solution and 50 µl of stop solution in the RAMCO assay.

2.5.2.3 Results

The average absorbency of each standard was calculated and plotted against the corresponding soluble transferrin receptor concentration (U/l in Eurogenetics) and a standard curve could be drawn. The average absorbency of each patient sample was

calculated and the concentration of soluble transferrin receptors could be read from the standard curve. These calculations could easily be performed by the use of computer programs which were commercially available.

2.5.3 Precautions and interactions

The kit had to be stored at 2-8 °C and reagents should be stored at this temperature as soon as possible after the completion of the assay. For the Eurogenetics assay the maximum number of strips in one run was limited to six. The reason for this was the short incubation time.

Human serum or plasma could be used for the soluble transferrin receptor measurements. No influence on the test results had been observed with anticoagulants as long as there is no dilution of the sample. Samples could be stored at 2-8 °C for a few days (7 for the RAMCO assay) or frozen at -20 °C for a longer time (6 months for the RAMCO assay). To avoid damage to the protein repeated freezing and thawing of the samples should be avoided.

Haemolysis ought to be prevented although for the RAMCO assay it has been claimed that moderate haemolysis does not interfere with the test.

2.6 Serum concentrations of ferritin and total iron

The concentration of ferritin in serum was determined using an Im_x bench-top immunoassay system (Abbott, USA) (17). This system combines two assay technologies in one instrument; the Microparticle Enzyme Immunoassay (MEIA) and the Fluorescence polarisation immunoassay. The first method was used to measure of the serum concentration of ferritin.

The reaction was run in a MEIA cell, which served as the solid phase element. The MEIA cells were loaded onto a carousel and inserted into the analyser. The serum sample (150 µl) was pipetted into the outermost well of the cell. The test specific reagent pack, which contains the antibody coated latex micro particles, was inserted into the system. The different assay components were automatically pipetted into the reaction well. After incubation, the reaction mixture was transferred to the glass fibre matrix. The unbound components were washed away and the alkaline phosphatase-labeled conjugate was added followed by the addition of the fluorogenic substrate 4-methylumberrifyl phosphate. The resulting 4-methylumbelliferone was detected by a front-surface fluorometer.

The total iron concentration in serum was determined on an AU 510 routine chemical analyser (Merck, Germany).

A serum aliquot was pipetted from a tube into a reaction vial in the instrument. Protein bound iron was released from transferrin by quanidinium chloride. The resulting free iron in the reaction vial was subsequently reduced to the ferrous (FeII) form by the action of ascorbic acid. The addition of ferrozin resulted in the formation of a reddish complex, which could be photometrically analysed. The intensity of this colour was directly related to the total iron concentration in the sample.

2.7 Identification of isotransferrins

Different isotransferrins were identified in an analytical electrophoresis system as had been described by Van Noort et al. (18).

Serum samples subjected to isoelectric focusing were first saturated with iron to occupy both iron binding sites. Complete saturation was ensured by mixing 50 µl of serum with 5 µl 0.6 mmol/l of $NaHCO_3$ and 3 µl 10 mmol/l Fe(III) citrate. The samples were left to incubate for 1 hour at room temperature.

The isoelectric focusing was performed on the Phast system (Pharmacia, Sweden) using an immobiline gel. To obtain a pH gradient between the electrodes of 5.0-6.0, a part of the immobiline DryPlate, pH 4-7 (Pharmacia, Sweden) was cut out to the same size as the Phast gel. Prior to isoelectric focusing the DryPlate was rehydrated by applying 1 ml 20% (v/v) glycerol on the surface of the gel for 90 minutes. To ensure an even heat transfer between the cooling plate and the gel, paraffin oil (50 µl) was applied between this plate and the gel backing. To obtain sharp bands without smears, pre-focusing was required.

Prepared serum samples of 0.3 µl were applied to the gel in the Phast system. The instrument settings are summarised in table 2.1.

Table 2.1 - Instrument settings.

sample applicator down at			x2 0 Vh		
sample applicator up at			x3 0 Vh		
sep x1	2,000 V	1.5 mA	1.5 W	15 °C	485 Vh
sep x1	200 V	0.3 mA	0.3 W	15 °C	15 Vh
sep x1	2,000 V	1.5 mA	1.5 W	15 °C	1,800 Vh

After focusing the gel was incubated with 100 µl rabbit anti-human transferrin at room temperature for 20 minutes. The gel was washed for 48 hours in 0.15 M NaCl at 4 °C. This solution was changed several times.

The washed gels were stained. The gels were incubated for 20 minutes with 20% TCA and stained with Coomassie Brilliant Blue R (1 g/l) at 50 °C for 10 minutes according to the instructions described in the PhastSystem Users Manual (Pharmacia, Sweden). The gels were destained in 30% methanol (v/v), 10% acetic acid (v/v) at room temperature.

Different transferrin bands were quantified in a densitometer (Ultroscan X laser, Pharmacia Sweden). The results were analysed using the Gelscan XL software (Pharmacia, Sweden).

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Chapter 3

The expression of transferrin receptors on erythroblasts in anaemia of chronic disease, myelodysplastic syndromes and iron deficiency

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3.1 Abstract

The presence of transferrin receptors on erythroblasts in patients with iron deficiency, anaemia of chronic disease (ACD) and myelodysplastic syndrome (MDS) was studied by two colour analysis on a flow cytometer. CD 71 was used to quantify the number of transferrin receptors and GLY-A to identify erythroblasts. In cases of iron deficiency the number of transferrin receptors was increased on part of the erythroblasts thus facilitating iron uptake by the cells. In patients with ACD or MDS, a decrease of the number of transferrin receptors on erythroblasts was found. This leads to the conclusion that the ineffective response to iron therapy in cases of ACD and MDS can be explained by a decline of transferrin receptors on the red cells.

3.2 Introduction

Iron is essential for life. Every cell needs iron for metabolism and growth. Iron is delivered to cells by transferrin, an 80.000 Dalton glycoprotein which is capable of binding two molecules of iron per mol protein (1). Iron uptake is mediated by the transferrin receptor which is located on the cell membrane of most cells. After binding of transferrin to the receptor, the transferrin-iron complex is taken up by the cell and iron release takes place. The apo-transferrin-receptor complex leaves the cytoplasm and transferrin re-enters the serum (2).

Iron is needed by all cells, and even more so by developing red blood cells, which need extra iron for the production of haemoglobin. In different conditions, abnormal iron uptake can be seen in bone marrow aspirates by specific staining techniques. For example in patients suffering from anaemia of chronic disease (ACD), large amounts of iron can be seen in the macrophages of the Mononuclear Phagocyte System (MPS) while hardly any iron is taken up by the erythroblasts. These findings are opposite to morphological findings in myelodysplastic syndrome in which a normal iron content in the MPS is seen while iron may accumulate in the erythroblasts. In these erythroblasts the iron is located in coarse dots around the nucleus while in normal cases iron appears as very fine dots in the cytoplasm. The question raises, whether the abnormal iron metabolism in different conditions can partly be explained by an abnormal binding of transferrin to its receptor, or by abnormal steps in the intracellular metabolism.

Much research has been done on the interaction of transferrin and its receptor in red blood cells. It has been established that in anaemia of chronic disorder iron uptake is decreased compared to normal individuals (3). In the myelodysplastic syndrome (MDS), erythron transferrin uptake has found to be independent of plasma iron concentration and higher than in normal individuals (4).

Flow cytometry can be used to study the expression of antigens on cell membranes like the presence of receptors and proteins. Monoclonal antibodies raised against these antigens are coupled to fluorochromes. Incubation of these monoclonal antibodies with the cells of interest will result in binding of the monoclonal antibody to its antigen giving a fluorescence signal which can be detected by a flow cytometer.

In this study, flow cytometry was used to detect the presence of transferrin receptors on erythroblasts. A monoclonal antibody directed against the transferrin receptor (CD71) was combined with an antibody specific for red blood cells active in synthesis of haemoglobin (Gly-A). The intensity of the fluorescence signal is related to the number of fluorescence molecules binding to the cell. In this way the number of transferrin receptors on erythroblasts could be compared in different patients. Correction for day-to-day instrument variation was performed by the use of fluorescence standard beads carrying a well defined number of fluorochromes on their surface.

3.3 Materials and Methods

All reagents used were of analytical grade. All samples were obtained with permission from the patient.

3.3.1 Patients

Bone marrow samples of patients from three groups with different forms of anaemia were studied. Bone marrow cells were obtained from the sternum or iliac crest. The diagnosis of ACD, iron deficiency or MDS was based on morphological criteria combined with clinical and chemical data.

For the diagnosis of ACD patients were classified according to the clinical syndrome and chemical data. In all patients a decreased haemoglobin concentration was found (range 9-11 g/dl). The iron content in serum was low (range 2-11 µmol/l) and in all patients elevated ESR (> 45 mm) levels were found as a sign of an inflammatory condition. In the bone marrow aspirate, the classic combination of high levels of stainable iron in the macrophages of the MPS and the absence of iron in erythroblasts was seen.

Iron deficiency was defined by morphological criteria like microcytic anaemia and absence of iron in morphological staining techniques. In most patients a low haemoglobin concentration and low iron content in serum was found. In one patient however, the haemoglobin concentration was still normal.

The diagnosis of myelodysplastic syndrome was based on the morphological criteria by the latest French American British (FAB) classification (5). All bone marrow aspirates were viewed by a panel of morphologists. Patients diagnosed as chronic myelo-monocytic leukaemia (CMMOL) were excluded from the study, so were patients with refractory anaemia with excess of blasts in transformation (RAEBt).

Bone marrow samples of healthy volunteers were studied as normal controls.

3.3.2 Immuno phenotyping

Bone marrow samples were obtained after informed consent. Samples were drawn in a citrate solution to prevent clotting: 1 ml sodiumcitrate (3.8%, w/v) was added to 3 ml bone marrow aspirate. Mononuclear cells were separated by density centrifugation using Lymphoprep solution (Nycomed, Oslo). After being washed and centrifuged twice with Diluid (Baker), containing 0.5 % BSA (w/v) and 0.05 % NaN_3 (w/v), cells were suspended in RPMI/fetal calf serum to a final concentration of 10 x 10^6 cells/l. Mononuclear cells were then incubated with two monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) and phycoerythrin, respectively for 30 min at 4°C at a concentration recommended by the manufacturer. The monoclonal antibodies used were LCA (CD 45), LeuM3 (CD 14), Leu4 (CD 3), Leu12 (CD 19), LeuM9 (CD 33), all purchased from Becton-Dickinson, OKM5 (CD 36) purchased from Ortho, IOA71 (CD 71) and GLY-A both purchased from Immunotech. Control mouse Ig's (IgG 1 and IgG2a) were also purchased from Becton-Dickinson. The cells were analysed on a FacStarplus flow cytometer equipped with Lysis II software (Beckton-Dickinson). The nucleated population was gated on a two-parameter forward light scatter and 90° light scatter cytogram. The percentage of erythroblasts expressing the surface antigen of interest (CD 71) was determined from a dual parameter dot plot (GLY-A-FITC/CD 71-PE). To measure the mean fluorescence intensity Quantum 25 fluorescence beads (kindly provided by Becton-Dickinson) were used as a standard. Beads with 0 - 19.0 x 10^5 molecules of equivalent soluble fluorochrome (MESF) per bead were analysed on the flow cytometer. In a histogram each bead can be seen as a peak having its maximum at another channel of fluorescence intensity. By plotting the mean channel and MESF for every bead, a standard curve was drawn. The mean fluorescence intensity of different antibodies was read as MESF from the standard curve by use of the mean channel of fluorescence intensity in the histogram. Determining the MESF of CD 71 on GLY-A positive cells gave information on the number of transferrin receptors on erythroblasts of different patients.

3.3.3 Cryopreservation

To a cell suspension containing 100 x 10^6 cells 5 ml of a cryopreservative was added dropwise at 4°C. The cryopreservative contains DMSO, RPMI and FCS (Gibco) in a final concentration of 10, 65 and 25% (w/v) respectively. DMSO is added as the last component after mixing with some of the RPMI and FCS. After adding the cryopreservatives the cells were transferred to -80°C for at least 4 hours. The cells were then brought into a container with liquid nitrogen pending use.

FCS was added to the thawing cells; cells were then spinned down at room temperature at 1,500 rpm for 5 min and washed twice with RPMI + 10% FCS.

3.3.4 Staining procedures

Bone marrow aspirates were examined on a slide stained with May Grünwald-Giemsa.

Iron content is estimated by the Prussian blue reaction.

Cytospin slides of bone marrow aspirates were stained before and after cryopreservation with a staining technique for erythroid cells (6).

3.4 Results

This paragraph describes the results obtained from this study. The first section describes the freeze-thaw stability and in the second section the results of the flow cytometric experiments are given.

3.4.1 Freeze-thaw stability

To determine the freeze thaw stability of erythroblasts, cytospin preparations before and after freezing were analysed. These slides were stained with May Grünwald-Giemsa and with a specific staining method for haemoglobin. With this last method, erythroblasts can be distinguished from other cells in a more accurate way as haemoglobin appears as a yellow brownish colour. In all cases we found a decreased number of erythroblasts on the slides after thawing compared to the original slide (table 3.1).

Table 3.1 - Relative number of erythroblasts before and after freezing.

sample number	before freezing	after thawing
1	11	8
2	8	2
3	6	2

To study the freeze-thaw stability of the transferrin receptor, the MESF in different samples was determined before and after freezing. The MESF per bead decreased after storage at -196°C (table 3.2).

Table 3.2 - Relative number of transferrin receptors on erythroblasts before and after a standard cryopreservation procedure.

sample	before		after	
	population	MESF	population	MESF
1	1	4.43	3	0.37
				1.37
				4.96
2	2	1.05	1	1.62
		4.66		
3	1	5.01	1	2.54

3.4.2 Transferrin receptors on erythroblasts

Using fresh bone marrow cells, the intensity of the fluorescence signal for CD 71 expressed as MESF/bead was measured on the erythroblasts in patients with anaemia of different aetiology. This method is an indirect way to estimate the number of transferrin receptors on the cells.

Table 3.3-3.5 show the results for different patients. Table 3.6 gives the results for healthy volunteers.

Table 3.3 - Relative number of transferrin receptors on erythroblasts in cases of ACD.

sample	diagnosis	population	MESF
1	ACD	1	3.2
2	ACD	2	2.17
			6.53
3	ACD	1	1.67
4	ACD	1	4.43
5	ACD	1	3.33
6	ACD	2	1.01
			3.79
7	ACD	1	2.39
8	ACD	1	2.65
9	ACD	1	2.51
10	ACD	1	2.72
11	ACD	1	3.03
12	ACD	1	4.91
13	ACD	1	3.54
14	ACD	1	3.44
15	ACD	1	4.35

Table 3.4 - Relative number of transferrin receptors on erythroblasts in cases of iron deficiency.

sample	diagnosis	population	MESF
1	iron deficiency	3	3.31
			6.43
			11.88
2	iron deficiency	2	0.27
			9.7
3	iron deficiency	3	3.53
			7.09
			12.28
4	iron deficiency	1	8.16
5	iron deficiency	2	2.29
			8.78
6	iron deficiency	2	1.97
			10.67
7	latent iron def.	1	6.31

Table 3.5 - Relative number of transferrin receptors on erythroblasts in MDS.

sample	diagnosis	population	MESF
1	RA	1	3.22
2	RAEB	3	0.57
			1.31
			3.46
3	RA	2	1.41
			5.03
4	RA	1	2.61
5	RA	1	2.61
6	RARS	1	2.51
7	RARS	2	1.08
			5.49
8	RAEB	1	3.34
9	RARS	1	4.2
10	RA	1	1.65
11	RARS	1	1.02
12	RA	1	1.09

Table 3.6 - Number of transferrin receptors on erythroblasts expressed as MESF/bead in healthy volunteers.

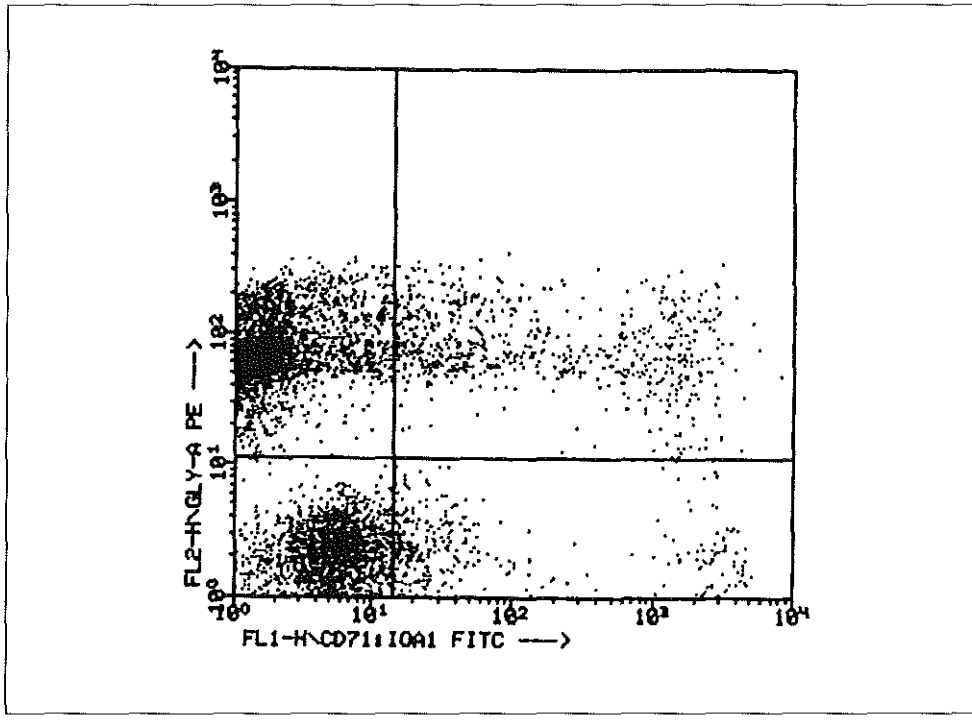
sample	diagnosis	population	MESF
1	volunteer	1	6
2	volunteer	1	5.65
3	volunteer	1	5.87
4	volunteer	1	5.25
5	volunteer	1	5.2

In some cases more than one population of cells could be gated expressing both the transferrin receptor as well as GLY-A (figure 3.1). The difference between these populations was the fluorescence intensity for CD 71, while the signal for GLY-A was constant.

In patients with iron deficiency more than one group of cells could be defined in every patient, part of the cells having a very high expression of CD 71 while the remaining cells usually showed normal expression.

In patients with anaemia of chronic disease and myelodysplastic syndrome, the fluorescence intensity in all cells was below normal values. In some cases an extra population was seen with an extremely low expression of CD 71.

Figure 3.1 - Example of a two-parameter dot plot in a patient, note: the two populations of GLY-A and CD 71 double-positive cells in the upper right quadrant.



3.5 Discussion

Iron is one of the essential elements in the human body. All cells need iron for growth and metabolism. Much research has been done on the interaction of the transferrin-iron complex and the transferrin receptor on different cell types. The erythroblast has been the cell of interest in many studies, since erythroblasts require iron for metabolism as well as for haemoglobin synthesis. Shortage of iron causes anaemia since iron is not available for incorporation into haemoglobin. In some types of anaemia the role of iron is less clear. Research has been done on the in vitro uptake of iron into erythroblasts of patients with ACD and MDS.

Recent progress in flow cytometry and the development of new monoclonal antibodies make it possible to study the number of transferrin receptors on different cell types and in different conditions. In this study, the expression of transferrin receptors on erythroblasts was studied by the use of two monoclonal antibodies: CD 71 for the detection of the transferrin receptor and GLY-A to define erythroblasts in the bone

marrow samples. Cells which were reacting with both monoclonal antibodies were the cells of interest, as they are the erythroblasts active in haemoglobin synthesis and expressing tansferrin receptors on its membrane. The number of transferrin receptors per cell was quantified using a standard curve derived from the fluorescence intensity of standard beads. The value read from the standard curve is directly related to the number of transferrin receptors on the erythroblast.

In this study, different groups of patients were compared. A constant MESF/bead has been found in healthy volunteers. In these normal cases only one well-defined population of GLY-A and CD 71 double-positive cells was found. It seems that in normal cases the number of transferrin receptors on erythroblasts is within very narrow limits, not only within the same sample but also between various persons. In patients with anaemia more variation was seen. In most patients, irrespective the cause of the anaemia, more than one group of erythroblasts can be seen carrying different numbers of transferrin receptors on the membrane. In cases of iron deficiency increased numbers of transferrin receptors on the cell membrane were found in at least part of the cells. This can be easily explained as an adaptation of the body to the state of iron deficiency. Increasing the number of transferrin receptors on the membrane facilitates the uptake of iron by the cell. Patient number 7 was an interesting case. In this patient, iron deficiency was still in an early phase, anaemia had not developed yet, but the erythroblasts already carried an increased number of transferrin receptors.

The findings in ACD and MDS are less easy to be explained. MDS is a heterogeneous condition characterised by anaemia, dyshaematopoiesis and varying numbers of blasts. Following the FAB-classification chronic melo-monocytic leukaemia is included in MDS. However, it is not certain whether this condition is truly a myeloproliferative disorder. RAEB(t) is also included in MDS according to the FAB-classification, but it can be seen as an early stage of acute leukaemia. Both patients diagnosed as chronic myelo-monocytic leukaemia and RAEB(t) were excluded from this study. All MDS patients in our study had in common a low haemoglobin concentration and abundant coarse dots in the Prussian blue iron stain. Some patients showed classic ringed sideroblasts. All patients exhibited less of transferrin receptors on erythroblasts than healthy controls. On part of the cells, the transferrin receptor was almost completely lacking. These erythroblasts will not be able to take up iron from transferrin as effectively as normal erythroblasts. This can contribute to the ineffective erythropoiesis in MDS and explains why in these patients iron supplement does not lead to a rise in haemoglobin concentration. Two patients were diagnosed as RAEB. No difference was seen in CD 71 expression when compared to RA or RARS. However, two patients is only a small number, so more experiments have to be done before a final conclusion can be drawn.

Similar date were obtained in cases of ACD. It is proved that in cases of ACD iron uptake in vitro is impaired compared to healthy controls (3). In this study we found that in all patients at least part of the erythroblasts express a very low number of transferrin receptors. This will lead to a lower uptake of iron from transferrin. In bone marrow samples of patients with ACD, abundant iron is seen in the macrophages of the MPS were it is stored. Iron is administrated routinely to many patients with ACD. This is not effective since the erythroblast can not take up the iron, so all iron is stored in the MPS leading to an iron overload in many cases.

Bone marrow samples of patients are not easy to obtain. MDS is a rare condition and for the diagnosis of ACD and iron deficiency usually no bone marrow sample is taken since the diagnosis can be made using other parameters. Therefore it would be interesting to study frozen cells so samples can be obtained from other hospitals as well. However, this study proved that not only erythroblasts but also the transferrin receptor can be destroyed in a standard cryopreservation procedure. This means that studies on the interaction of transferrin and its receptor can only be done on fresh material.

3.6 References

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Chapter 4

Analytical and clinical implications of soluble transferrin receptors in serum

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based upon: Eur J Clin Chem Clin Biochem (1996); 34:645

and: Eur J Clin Chem Clin Biochem (1997); 35:793

4.1 Abstract

In this study the analytic performances of recently introduced assays for soluble transferrin receptors in serum were tested. The Ramco transferrin assay was compared with the Eurogenetics assay. In a small clinical study serum samples of patients with anaemia of chronic disease, iron deficiency and myelodysplastic syndrome were analysed as well as sera obtained from healthy volunteers.

In a limited study the Orion transferrin receptor assay was compared to the Ramco assay. This study included an analysis of the analytical performances as well as a limited clinical study to compare the clinical usefulness of the different assays.

The analytical performances of the Ramco and Orion assays were found to be acceptable. In the Eurogenetics test however, inter assay imprecision and the end of run drift were unacceptable high.

We were able to confirm that in patients with uncomplicated iron deficiency the concentration of soluble transferrin receptors is higher than in healthy volunteers. In cases of anaemia of chronic and inflammatory disease the levels of soluble transferrin receptors in serum are slightly, but not significantly, higher than in normal subjects.

Measurement of soluble transferrin receptors in serum provides a good differentiation between anaemia of chronic disease and iron deficiency. However it was also shown that the clinical conclusion drawn may depend on the test kit used, as result of a lack of standardisation. For diagnosis and follow up of patients this is unacceptable. The time for international standardisation has come to make results in the follow up of individual patients as well as results derived from clinical studies comparable.

4.2 Introduction

Iron is one of the most important elements in the human body. It is essential for cellular growth and metabolism. However, an excess of iron can lead to intracellular damage to DNA and proteins by producing free radicals in combination with oxygen (1). To prevent damage from these radicals iron is transported complexed to transferrin.

The transferrin receptor is a transmembrane protein present in all body cells (1,2). It consists of two identical domains. Each domain is capable of binding two molecules of transferrin. The affinity of the receptor for transferrin depends on the saturation of transferrin with iron and is especially high for completely saturated transferrin. The number of cellular transferrin receptors depends on the cell's need for iron. The production of the transferrin receptor is regulated on the level of RNA by the

intracellular iron content. Depending on this concentration either the iron storage protein ferritin or the transferrin receptor is produced. Various proteins, the so called iron binding proteins, can influence these processes. An excess of iron binding to the iron responsive elements facilitates the translation of RNA coding for the ferritin molecule. In intracellular iron deficiency, a decreased binding of iron to these proteins will lead to the translation of that part of the RNA which codes for the transferrin receptor.

Soluble transferrin receptors in serum are found as a truncated fragment of transmembrane transferrin receptors (3,4). The level of soluble transferrin receptors is directly proportional to the total mass of tissue receptors (2). Since most of the tissue receptors can be found on erythroid precursor cells, the concentration of transferrin receptors in serum is directly proportional to erythroid activity (5). In cases of erythroid hyperplasia as in iron deficiency and haemolytic anaemia, the level of soluble transferrin receptors is raised. In cases of bone marrow aplasia the level is sharply reduced.

Recently the concentration of soluble transferrin receptors in serum has been postulated as a new quantity in the diagnosis of anaemia caused by iron deficiency (5,6,7,8). A possible role for soluble transferrin receptors in the differentiation of anaemia of chronic disease from iron deficiency could be expected and has been described (9,10,11).

In the present study, the analytical performances of recently introduced soluble transferrin assays were tested. Serum samples from patients with anaemia of chronic and inflammatory diseases, iron deficiency and myelodysplastic syndromes were analysed as well as sera obtained from healthy volunteers. A bone marrow aspirate was taken from every patient in order to establish the amount of stainable iron, which serves as the gold standard for total body iron stores.

4.3 Materials and methods

This paragraph describes the materials and methods used in these experiments. All reagents were of analytical grade. All serum and bone marrow samples were taken after informed consent.

4.3.1 Materials

4.3.1.1 Test procedure

The Ramco transferrin receptor (TfR) kit was obtained from DPC, Apeldoorn, The Netherlands. The Eurogenetics assay was obtained from Eurogenetics, Tessenderlo, Belgium. The Orion assay was purchased from Imphos, Amersfoort, The Netherlands.

All sTfR kits are enzyme immunoassays (EIA) based upon the double antibody sandwich method. They differ from each other in dilution the samples and in incubation time. In the Ramco test samples are prediluted 100-fold and in the Eurogenetics assay 10-fold. In the Orion assay no predilution step is required. The incubation time in the Ramco and Orion tests is one hour and in the Eurogenetics test only 20 minutes. Results are expressed as kU/l in the Eurogenetics assay and in mg/l in the Ramco and Orion tests.

The assay procedures were followed according to the instructions from the manufacturers.

4.3.1.2 Apparatus

Precision pipettes: reference pipettes were used obtained from Eppendorff, Germany.

Microplates were washed in a microtitration plate washer 1296-024 obtained from Wallac, Finland.

Microtiter plates were analysed in the microplate absorption photometer type ht II obtained from Anthos, Austria.

4.3.1.3 Patients

Serum samples and freshly drawn bone marrow aspirates of patients with 3 forms of anaemia were studied. Serum samples were stored at -80 °C until analysis.

Bone marrow cells were obtained from the sternum or iliac crest and analysed immediately. The diagnosis of anaemia of chronic disorder, iron deficiency or myelodysplastic syndrome was based on morphological criteria combined with clinical and chemical data.

For the diagnosis of anaemia of chronic disorder (17 patients), patients were classified based on the clinical syndrome combined with:-

(a) a low haemoglobin concentration;
(b) a low iron content in serum;
(c) the classic combination in bone marrow aspirates of high levels of stainable iron in the macrophages of the MPS and the absence of iron in erythroblasts.

Iron deficiency (10 cases) was defined by the combination of:-

(a) a low haemoglobin concentration, low iron content in serum;
(b) a low ferritin concentration;
(c) morphological criteria like microcytic anaemia and absence of bone marrow iron in morphological staining techniques.

The diagnosis of myelodysplastic syndrome (10 cases) was based on the morphological criteria by the latest French American British (FAB) classification (12).

All bone marrow aspirates were judged by a panel of trained morphologists. Patients diagnosed with chronic myelo-monocytic leukaemia were excluded from the study, as well as patients with refractory anaemia with excess of blasts in transformation (RAEBt).

Serum samples with a concentration of soluble transferrin receptors ranging from 2.6-28 mg/l in the Ramco assay were selected to test the clinical value of the Orion assay. All samples were re-analysed with both assays simultaneously.

Serum samples from 62 healthy volunteers were taken as normal controls.

4.3.1.4 Staining procedures

Bone marrow aspirates were examined on a slide stained with May Grünwald-Giemsa.

Iron content was estimated by the Prussian blue reaction.

4.3.1.5 Statistics

For statistical analysis a Student-test was performed.

Regression analysis according to Bablock and Passing was performed using commercial software Eval-kit 3.1, CKCHL, Elisabeth Ziekenhuis, Tilburg, The Netherlands.

4.3.2 Methods

4.3.2.1 Intra assay imprecision

To determine the intra assay imprecision ten replicate measurements on serum samples were performed.

4.3.2.2 Inter assay imprecision

Duplicate measurements on 3 serum samples were performed in order to determine the intra assay imprecision. Each sample was analysed in five different assays over a period of five weeks. Each serum was divided in five aliquots before freezing to prevent freeze-thaw instability of the receptor.

The inter assay imprecision of the Orion test kit was limited to duplicate measurements of 2 samples. Each sample was analysed in two different assays on the same day. Additionally the end of run drift was determined by analysing the same 2 samples in the first wells as well as in the last wells of the microplate in two runs.

Additionally the end of run drift was determined by analysing the same samples in the first wells as well as in the last wells of the microplate or in the last wells of six strips (Eurogenetics).

4.3.2.3 Analytical sensitivity

The zero standard was determined ten times along with the set of standards and controls. The lowest detectable transferrin receptor concentration was considered as the mean extinction of the zero standard plus two times the standard deviation.

4.3.2.4 Assay linearity

Serial dilutions of serum samples were measured. Serum samples were diluted with zero standard from 1-fold to 10-fold.

4.3.2.5 Method comparison

Regression analysis was performed according to the procedure described by Passing and Bablock (13).

4.3.2.6 Reference values

To find the normal values, 62 sera of healthy volunteers were analysed. The normal range was defined as the mean value ± 2 SD.

4.4 Results

This section describes the results obtained from this study. In the first part the analytical performances of all tests are described. The second part gives the results of the clinical study.

4.4.1 Analytical performances

4.4.1.1 Intra assay imprecision

In the three samples a mean serum transferrin receptor concentration of 1.9 mg/l, 6.2 mg/l and 12.1 mg/l was found. The Orion assay was tested with eight samples with a mean transferrin receptor concentration ranging from 0.6 - 9.0 mg/l.

Using these samples an intra assay imprecision of respectively 5.4%, 3.5% and 4.5% was found in the Ramco assay. In the Eurogenetics assay a variability of respectively 4.7%, 7.4% and 11.1% was found. The intra-assay imprecision in the Orion assay ranged from 3.9% to 6.1%.

4.4.1.2 Inter assay imprecision

Three serum samples were used with a mean concentration of soluble transferrin receptors of 2.3 mg/l, 6.0 mg/l and 12.0 mg/l respectively.

Using these samples, an inter assay imprecision of 9.0%, 5.5% and 5.4% was found in the Ramco assay. In the Eurogenetics assay these values were 23.7%, 14.7% and 19.5%.

A mean end of run drift of 4.1%, 5.5% and 10.6% in the Ramco assay and a mean end of run drift of 13.9%, 10.9% and 14.4% in the Eurogenetics assay were found. In individual runs the end of run drift after 6 strips could rise up to 24% in the Eurogenetics assay.

In the Orion assay the inter assay imprecision was 4.0% and 6.2% and no end of run drift was found.

4.4.1.3 Analytical sensitivity

The detection limits of TfR are 0.2 mg/l in the Ramco assay and 31 kU/l in the Eurogenetics assay.

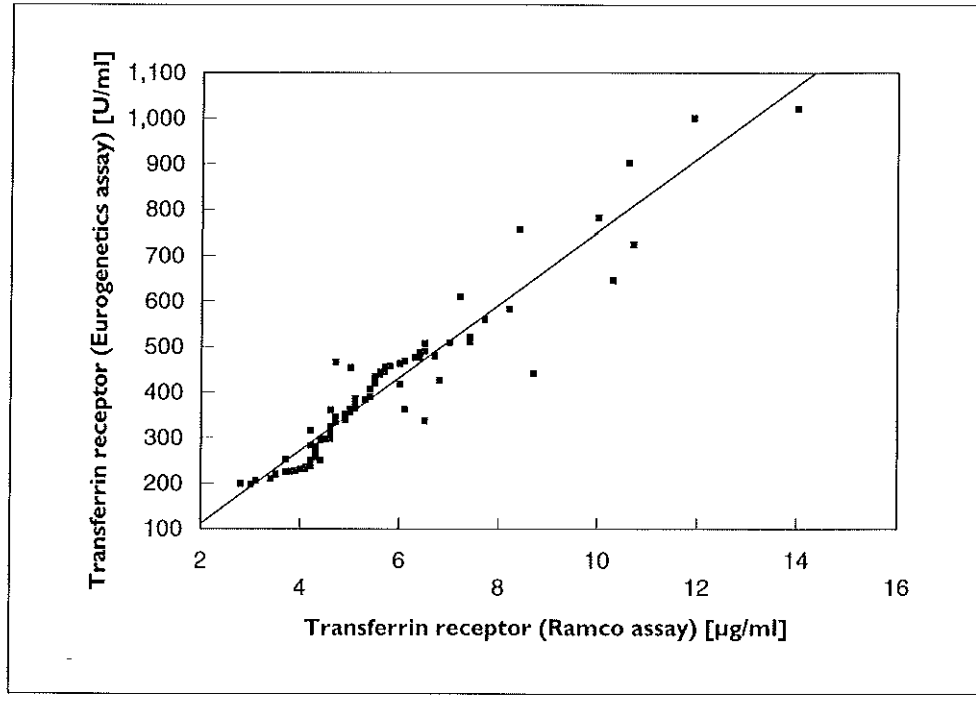
4.4.1.4 Assay linearity

In the Ramco assay the recovery after dilution was 108% for 1-fold to 10-fold diluted samples. In Eurogenetics the recovery was progressive from 114% in an 1-fold to 126% in a 10-fold dilution.

4.4.1.5 Method comparison

In regression analysis a linear correlation between the Ramco and Eurogenetics tests was found, r=0.863 (figure 4.1). A linear correlation was also found between the Ramco and the Orion test (r=0.700).

Figure 4.1 - Method comparison by regression analysis performed according to Passing and Bablock. Results of the Ramco assay are expressed as µg/ml x 100, in the Eurogenetics assay as U/ml.



4.4.2 Clinical significance

For the Ramco assay we found a normal range of 4.94 +/- 1.64 mg/l, for the Orion assay 3.1 - 4.5 mg/l. In the Eurogenetics assay the normal range was 351 +/- 200 U/ml.

With Ramco assay 17 sera were analysed of patients with anaemia of chronic and inflammatory diseases, 10 sera of patients with proven iron deficiency and 10 sera of patients with MDS. Results are shown in table 4.1.

In iron deficiency the mean transferrin receptor concentration was significantly higher than in normal subjects. In ACD the mean concentration was significantly lower than in iron deficiency and differs not significantly from normal subjects. In MDS the range was very wide but excluding the two most extreme values resulted in a mean concentration which was not significantly different from normal subjects.

Table 4.1 - Serum transferrin receptors in normal subjects and in patients with iron deficiency, myelodysplastic syndrome and anaemia of chronic and inflammatory disease.

diagnosis	cases	transferrin receptor mg/l	range	p-value
healthy volunteers	62	4.94 ± 0.82	3.4 - 7.0	.....
iron deficiency	10	12.23±2.03	10.0 - 15.8	< 0.01
myelodysplastic syndromes	10	11.61±11.63	4.2 - >40	< 0.01
anaemia of chronic disease	17	5.84 ± 1.95	3.0 - 8.7	< 0.01

The clinical value of the Orion test kit was tested by analysing 38 serum samples with both the Orion and the Ramco test kits. The upper limit of the reference interval was exceeded in 22 of 38 patients using the Ramco assay. However, the upper reference value was exceeded in all patients when using the Orion test kit, leading to a different clinical conclusion in 16 cases. The deviation which was found was not consistent: in 10 cases the concentration of sTfR in the Ramco assay exceeded the value found by the Orion assay, while in 16 cases the opposite was found. In 12 cases both values exceeded 10 mg/l. Since the highest standard in the Orion test kit is 10 mg/l, the true value of these samples could not be measured with this test kit without predilution.

4.5 Discussion

The analytical performances of three commercial available assays for soluble transferrin receptors in serum were studied. In addition the clinical significance of the results was assessed.

The test kits distributed by Eurogenetics and Orion were easier to use when compared to the Ramco assay but the analytical performances of the Eurogenetics assay were unacceptable. The combination of a high inter assay imprecision and a high end of run drift makes the assay useless in a routine laboratory, although increasing the incubation time to one hour and increasing the dilution of the samples might improve the results. The manufacturer recommends to test only 6 strips in one run. However an end of run drift of 24% within the first 6 strips was still found. Analysing only 6 strips in one run makes the test much more expensive than the Ramco test because a standard curve has to be established in every run.

The analytical performance of the Ramco and Orion assays were better. We found intra and inter assay imprecisions that were slightly higher or the same as those given by the manufacturer.

The highest standard in the Orion assay has a concentration of 10 mg/l, making it unsuitable for measuring samples which exceed 10 mg/l. Introducing an extra high level standard to the set of standards may solve this problem.

Both the Ramco and the Orion test kit express their results in mg/l, suggesting comparable results, but the results derived from both assays were not comparable. In 16 out of 38 cases this led to a different clinical conclusion. In contrast, the Eurogenetics assay expresses the results in kU/l. In the past, standardisation of the soluble transferrin receptor assays has been problematic, because purified transferrin receptors were not available. The lowest standards in the Orion test kit are derived from pooled human serum, while the highest standard consists of purified placental transferrin receptor. The affinity of the monoclonal antibody can be different for receptors derived from different tissues. The set of standards in the Ramco test kit is composed of purified placental transferrin receptor. This guarantees a comparable affinity of the monoclonal antibody for every standard.

The diagnosis of an uncomplicated iron deficiency is usually straightforward. Serum ferritin is a reliable blood constituent for predicting bone marrow iron stores. Prediction of the iron content in bone marrow from the ferritin concentration in serum is less reliable in inflammatory disease, in cancer and liver disease. In these cases serum ferritin is often raised as a result of the acute-phase reaction. Serum ferritin parallels other proteins such as C-reactive in the acute-phase reaction. Often a bone marrow aspirate has to be taken to establish the diagnosis of anaemia of chronic

disease. Therefore it would be valuable to have a new quantity for the differentiation of iron deficiency from anaemia of chronic and inflammatory disease. It has been postulated that the soluble transferrin receptors in serum could be important in this differentiation (9).

In earlier publications the concentration of sTfR has been found to be an important tool in the assessment of iron status. In this study we were able to confirm that in patients with uncomplicated iron deficiency the concentration of soluble transferrin receptors is significantly higher than that in healthy volunteers. In cases of anaemia of chronic and inflammatory disease the levels of soluble transferrin receptors in serum are slightly, but not significantly, higher than in normal subjects. By measuring soluble transferrin receptors in serum a good differentiation between anaemia of chronic disease and iron deficiency is possible.

An other diagnostic problem is caused by anaemia as part of myelodysplastic syndromes. Myelodysplastic syndrome is a heterogeneous group of disorders in which anaemia is often the presenting symptom. In the early phases many patients receive iron to correct their haemoglobin concentration, leading to a delay in diagnosis and accumulation of iron in multiple organs. In this study soluble transferrin receptors in the sera of patients with myelodysplastic syndrome were analysed. The results varied enormously. In most patients normal levels were seen. However, some individual patients showed extremely elevated values, even far above the upper limit found in severe iron deficiency. These results indicate that in myelodysplastic syndrome investigation of the bone marrow is still required for diagnosis, and soluble serum transferrin receptor analysis is not helpful.

In conclusion, the results in this study have shown that the analytical performances of the Ramco and Orion assays were acceptable, whereas those of the Eurogenetics assay were not. In the latter assay the inter assay imprecision and the end of run drift were unacceptable high. Using these commercial test kits the small clinical study confirms previously published data showing that soluble transferrin receptors are elevated in uncomplicated iron deficiency. Measurement of the concentration of transferrin receptors in serum can help in the differentiation of subjects with anaemia of chronic and inflammatory disease from those with iron deficiency. In the diagnosis of myelodysplastic syndrome the soluble transferrin receptor concentration is of no help and a bone marrow aspiration is always required. However it was also shown that the conclusion drawn may depend on the test kit used, as result of a lack of standardisation. For diagnosis and follow up of patients this is unacceptable. The time for international standardisation has come to make results in the follow up of individual patients as well as results derived from clinical studies comparable.

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Chapter 5

Relationship between soluble transferrin receptors in serum and membrane-bound transferrin receptors

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5.1 Abstract

The expression of transferrin receptors on the cell membrane of erythroblasts was analysed with flow cytometry in patients with different forms of anaemia. At the same time the concentration of soluble transferrin receptors (sTfRs) was analysed in serum. It was shown that only in iron deficiency a high concentration of sTfRs in serum could be explained by an increased expression of transferrin receptors on the erythroblastic membrane. In anaemia of chronic disease and myelodysplasia a discrepancy between a low expression on the cell membrane and normal or elevated serum values was seen.

From this study we conclude that the concentration of sTfRs in serum does not only depend on the expression of transferrin receptors on the erythroblasts but also on the erythroid proliferation activity.

5.2 Introduction

Iron is a key element in cell metabolism and growth in all cell types (1). Erythroid cells have a special requirement for iron due to the synthesis of haemoglobin. In serum, iron is transported complexed to transferrin, an 80 kDalton glycoprotein which is capable of binding two molecules of iron per transferrin molecule. The transferrin-iron complex binds to the transferrin receptor on the cell membrane thereby making iron uptake possible (1,2).

The transferrin receptor is a transmembrane protein present in all body cells. The number of transferrin receptors per cell depends on the requirement for iron and is regulated at the level of RNA (1). In intracellular iron deficiency transferrin receptors are produced, whereas in the state of intracellular iron overload the storage protein ferritin comes to expression (3). The transferrin receptor can be detected on the cell membrane by flow cytometry by using the monoclonal antibody CD71. Using another monoclonal antibody for distinguishing different cell types, the presence of transferrin receptors on a special cell type can be studied. The combination of GLY-A and CD 71 gives information on the number of transferrin receptors on erythroid cells (4).

In serum soluble transferrin receptors (sTfR) can be found as truncated fragments of the intact cellular receptors. These fragments appear in serum due to shedding or cleavage (2,5,6). The level of transferrin receptors in serum has been found to be directly proportional to the total amount of tissue receptors (2). Since the majority of tissue receptors can be found on erythroid cells, this is directly proportional to the erythroid mass. In cases of erythroid hyperplasia a rise in the levels of soluble transferrin receptors can be expected. This has been found to be true in iron deficiency and haemolytic anaemia (7,8,9,10). Likewise, in cases of bone marrow aplasia the level is sharply reduced. In patients with ineffective erythropoiesis, as in

myelodysplastic syndromes, a rise in the level of soluble transferrin receptors could be expected because of the increase of the erythroid compartment. However this relationship has not been found (11,12).

In the present paper we wish to describe the relationship between the expression of membrane bound transferrin receptors on erythroblasts and the level of sTfRs in serum. Soluble transferrin receptors in serum were quantified by an enzyme immune assay. The expression of membrane transferrin receptors was studied by flow cytometry.

5.3 Materials and Methods

This paragraph gives an overview of the methods used in this study. All reagents were of analytical grade. All patients samples were obtained with permission.

5.3.1 Patients

Fresh bone marrow aspirates were obtained from the sternum or iliac crest of 35 patients who were referred to the laboratory for analytic tests of anaemia as well as from 4 healthy volunteers. A serum sample was taken at the same time or just before the bone marrow aspiration. Exclusion criteria were: blood transfusion just before the bone marrow punction and megaloblastic anaemia due to deficiency of vitamin B12 or folic acid. All bone marrow aspirates were viewed by a panel of trained morphologists and repetitive counting of at least 500 cells were performed.

Patients were classified based on morphological criteria combined with clinical and chemical data as anaemia of chronic disease (17), iron deficiency (10) or myelodysplastic syndrome (8).

The diagnosis anaemia of chronic disease (ACD) was based on clinical and laboratory data. The laboratory data were a low haemoglobin concentration, low iron content in serum and the classic combination in bone marrow aspirates of high levels of stainable iron in the macrophages of the MPS and the absence of iron in erythroblasts.

Iron deficiency was diagnosed by the combination of low haemoglobin concentration, low iron content in serum and morphological criteria like microcytosis and absence of iron using morphological staining techniques.

The diagnosis of myelodysplastic syndrome was based on the morphological criteria by the latest French American British (FAB) classification (13). In this study only patients with refractory anaemia (RA), RA with an excess of blasts (RAEB) and refractory anaemia with ring sideroblasts (RARS) were included.

All bone marrow samples were analysed within 12 hours of aspiration. Serum samples were stored at -80 °C until they were analysed.

5.3.2 Apparatus

Flow cytometer: FacStar^{plus} flow cytometer (Becton Dickinson, USA) was used to analyse the cells. This flow cytometer was equipped with Lysis II software.

Precision pipettes: reference pipettes were obtained from Eppendorff, Germany.

A microtitration plate washer 1296-024 obtained from Wallac, Finland was used for washing the microplates.

Microplates were analysed in the microplate absorption photometer type ht II, Anthos Austria.

5.3.3 Staining procedures

Bone marrow aspirates were stained with the May Grünwald-Giemsa stain and examined on a slide.

Iron content was estimated by the Prussian blue reaction.

5.3.4 Soluble transferrin receptors in serum

Soluble transferrin receptors in serum were quantified with the Ramco transferrin receptor kit, an enzyme immunoassay which was purchased from DPC, Apeldoorn, The Netherlands. The assay procedure was performed according to the instructions of the manufacturer.

5.3.5 Immuno phenotyping

Three millilitres of bone marrow aspirate were drawn in 1 ml sodium citrate (3.8%, w/v) to prevent clotting. Mononuclear cells were isolated by density centrifugation using Lymphoprep solution (Nycomed, Oslo). Cells were suspended in RPMI/ foetal calf serum to a final concentration of 10 x 10^6 cells/l, after being washed twice with Diluid (Baker), containing 0.5 % BSA (w/v) and 0.05 % (w/v) NaN_3.

The mononuclear cells were then incubated with two monoclonal antibodies conjugated with fluorescein isothiocyanate and phycoerythrin respectively for 30 min at 4°C at a concentration recommended by the manufacturer. Monoclonal antibodies used were LCA (CD 45), LeuM3 (CD 14), Leu4 (CD 3), Leu12 (CD 19), LeuM9 (CD 33), all purchased from Becton-Dickinson, OKM5 (CD 36) purchased from Ortho,

IOA71 (CD 71) and GLY-A both purchased from Immunotech. Control mouse immune globulins (IgG 1 and IgG2a) were also purchased from Becton-Dickinson.

The nucleated population was gated on a two-parameter forward light scatter and 90° light scatter cytogram, the percentage of erythroblasts expressing the surface antigen of interest (CD 71) was determined from a dual parameter dot plot (GLY-A-FITC/CD 71-PE).

Transferrin receptors on erythroblasts were quantified as described (4). A standard curve was derived by plotting the mean channel and the fluorescence intensity (expressed as MESF) for every bead of Quantum 25 fluorescence beads (kindly provided by Becton-Dickinson). Determining the MESF of CD 71 on GLY-A positive cells gives information on the number of transferrin receptors on erythroblasts of different patients.

5.4 Results

A reference range for sTfRs in serum was established by analysing sera of 62 healthy volunteers. The normal range was defined as the mean value ± 2 SD and was found to be 4.94 ± 1.64 mg/l.

Figure 5.1 shows the results of the analysis of sTfRs in serum. From this diagram it can be seen that in iron deficiency the concentration of sTfRs in serum was increased above the upper limit of the reference range. In MDS normal levels of sTfRs were found in most patients. In two cases values were found that were far above the upper limit of the test. In anaemia of chronic disease the level of sTfR in serum was slightly elevated in 6 out of 17 patients.

Figure 5.1 - Concentration of soluble transferrin receptors in serum in healthy volunteers, iron deficiency, anaemia of chronic disease and myelodysplasia.

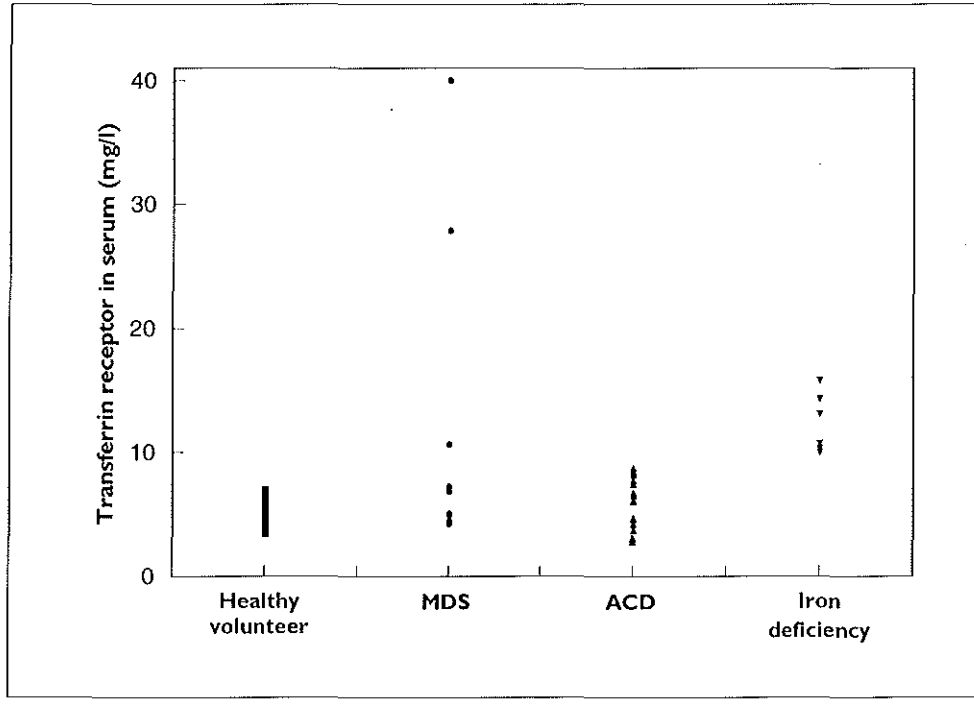
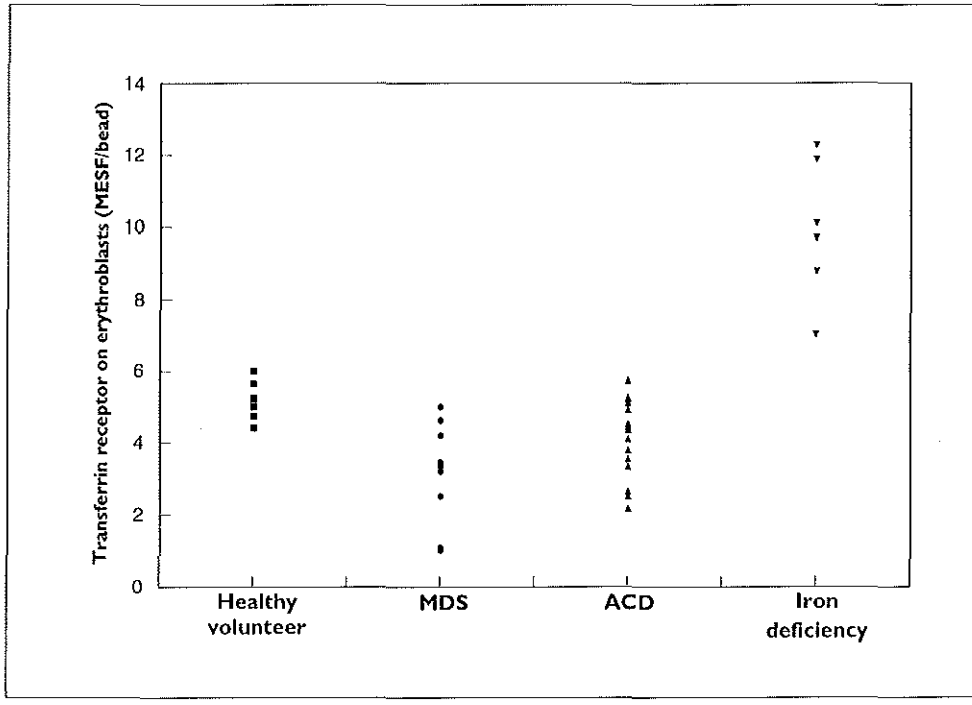


Figure 5.2 shows the expression of transferrin receptors on the erythroblastic membrane. In some patients more than one population of cells could be identified showing different fluorescence intensity for CD 71. This reflects a difference in the number of transferrin receptors for these populations of cells (4). In all cases it was possible to identify one major population which is indicated in figure 5.2. The expression of transferrin receptors on the erythroblastic membrane was increased in all cases of iron deficiency. In MDS the expression of transferrin receptors on the cell membrane was sharply reduced in some of the patients and comparable to healthy volunteers in the remaining patients. In ACD the expression of transferrin receptors on the cell membrane was reduced in most patients.

Figure 5.2 - Expression of transferrin receptors on the erythroblastic membrane in bone marrow of healthy volunteers, iron deficiency, anaemia of chronic disease and myelodysplasia.



5.5 Discussion

Transferrin is one of the most extensively studied proteins of the iron metabolism. It transports an element essential for cell metabolism between the sites of absorption and storage and the sites of utilisation. The interaction of transferrin and the transferrin receptor is essential for the influx of iron into the cell.

In earlier studies it was shown that in cell culture transferrin receptors are released from erythroid cells to the serum at the middle to late phases of maturation, mainly from polychromatic erythroblasts (14). In human sera sTfRs can be found as truncated fragments of membrane bound receptors. In many studies the relationship between the concentration of sTfRs has been analysed in relation to clinical conditions. The increased concentration in iron deficiency was found to be a useful parameter in differentiating it from anaemia of chronic disease (15).

Transferrin receptors can be found on the cell membrane in almost all cell types. However the concentration is highest on erythroid cells, since these cells not only require iron for metabolism but also for incorporation in haemoglobin. It is for this reason that the concentration of sTfRs in serum is mainly influenced by the expression of transferrin receptors on erythroid cells. On theoretical grounds it can be expected that the level of sTfRs in serum does not only depend upon the expression of transferrin receptors on individual cells but also on the total mass of erythroid cells. Evidence for this hypothesis can indirectly be gained from clinical studies. For example in bone marrow aplasia the level of sTfRs is decreased. In iron deficiency an increased expression of transferrin receptors on the cell membrane can be found, reflecting the increased requirement for iron by these cells (8,9).

In this study the level of sTfRs in serum was analysed in different clinical conditions in relation to the expression of transferrin receptors on the erythroblastic cell membrane. A direct relationship between the expression of transferrin receptors on the erythroid membrane and the level of soluble receptors in serum was only found in iron deficiency. In this condition the increased expression of transferrin receptors on erythroblasts leads to a direct rise in the concentration of sTfRs in serum.

In ACD the expression of transferrin receptors on erythroid cells is subnormal as well as the in vitro uptake of iron (4,16). In this study in 6 out of 17 patients the level of sTfRs in serum was increased while the expression of transferrin receptors on the cell membrane was reduced in most patients. This can not be explained by an absolute increase of erythroid mass since bone marrow cellularity and erythroid activity were within normal limits. A possible explanation could be an increased shedding of transferrin receptors from the cell membrane of maturating erythroid cells.

In patients with MDS normal levels of sTfRs were found in some sera whereas on the erythroid cell membrane the concentration of soluble transferrin receptors was sharply reduced. This could be explained by an increased cellularity and erythroid hyperplasia in the bone marrow aspirations reflecting an abundant amount of ineffective erythropoietic cells carrying decreased numbers of transferrin receptors on the cell membrane. In two cases an extremely elevated concentration of sTfRs was found in serum. The expression of transferrin receptors on the cell membrane was extremely low in both patients. Examination of the bone marrow aspirations revealed an extreme bone marrow hyperplasia with an abundant erythroid mass.

Recently, tests have been introduced for the detection of sTfRs in serum. These tests were brought into the market for the differentiation of iron deficiency from the anaemia of chronic disease. In this study the results in these tests were compared to the total erythroid activity and the expression of transferrin receptors on the erythroid cell membrane.

In conclusion it was found that only in uncomplicated iron deficiency can the concentration of sTfRs be correlated with bone marrow parameters. However the diagnosis of an uncomplicated iron deficiency is usually straightforward and the concentration of sTfRs does not provide additional information. In all other conditions the relationship between the level of sTfRs and membrane bound receptors was less clear and was also dependent on the total erythroid mass. This makes the interpretation of the level of sTfRs in serum difficult and a matter of debate.

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Chapter 6

Evaluation of the iron status of the newborn by soluble transferrin receptors in serum and characterisation of the transferrin microheterogeneity in newborns

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6.1 Abstract

The concentration of soluble transferrin receptors in serum has proven to be a reliable predictor of iron status in adults. Its high sensitivity for iron deficiency combined with a small sample size (10 µl) makes it an interesting parameter for the assessment of iron stores in newborn infants.

In the present study we investigated the usefulness of the concentration of soluble transferrin receptors in serum in the assessment of iron metabolism in the newborn. Infants born after an uncomplicated labour were compared to infants in the intensive care unit. The molecular structure of transferrin was studied to obtain information upon the interaction between transferrin and its receptor.

The concentration of soluble transferrin receptors in serum was found to be elevated compared to normal adults and independent of iron metabolism. The concentration of soluble transferrin receptors did not correlate with serum iron and ferritin concentration. In contrast to what was found in other studies, no relationship could be demonstrated between soluble transferrin receptors and birth weight or gestational age.

The molecular structure of transferrin in infants, showed a shift towards the less sialylated isotransferrins.

6.2 Introduction

Iron stores at birth have been shown to correlate very well with iron stores at 6, 9 and 12 months of age (1). In infants born at term the iron stores are usually sufficient for the first months of postnatal growth (2). Premature infants are more likely to develop iron deficiency anaemia since the total body iron at birth is lower than in full term infants. In addition, postnatal growth is faster than in infants born at term and susceptibility to infections puts an extra demand on the iron reserve (3). Iron deficiency in infants can lead to several systemic abnormalities, anaemia often being the first (2). However, the most serious result of iron deficiency at an early age is alteration in cognitive performance and motor development. Several studies have shown impaired mental and motor development of infants with iron deficiency compared to their peers with normal iron status (2,4-6).

Should the state of iron deficiency be cured as soon as the diagnosis has been made, iron overload should be prevented as well. Recent studies in newborn infants have shown that detectable levels of non-proteinbound iron can be found in up to 25% of healthy infants (7-9). Non-proteinbound iron is rapidly oxidised thereby possibly catalysing the conversion of O_2 and H_2O_2 into the highly reactive hydroxyl radical (10-14).

This ambiguous relationship of man with iron has proven the clinical importance of the assessment of iron status. In adults the serum ferritin concentration is the method of choice to estimate the iron stores. Recently the iron concentration of ferritin has been described to be a better indicator of iron reserve (15). In infants the erythrocyte ferritin concentration has been shown to be a reliable parameter of iron stores (16). However, all of these methods require large sample volumes, which makes them less useful in (premature) infants.

The cellular uptake of iron is mediated by the transferrin receptor. Iron is brought to the cell complexed to transferrin. The affinity of the transferrin receptor for transferrin depends both on the pH and the iron saturation of transferrin. Apart from that, the microheterogeneity of transferrin may also play a role.

Recently, the concentration of soluble transferrin receptors in serum has proven to be a reliable predictor of iron status. This parameter has been shown to reflect the cellular need for iron and is elevated in cases of cellular iron deficiency (17-20). Its high sensitivity for iron deficiency combined with a small sample size (10 µl) makes it an interesting parameter for the assessment of iron stores in newborn infants. The aim of the present study was to investigate the usefulness of the concentration of soluble transferrin receptors in serum in the assessment of iron metabolism in the newborn. This parameter was studied in relation to serum ferritin concentration and serum iron concentration as well as in relation to parameters such as gestational age, birth weight and birth asphyxia.

The transferrins are a heterogeneous group of proteins showing different degrees of sialylation and galactosylation. Different forms of human transferrin have been described in biological fluids. In serum from healthy individuals the tetra-sialo transferrin is the main component. Variation in the microheterogeneity pattern of transferrin can be found in several pathological conditions. A highly aberrant pattern has been described in children suffering from a rare syndrome dominated by neurological abnormalities (21). In serum from these children the di- and a-sialo fractions are present in high concentrations. This syndrome is known as the carbohydrate-deficient glycoprotein syndrome. However, little is known about the sialylation pattern of transferrin during normal foetal development. In the present study the sialylation of transferrins was studied in a group of newborns.

6.3 Materials and Methods

This section summarises the methods used in this study. All samples from the delivery room were transported to the laboratory as soon as possible. Parents agreed that their babies participated in the study.

6.3.1 Patients

The study population consisted of 133 infants, 98 healthy infants with a gestational age of 34 weeks or more born after uncomplicated labour and 35 infants with a gestational age ranging from 27 to 40 weeks who were admitted to the neonatal intensive care unit for prematurity or other neonatal complications such as birth asphyxia. Parents agreed that their baby participated in the study and the study design was approved by the medical ethics committee of the hospital.

Neonatal blood was taken from the umbilical cord, just after clamping. Blood samples were centrifuged immediately and serum was stored at -80 °C until analysis. In all samples the serum iron concentration and the concentration of soluble transferrin receptors was assayed. To limit the sample volume the ferritin concentration was only assayed in the samples taken from healthy infants. The microheterogeneity pattern of transferrin was assayed in 10 healthy children.

Intensive care patients were divided into a moderately asphyxiated group (n=15, arterial cord blood pH <= 7.18) and a non asphyxiated group (n= 18, arterial cord blood pH > 7.18). Severely asphyxiated children were not included in the study.

In addition, when blood was withdrawn from the patients in the neonatal intensive care unit for clinical purposes during the first 5 days of life a small additional sample was taken to study the day to day variation of the soluble transferrin receptor concentration. For this study repeated samples were taken from 20 patients. These blood samples were taken from an indwelling arterial catheter and the serum samples were also stored at -80 °C until analysis. Patients receiving a blood transfusion in the first days of life were not included in the study.

Serum samples were taken from 64 adult healthy volunteers to obtain the normal values for soluble transferrin receptors in adults.

6.3.2 Test procedures

All chemicals used were of analytical grade.

Pipetting was performed with reference pipettes that were obtained from Eppendorff, Germany.

Soluble transferrin receptors in serum were quantified with the Ramco transferrin receptor assay. This is an enzyme immunoassay which was purchased from DPC, Apeldoorn, The Netherlands. The assay was performed according to the instructions of the manufacturer. A microtitration plate washer 1296-024 obtained from Wallac, Finland, was used for washing the plates and microplates were analysed in the microplate absorption photometer type ht, Anthos, Austria.

The iron concentration of serum was assayed by means of the AU 510 analyser (Merck, Germany).

The ferritin concentration was analysed on a IM_x immune assay system (Abbott, USA) using the ferritin assay which was also purchased from Abbott, USA.

The blood gas analysis was performed on a ABL-510 blood gas system.

Transferrin microheterogeneity was studied in 10 newborns born after an uncomplicated labour. Different transferrins were separated by analytical isoelectric focusing on an immobiline gel (pH 4-7) in the PhastSystem as has been described in detail in chapter 2.

To prevent interference of incompletely iron-saturated transferrin with the test results, serum samples were saturated with iron before the analysis was performed: 50 µl of serum was incubated with 2 µl 10 mmol/l Fe(III)citrate and 2 µl 0.5 mol/l $NaHCO_3$.

6.3.3 Statistics

The association between the concentration of soluble transferrin receptors in serum and other variables was calculated using Spearman's rank coefficient of correlation.

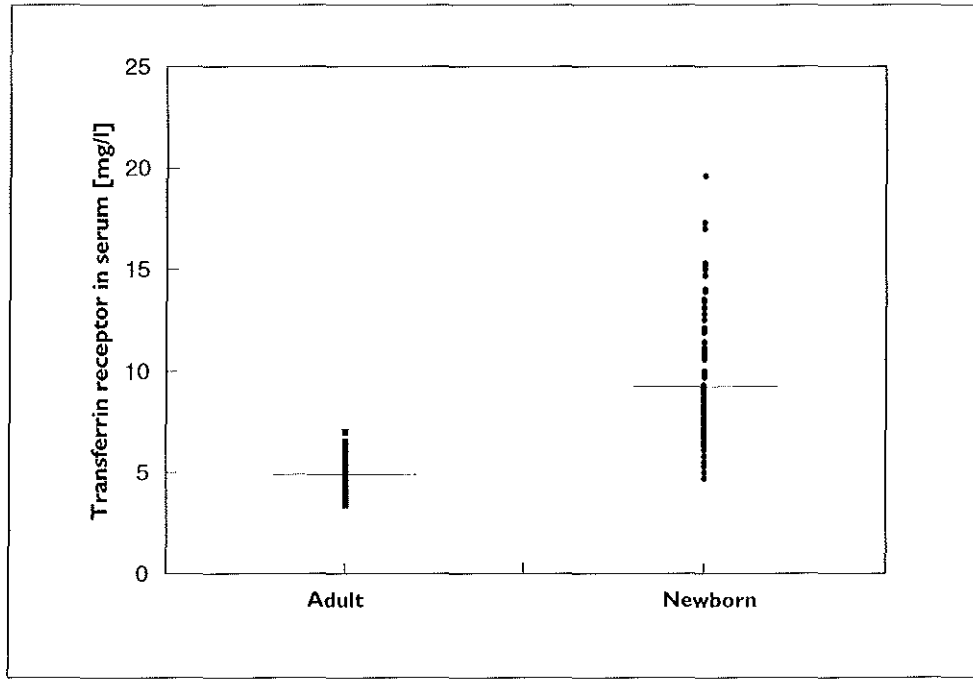
As the concentration of soluble transferrin receptors does not show a normal distribution a Mann-Whitney test was performed to study the difference between groups.

All statistical analysis was performed using commercial soft ware (SPSS, Chicago, Illinois).

6.4 Results

Figure 6.1 shows the distribution of soluble transferrin receptor concentration in serum for adults and newborns. The median concentration of soluble transferrin receptors found in the adult population was 4.90 mg/l (10th-90th percentile: 3.96-6.18 mg/l). In the group of newborn infants a median concentration of 9.25 mg/l (10th-90th percentile: 6.30-14.63 mg/l) was found, which is significantly higher than in adults ($p < 0.0001$).

Figure 6.1 - Soluble transferrin receptor concentration distribution between adults and newborns.



The concentration of soluble transferrin receptors in serum was studied in relation to cord blood pH as an indicator of asphyxia. The median concentration of soluble transferrin receptors in moderately asphyxiated children (cord blood pH <=7.18) was 10.45 mg/l (10th - 90th percentile: 5.83 - 14.21 mg/l). In the non asphyxiated group, (cord blood pH > 7.18) the median concentration of soluble transferrin receptors was 9.10 mg/l (10th - 90th percentile: 6.30 - 15.04 mg/l). This difference was not significant ($p = 0.424$). The difference between the concentration of soluble transferrin receptors in serum in infants in intensive care and healthy children was not significant ($p = 0.082$).

The day to day variation in soluble transferrin receptors in 20 intensive care unit infants was studied during the first 5 days of life. The median concentration of soluble transferrin receptors on the first day of life was 10.40 mg/l (10th - 90th percentile: 4.50 - 16.70 mg/l). On the fifth day of life a median concentration of 9.30 mg/l (10th - 90th percentile: 2.7 - 15.2 mg/l) was found. This difference was not significant ($p = 0.683$).

However, in individual patients a sharp decrease or increase was noted (10.7 and 12.2 mg/l falling to 2.4 and 2.2 mg/l respectively and 15.6 mg/l rising to 21.3 mg/l).

The concentration of soluble transferrin receptors in serum was studied in relation to two parameters of iron metabolism: the total iron concentration in serum (figure 6.2) and the concentration of ferritin in serum (figure 6.3). The serum concentrations of soluble transferrin receptor and iron did not correlate ($r = -0.068$). Only a slight negative correlation could be seen for the concentration of soluble transferrin receptors in serum and the concentration of serum ferritin ($r = -0.380$).

Figure 6.2 - Relationship between the serum concentrations of soluble transferrin receptors and total iron.

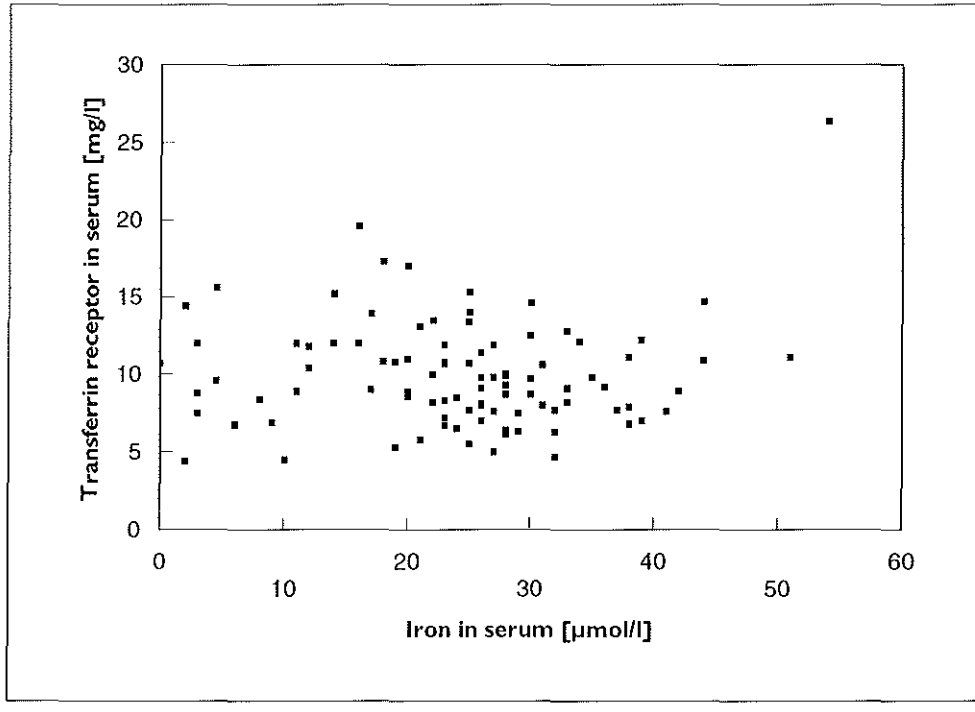
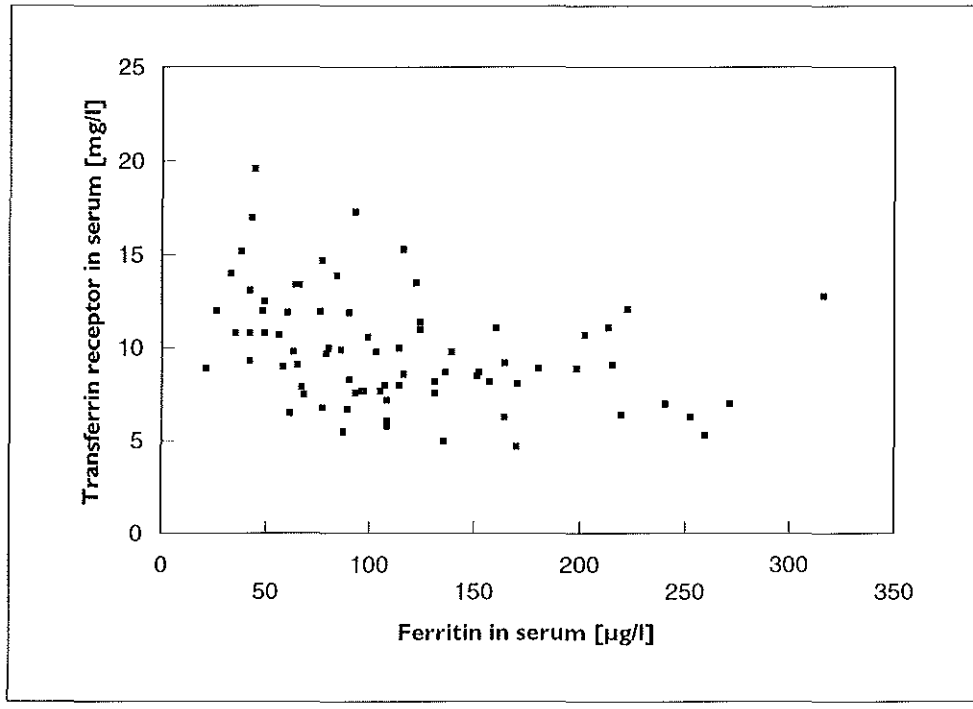


Figure 6.3 - Relationship between the serum concentrations of soluble transferrin receptors and ferritin.



The relationship between the concentration of soluble transferrin receptors in serum and gestational age is shown in figure 6.4. No direct relationship could be found between these two parameters ($r = 0.04$). The same was found for the concentration of soluble transferrin receptors in serum in relation to birth weight (figure 6.5, $r = -0.110$).

Figure 6.4 - Concentration of soluble transferrin receptors in serum in relation to gestational age.

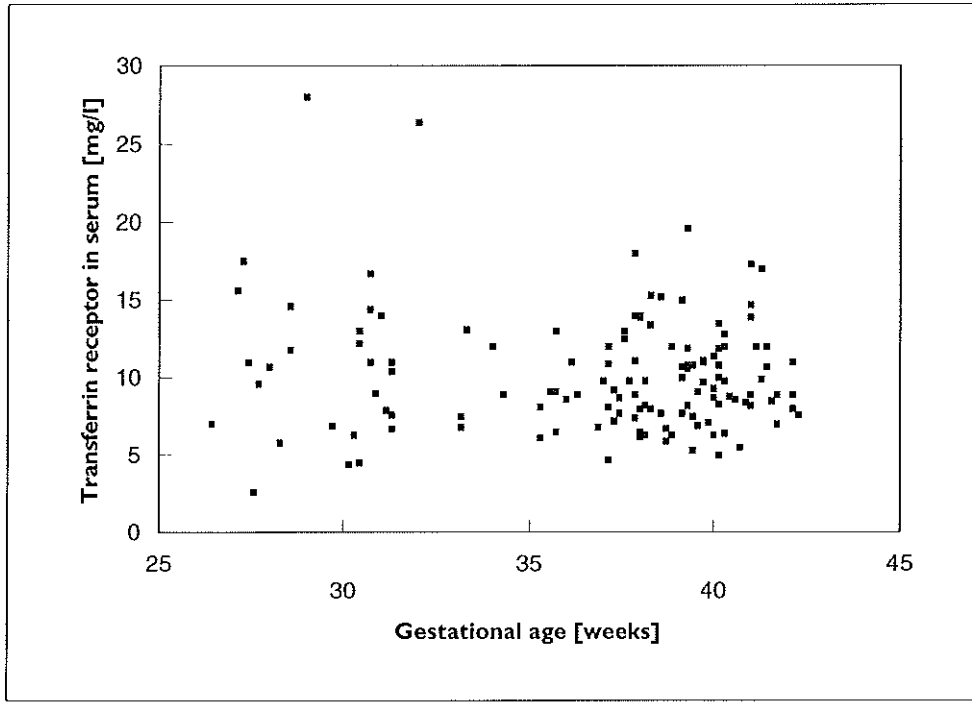


Figure 6.5 - Concentration of soluble transferrin receptors in serum in relation to birth weight.

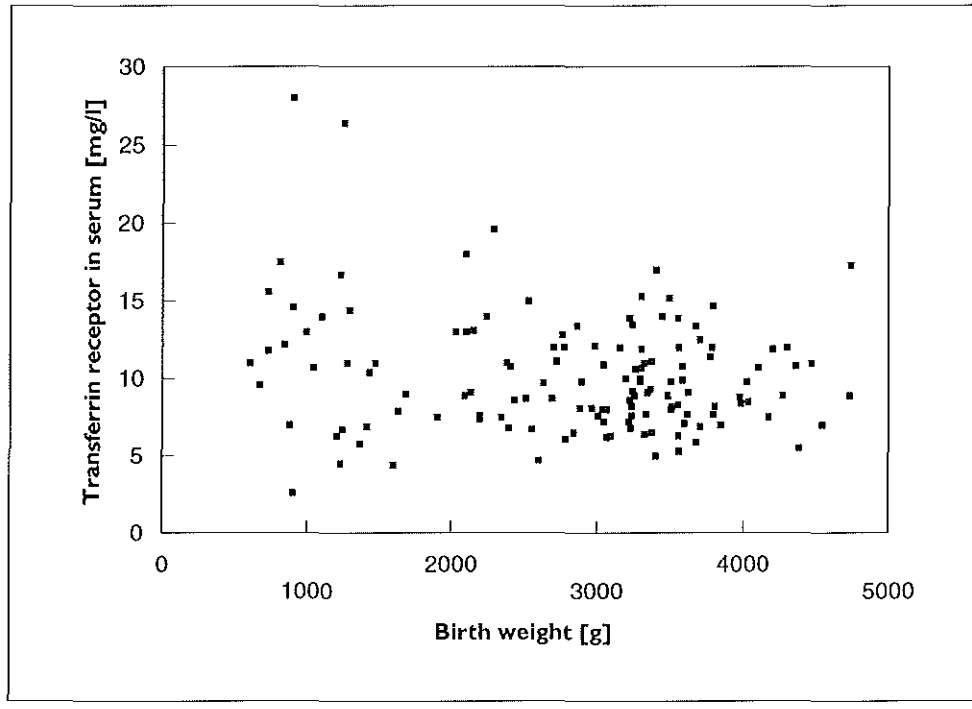


Table 6.1 summarises the mean percentages for 6-,5-,4-,3-,2-,1- and 0-sialo transferrin given as a percentage of the total amount of transferrin. Results are compared with the reference values for an adult population (lower row). As can be seen from this table the percentage of highly saturated transferrin in newborns is significantly lower compared to adult values especially the 5-, and 6- sialo transferrin. At the other end of the spectrum a-sialo and 1-sialo transferrin can be detected in newborns, while in adults these fractions are undetectable.

Table 6.1 - Mean percentages of different iso-forms of transferrin in newborns and adults.

patients	6-sialo	5-sialo	4-sialo	3-sialo	2-sialo	1-sialo	0-sialo
newborns	3.1	13.8	65	9.1	4.2	3	1.9
adults	5	20	63	9	3	nd	nd

6.5 Discussion

The transferrin receptor is a transmembrane glycoprotein which is involved in the cellular iron uptake. Transferrin receptors can be found on the cell membrane of almost all cell types but the expression on the cell membrane is highest on rapidly proliferating cells. In serum, fragments of these receptors can be found (22). The concentration of soluble transferrin receptors in serum has been shown to be directly influenced by the total amount of tissue receptors. In adults the erythropoietic cells are some of the most rapidly proliferating cells in the body and not only require iron for metabolism but also for incorporation into haemoglobin. It is for this reason that the concentration of soluble transferrin receptors in serum is mainly influenced by the expression of transferrin receptors on erythroid cells.

In foetal life, rapid cell proliferation and tissue growth is not only restricted to the erythropoietic cells in bone marrow. Therefore the concentration of soluble transferrin receptors in serum can be expected to depend upon the expression of transferrin receptors not only on erythropoietic cells but on all cell types. As a result, its concentration will be elevated compared to the adult population. In addition, the concentration of soluble transferrin receptors in serum could be expected to depend upon gestational age or birth weight. In this study the concentration of soluble transferrin receptors in serum was indeed found to be significantly higher in infants compared to the adult population. However, no relationship could be demonstrated between the concentration of soluble transferrin receptors in serum and gestational age or birth weight. This is in contrast to what was reported from a recent study (23). In this study a relationship was described between the concentration of soluble transferrin receptors and gestational age. However, the patients used in both studies were not comparable. In the present study all samples were taken after birth so were comparable to each other. This is in contrast to the other study in which samples were obtained by different techniques. The pre term serum samples were taken by antenatal umbilical cord blood sampling from foetuses in a rather stable physiological condition while samples from full term infants were obtained after birth. This may lead to differences in sample composition making it difficult to interpret results. Apart from that, careful examination of the results in this previous study shows that only a slight correlation has been described between the concentration of soluble transferrin receptors and gestational age ($r = 0.366$, $p = 0.06$) and the variation is high. The conclusion drawn from this study that the concentration of soluble transferrin receptors can be of use in the clinical assessment of growth retardation seems to be premature and can not be confirmed by our data.

In our study no difference in the concentration of soluble transferrin receptors could be demonstrated in the healthy population compared to their asphyxiated peers. This latter finding implicates that the concentration of soluble transferrin receptor in serum, in contrast to the concentration of ferritin in serum does not response to acute perinatal distress.

In recent clinical studies the concentration soluble transferrin receptors has shown to reflect the cellular need for iron and has proven to be a sensitive parameter to differentiate anaemia of chronic disease from iron deficiency. In the state of iron deficiency the concentration of soluble transferrin receptors is elevated and has been shown to be inversely related to the ferritin concentration and serum iron concentration (24). So far, most studies have been performed on serum samples derived from adults with different clinical conditions.

The gold standard for the assessment of iron stores is the amount of stainable iron in a bone marrow aspiration. However, in infants bone aspirations are not easy to obtain. The concentration of soluble transferrin receptors could be a promising parameter to evaluate the iron status in infants because of its high sensitivity while only a small sample volume is required. In the present study the concentration of soluble transferrin receptors was studied in the newborn infant in relation to different serum parameters of iron metabolism. Bone marrow aspirations could not be studied for ethical reasons. Because the gold standard was lacking, no firm conclusions could be made. The concentration of soluble transferrin receptors in serum did not show any correlation with either the ferritin or the serum iron concentration. This finding confirms the data from earlier studies (23,25). Shortly after birth the concentration of soluble transferrin receptors seems to be a parameter which is independent of iron metabolism and can not be used for the assessment of iron status. This is confirmed by the observed day to day variation during the first days of life. However, additional studies are required to select the age from which the soluble transferrin receptor concentration in serum correlates with the iron status.

Both transferrin and its receptor carry carbohydrate chains. The role of these carbohydrate chains has not been clarified yet. The microheterogeneity pattern of transferrin is modified during certain conditions, probably influencing the interaction of transferrin and the receptor (26,27). In this study the microheterogeneity of transferrin in newborn infants was studied. A shift towards the less sialylated fractions could be observed. Especially the 5- and 6- sialo transferrins were decreased. In the technique of isoelectric focusing the different iso-transferrins are separated depending on their isoelectric point. The isoelectric point is mainly defined by the number of sialic acid residues attached to the terminal carbohydrate residue.

The shift towards the less sialylated transferrins that was observed in infants might reflect a lower degree of branching of the oligosaccharide chains in the N-terminal domain. However, this can also be caused by a desialylation of the glycans. More research is needed on the structure of these glycans but is limited by the volume of the sample that can be obtained.

In adults it has been shown that different isoforms of transferrin have a different affinity for the transferrin receptor. In adults it was shown that the affinity of transferrin for its receptor is not influenced by the degree of glycosylation. However, the iron donation from completely deglycosylated transferrin is decreased compared to fully glycosylated transferrin (27). In a hypothetical model one could presume that in newborns this deglycosylated transferrin might act as an iron scavenger by binding excess of iron without accumulation in the cell.

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Chapter 7

General discussion

7.1 General discussion

Iron is one of the most interesting elements in the human body. It plays a key role in many biochemical processes simply because of its property that in an aqueous environment it rapidly changes between its two common oxidation states by the uptake of release of an electron. Iron is also an essential element so its availability depends on the balance between the daily intake and output.

In man, depletion of iron stores can lead to several systemic abnormalities, anaemia often being the first. On the other hand, iron overload is just as harmful since iron acts in its unbound state as a transition metal in the production of highly reactive oxygen and hydrogen radicals (1).

The ambiguous relationship of man with iron is strained to an even more critical balance in infants. Iron deficiency during infancy or childhood may lead to irreversible damage affecting long term developmental outcome (2). Newborn infants are highly susceptible to the adverse effects of iron. In the brain tissue of these patients high levels of superoxide and hydrogen peroxide can be found. These oxygen species are produced during the process of reperfusion and reoxygenation in perinatal asphyxia. In newborn infants detectable levels of unbound iron have been found (3,4), catalysing the conversion of these molecules to the highly reactive hydroxyl radical. It is clear that the developing brain of the newborn may be damaged by the adverse effects of these hydroxyl radicals (5).

7.1.1 Transferrin receptors on erythroblasts

Patients presenting with anaemia are often treated with iron for a couple of weeks before the final diagnosis is established. Those patients in whom the anaemia is not caused by iron deficiency will fail to respond to iron therapy. Iron will accumulate and eventually the adverse clinical effects of iron overload will develop without correcting the anaemia.

The cellular uptake of iron is mediated by the transferrin receptor (5). An optimal iron uptake by the cell requires an undisturbed binding of transferrin and its receptor. During the last decade, there has been increasing interest in the interaction of transferrin and its receptor. Under normal circumstances, the expression of cellular transferrin receptors is inversely related to the intracellular iron content (6). The molecular structure of the transferrin itself, especially the function of the glycans has been the target of research as well (7).

Using flow cytometry the expression of transferrin receptors can be quantified on different cell types. Studying this expression on erythroblasts in patients with different types of anaemia may select a subpopulation of patients who are unresponsive to iron therapy. The assessment of the expression of membrane-bound transferrin receptors on erythroblasts by flow cytometry is a sensitive method and gives direct information on the ability of iron uptake by the cell of interest. However, this technique is labour intensive and expensive. More important, this technique can only be used on freshly drawn bone marrow cells, since the membrane bound transferrin receptor has been shown to be destroyed in the process of freezing and thawing.

As shown in chapter 3, a decline in transferrin receptors on the cell membrane can be found in some clinical conditions. This will result in a diminished uptake of iron even though the intracellular iron content is decreased. Treatment of these patients with iron supplements will not correct the low haemoglobin concentration but will lead to accumulation of iron in several organs. This can be easily seen in Prussian Blue stained bone marrow aspirations. The diminished expression of membrane bound transferrin receptors in anaemia of chronic disease confirms the classical finding of abundant iron in the MPS of bone marrow in combination with an (almost) complete absence of sideroblasts.

7.1.2 Soluble transferrin receptors in serum

The soluble transferrin receptor in serum is considered to be a truncated fragment of the membrane bound receptor reflecting the total amount of transferrin receptors on different cells, especially erythropoietic cells (8). During the last decade many studies have been published describing the clinical significance of the concentration of soluble transferrin receptors in serum. However, all studies were performed in a research setting, since no commercial assays were available until recently. In the last two years different commercial tests have been released on the market for the detection of soluble transferrin receptors in serum. The analytical performance of the various test kits was in general acceptable for use in daily practice. However, one assay could not be introduced into a clinical laboratory without major adjustments to the test procedure.

Care has to be taken in the interpretation of the results obtained from the different assays. As has been described in chapter 4, the lack of international standardisation of these assays makes comparison of the results obtained from different assays impossible. The expression of the concentration of soluble tansferrin receptors in mg/l by various test kits suggests some standardisation. However, the origin of the purified transferrin receptor used as a standard differs not only between different test kits but may even vary within the same assay. This may cause problems in the clinical follow up of patients attending different laboratories.

All tests introduced so far are enzyme linked immune assays (Elisa's). Apart from being a labour-intensive and expensive method this implies that the assay will only be done when sufficient samples have been collected which will result in delayed reporting of the results to the clinician. This limits the value of this test in a diagnostic setting.

7.1.3 Clinical significance of sTfR in serum

The two groups of patients who will benefit most from a new and simple test for the assessment of iron metabolism are (newborn) infants and patients of all ages presenting with anaemia of unidentified origin. It is for this reason that much research has been done on the clinical significance of the assay of sTfR in serum. In several studies it has been proven that the concentration of sTfR in serum has a high sensitivity in the diagnosis of iron deficiency (9). Support for this hypothesis was found in chapter 5 of this thesis in which a direct relationship was described between an elevated expression of transferrin receptors on the erythroblastic membrane and an increased serum concentration of soluble receptors in iron deficiency. However, the diagnosis of uncomplicated iron deficiency is usually straightforward and can be established by the combination of a low haemoglobin concentration, MCV, total serum iron concentration and low ferritin concentration in serum (10).

The diagnosis of anaemia of chronic disease (ACD) is more complex. In clinical practice it is often difficult to differentiate this from anaemia caused by other factors (11). Those cases in which the anaemia of chronic disease is complicated by iron deficiency are especially difficult to diagnose. In chapter 3 it was found that the expression of transferrin receptors on erythroblasts in these patients was reduced compared to the healthy state. As has been described in chapter 5, no direct relationship can be found between the erythroblastic expression of transferrin receptors and the concentration of sTfR in serum. The discrepancy between a low expression of transferrin receptors on the erythroblastic membrane despite a normal or elevated serum concentration of the soluble form in some patients with ACD can be explained by shedding of the membrane receptor into the circulation. This limits the diagnostic value of the concentration of sTfR in ACD. In those patients in whom iron stores are sufficient this concentration can give valuable additional information. However, if iron stores are depleted the concentration of sTfR in serum will be the result of the combination of an increased concentration as a result of the iron deficiency and a normal or increased concentration as a result of chronic disease. The resultant concentration will depend upon the severity of each condition.

In patients who are suspected of having myelodysplasia the concentration of sTfR does not contribute to an earlier diagnosis. A bone marrow aspiration is still required to make the diagnosis. The diagnosis of myelodysplasia always has to be considered in every patient who does not respond to iron therapy after three months. The diagnosis requires the assistance of an experienced morphologist.

Since both iron deficiency as well as iron overload may lead to permanent damage to cellular proteins and DNA, iron stores in infants have to be kept within narrow limits. Iron stores at birth have been shown to correlate with iron stores later in childhood (12,13). In small children the concentration of sTfR could be an interesting parameter for the diagnosis of iron deficiency, especially in those cases where only a limited sample volume is available. If in these patients the diagnosis of iron deficiency is suspected on the basis of a low haemoglobin concentration, the suspected diagnosis can be confirmed by assaying the sTfR concentration in the EDTA-plasma sample. This way a second venous blood sampling to obtain a serum sample for assessment of the ferritin concentration is prevented. However, the concentration of sTfR was found to be independent of iron metabolism in newborns (chapter 6). The relationship between the sTfR concentration in the newborn and gestational age or birth weight which was earlier described by Carpani (14) could not be confirmed. Apart from that, no extensive studies have been performed to establish age related reference values.

7.1.4 Transferrin and its receptor in newborns

The key process in the cellular iron uptake is the interaction of transferrin, the iron transporting protein, with its receptor.

During the last decade extensive studies have been performed on the glycan composition of transferrin. The importance of the glycans for the function of the proteins has not been clarified yet (15). However, some clinical conditions have been shown to correlate with a different glycosylation pattern of the glycoproteins. For example, in the carbohydrate-deficient glycoprotein syndrome a highly aberrant glycosylation pattern has been shown to correlate with serious neurological abnormalities. In other clinical conditions, like pregnancy, a more physiological shift towards higher or less glycosylated proteins can be found (16). This might be an adaptation to an altered physiological state. Previous studies have shown that even though the affinity of transferrin for its receptor is not influenced by the degree of glycosylation of the protein, the iron uptake from aglyco-transferrin is decreased. In chapter 6 we describe a shift towards less sialylated transferrin in the newborn. Since studies in newborns are always limited by the available sample volume we were not able to identify the structure of the glycans in relation to gestational age. If we assume that the elevated asialo-transferrin concentration in newborns is representing an actual elevation of the aglyco-transferrin concentration this might act as a protection

mechanism for adverse effects of the non-proteinbound iron which can be found in newborns. In this hypothetical model deglycosylated transferrin serves as an iron scavenger clearing the circulation from the non-proteinbound iron from the circulation. This iron is then sequestered without taking too much iron to the cell.

The transferrin receptor is also a glycoprotein. This raises the question whether the transferrin receptor shows the same pattern of glycosylation as transferrin, and if so whether the affinity of the transferrin receptor for transferrin is influenced by this glycosylation pattern.

7.2 Conclusions

From this study it can be concluded that:-

(a) the ineffective response to iron therapy in cases of ACD and MDS can be explained by a reduction of transferrin receptors on the erythroblastic membrane;

(b) the concentration of soluble transferrin receptors in serum is directly related to the erythroblastic membrane expression only in iron deficiency;

(c) the discrepancy between a low expression of transferrin receptors on the erythroblastic membrane despite a normal serum concentration of the soluble form in some patients with ACD can be explained by shedding of the membrane receptor into the circulation;

(d) the concentration of sTfR in a diagnostic setting is of limited use;

(e) the concentration of soluble transferrin receptors in serum in the newborn is a parameter independent of iron metabolism, birth weight and gestational age;

(f) the shift towards the less sialylated iso-transferrins in the newborn may be part of an adaptation process by which unbound iron in the newborn is sequestered.

7.3 Future research

As this study proceeded, more questions have appeared which can only be answered by future research. Over the years, the laboratory techniques will evolve making it possible to further explore the field.

The recent developments in flow cytometry have made it possible to use three colour or even four colour fluorescence techniques in a routine setting. This increases the possibilities. In the last few years apoptosis has been an interesting new topic in many flow cytometric studies. This raises the possibility of studying apoptosis of the erythroblasts in MDS in relation to the transferrin receptor expression on its membrane. It would be interesting to perform this study to follow the response of individual patients who are treated with iron chelation therapy. Combining the results with the serum levels of IL 6 and EPO gives additional information. This might lead to some understanding of why some patients can be treated with erythropoietin to correct anaemia, while others become less dependent on red cell transfusions as a result of iron chelation.

In newborns more research is required on different aspects of iron metabolism. It would be interesting to identify the age-related glycan structure on transferrin. Apart from these issues it will be necessary to assess age-related reference values of soluble transferrin receptors for newborns and children of any age.

7.4 References

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Chapter 8

Summary - samenvatting

8.1 Summary

Iron is essential for life. It has its main function in the transport of oxygen through the body. Apart from that, iron is a key element in many biochemical processes in cell metabolism.

The iron balance in the human body almost completely depends on the dietary intake. The iron status is regulated at the level of iron uptake instead of at the level of iron excretion. Under normal circumstances the iron intake matches the daily losses but in some conditions this balance is disturbed due to an increased loss. In certain age groups in which rapid growth and development put an extra demand on iron stores iron deficiency easily develops. Other groups of patients at risk of developing iron deficiency are females in the fertile period and during pregnancy. Iron deficiency can lead to several systemic abnormalities, anaemia often being the first. The most serious results of iron deficiency at an early age are impairment of cognitive performance and motor development. Some studies have shown that these abnormalities persist despite extended oral iron therapy with adequate haematological response.

Should the state of iron deficiency be cured as soon as the diagnosis has been made to prevent long term consequences, iron overload should be prevented as well. Non-proteinbound iron acts as a transition metal catalysing the formation of highly reactive free radicals which can lead to permanent damage to intracellular proteins and DNA.

In the daily clinical practice of the general practitioner anaemia is one of the most frequent diagnoses. In many cases the anaemia is caused by iron deficiency. The diagnosis of iron deficiency is usually straightforward, requiring minimal diagnostic skills can be proven if a short period of iron suppletion leads to an increased haemoglobin concentration. Sometimes, however, the symptoms are masked by other diseases or the laboratory tests are disturbed as a result of other deficiencies or superimposed infections. In these patients it may be difficult to differentiate iron deficiency from other causes of anaemia in which the administration of iron may cause adverse effects.

The cellular uptake of iron is mediated by the transferrin receptor. The transferrin receptor is a transmembrane glycoprotein present on most cells. After the binding of transferrin to the receptor, the transferrin-iron complex is taken up by the cell and iron release takes place. The number of transferrin receptors on the cell membrane depends on the intracellular iron concentration. Soluble transferrin receptors can be found in serum. The concentration of these soluble transferrin receptors is related to the number of membrane-bound receptors and has been described as being a sensitive parameter of iron status.

This thesis describes a study of transferrin receptor expression on the erythroblastic cell membrane in relation to soluble transferrin receptors in serum. Different groups of patients were studied.

Chapter 1:

This chapter describes a summary of the latest views on iron metabolism. The different processes in iron uptake, iron transport and iron storage are described. An overview is given of the main causes of anaemia. Special attention is paid to iron metabolism during pregnancy and foetal development.

Chapter 2:

The techniques used in the experiments in this thesis are described in this chapter.

Chapter 3:

The presence of transferrin receptors on erythroblasts in patients with iron deficiency, anaemia of chronic disease and myelodysplastic syndrome is studied by two colour analysis on a flow cytometer. CD 71 is used to quantify the number of transferrin receptors and GLY-A to identify erythroblasts. In this study an explanation is found for the decreased iron uptake in erythroblasts despite sufficient storage pool iron.

Chapter 4:

In this chapter the analytic performances of recently introduced assays for soluble transferrin receptors in serum are described. In a small clinical study serum samples of patients with anaemia of chronic disease, iron deficiency and myelodysplastic syndrome are analysed as well as sera obtained from healthy volunteers. The problems in international standardisation are discussed.

Chapter 5:

It has been described in the literature that the concentration of soluble transferrin receptors in serum is directly related to the total amount of tissue receptors. In this chapter this relationship is studied. The expression of transferrin receptors on the cell membrane of erythroblasts in patients with different forms of anaemia is analysed with flow cytometry. At the same time the concentration of soluble transferrin receptors (sTfRs) is analysed in serum. The relationship described in the literature between the concentration of soluble transferrin receptors in serum and the number tissue receptors can not be confirmed for all diagnoses.

Chapter 6:

It has been known for a long time that iron deficiency in children may effect physical and cognitive development irreversibly. On the other hand, iron overload is just as harmful. In adults the concentration of soluble transferrin receptors in serum has proven to be a reliable predictor of iron status.

In the present study we investigate the usefulness of the concentration of sTfR in serum in the assessment of iron metabolism in the newborn. Infants born after an uncomplicated labour are compared to infants in the intensive care unit.

The interaction of transferrin and its receptor requires a structurally normal protein. In this chapter we also describe a study concerning the glycan structure of the transferrin molecule.

Chapter 7:

This chapter contains the general discussion and conclusions that can be drawn from the studies presented in the previous chapters.

8.2 Samenvatting

IJzer is van levensbelang, niet alleen omdat het onmisbaar is voor het transport van zuurstof door het lichaam, maar ook door de sleutelrol die het bekleedt in de stofwisseling van de cel. IJzer is een essentieel element, wat betekent dat de mens voor de toevoer volledig afhankelijk is van de voeding. De opname en uitscheiding van ijzer is in het ideale geval vrijwel in evenwicht en beweegt zich binnen nauwe grenzen. Door de toegenomen behoefte aan of het verhoogde verlies van ijzer tijdens onder meer groei, zwangerschap, borstvoeding en menstruatie kan gemakkelijk een tekort ontstaan. Dit kan aanleiding geven tot blijvende schade, zich uitend in bijvoorbeeld concentratiestoornissen en leerproblemen bij kinderen.

Even schadelijk als een ijzertekort is een teveel aan lichaamsijzer. Het ijzer is in staat om de vorming van zeer reactieve elementen te bevorderen, welke de cellulaire eiwitten en het DNA kunnen beschadigen.

Bloedarmoede komt in de dagelijkse praktijk van de huisarts veelvuldig voor. In veel gevallen is de oorzaak een gebrek aan ijzer en is dit gemakkelijk vast te stellen met minimale diagnostiek en omdat de klachten van de patiënt al in die richting wijzen. Een proefbehandeling met ijzer leidt dan al tot het gewenste resultaat, namelijk een correctie van de bloedarmoede. Soms echter, wordt het ijzergebrek gemaskeerd omdat bijkomende ziekte de diagnostiek vertroebelt of is het tekort aan ijzer niet de enige oorzaak van de bloedarmoede. Daarnaast kan bloedarmoede ook een heel andere oorzaak hebben, waarbij behandeling met ijzer juist schadelijk kan zijn. In deze gevallen is vaststellen van de oorzaak van de bloedarmoede gecompliceerd. Het is dan ook van belang om in een vroeg stadium de juiste oorzaak van de bloedarmoede adequaat vast te stellen. Het zal duidelijk zijn dat dit eens te meer geldt voor jonge kinderen.

Voor de opname van ijzer in de cel is de "transferrinereceptor" essentieel. Dit eiwit bevindt zich aan het oppervlak van de cellen fungeert als "herkenner" van het transferrine, het ijzertransporterend eiwit in de bloedbaan. Door koppeling van het transferrine aan de transferrinereceptor kan het ijzer worden opgenomen in de cel. Het aantal transferrinereceptoren op het celoppervlak zou een maat zijn voor de behoefte aan ijzer.

Het onderzoek dat beschreven wordt in dit proefschrift heeft de transferrinereceptor als centraal thema. Er is zowel gekeken naar transferrinereceptoren op de celmembraan van de jonge voorlopercellen in het beenmerg als naar de concentratie van oplosbare transferrinereceptoren in de bloedbaan. Deze oplosbare receptoren zijn fragmenten van de receptoren op de celmembraan en komen in de bloedbaan door afsplitsing van de cel. Verschillende patiëntengroepen werden bestudeerd.

Hoofdstuk 1:

In dit hoofdstuk wordt een samenvatting gegeven van de meest recente inzichten op het gebied van de ijzerstofwisseling. Hierbij komen de verschillende stappen in het proces van ijzeropname in het lichaam, ijzertransport door het lichaam en de opname van ijzer door de cel en de opslag in de cel aan de orde. Speciale aandacht wordt gegeven aan de ijzerstofwisseling tijdens de zwangerschap en bij de pasgeborene. Tevens wordt een beschrijving gegeven van de meest voorkomende oorzaken van bloedarmoede.

Hoofdstuk 2:

In dit hoofdstuk wordt een beschrijving gegeven van de methoden en de technieken die gebruikt zijn in dit onderzoek.

Hoofdstuk 3:

De transferrinereceptoren op het oppervlak van de jonge rode voorlopercellen in het beenmerg werden in kaart gebracht bij patiënten met verschillende vormen van bloedarmoede. De transferrinereceptoren op het celoppervlak werden gemeten met behulp van flowcytometrie, een techniek waarmee bepaalde celkenmerken kunnen worden zichtbaar gemaakt. Er wordt gebruik gemaakt van een fluorescerende stof die gebonden is aan een antistof welke specifiek reageert met een membraaneiwit.

In deze studie, die een beperkt aantal patiënten omvatte werd een verklaring gevonden voor de bevinding dat patiënten met bloedarmoede als gevolg van een chronische ziekte geen ijzer in kunnen bouwen, ondanks dat er een voldoende voorraad ijzer aanwezig is. Bij patiënten met myelodysplasie kon de ineffectieve groei van de jonge rode cellen zichtbaar gemaakt worden.

Hoofdstuk 4:

In dit hoofdstuk worden de analytische prestaties beschreven van de commerciële testkits die momenteel verkrijgbaar zijn en waarmee de concentratie van de oplosbare transferrinereceptoren gemeten kan worden. De verschillende kits bleken nogal verschillend te presteren. In één geval bleek dat de bepaling nog niet geschikt was voor gebruik in de routine. Er werd uitgebreid aandacht besteed aan de noodzakelijke internationale standaardisering van de bepalingen. Met de verkrijgbare testen werd ook een korte klinische studie verricht, waarbij nagegaan werd of de bepaling van de oplosbare transferrinereceptoren in het bloed kon bijdragen in het onderscheiden van de verschillende vormen van bloedarmoede.

Hoofdstuk 5:

Volgens de internationale literatuur zou de concentratie van oplosbare transferrinereceptoren in de bloedbaan direct afhangen van de hoeveelheid van deze receptoren op het celoppervlak. In dit hoofdstuk werd beschreven hoe bij de verschillende patiëntengroepen de concentratie van transferrinereceptoren in de bloedbaan zich verhoudt tot het aantal transferrinereceptoren op het celoppervlak. De veronderstelde directe relatie was niet bij alle diagnosegroepen even duidelijk aanwezig.

Hoofdstuk 6:

Bij pasgeborenen en bij te vroeg geborenen is nagegaan of de bepaling van oplosbare transferrinereceptoren een plaats verdient in de evaluatie van de ijzerstatus van het kind. Daar er slechts een zeer klein monstervolume nodig is voor de bepaling is deze mogelijk interessant. Voor de binding van transferrine aan de transferrinereceptor is de moleculaire structuur van het transferrine zelf ook van belang is. Omdat hierover bij gezonde kinderen niets bekend was is deze structuur ook bestudeerd.

Hoofdstuk 7:

In dit hoofdstuk worden de conclusies uit de overige hoofdstukken samengevat en worden aanbevelingen gegeven voor verder onderzoek.

List of abbreviations

ACD	: anaemia of chronic disease
CD	: cluster of differentiation/cluster of designation
CFU-GEM	: colony forming unit: granulocyte-erythroblast-megakaryocyte
CFU-GM	: colony forming unit: granulocyte-macrophage
CMML	: chronic myelo-monocytic leukemia
DNA	: desoxyribo nucleic acid
EIA	: enzyme immuno assay
ELISA	: enzyme linked immuno sorbent assay
EPO	: erythropoietin
ESR	: erythrocyte sedimentation rate
FAB	: French American Britisch cytomorphological classification of acute leukemias
Fe	: iron
FITC	: fluorescein isothiocyanate
FSC	: forward scatter signal
GLY-A	: glycophorin A
HRP	: horse radish peroxidase
Ig	: immunoglobulin
IRE	: iron responsive element
IRE-BP	: iron responsive element binding protein
LDH	: lactate dehydrogenase
MCV	: mean corpuscular volume
MCH	: mean corpuscular haemoglobin
MDS	: myelodysplastic syndrome
MEIA	: microparticle enzyme immunoassay
MESF	: molecules of equivalent soluble fluorochrome
MPS	: mononuclear phagocytic system
mRNA	: messenger RNA
Mw	: Molecular weight
IQ	: intelligence quotient
kDa	: kiloDalton

PE	: phycoerythrin
PM	: photomultiplier
RA	: refractory anaemia
RAEB	: refractory anaemia with excess of blasts
RAEB-t	: refractory anaemia with excess of blasts in transformation
RARS	: refractory anaemia with ring sideroblasts
RNA	: ribo nucleic acid
RPMI	: Roswell Park Memorial Institute
sd	: standard deviation
SSC	: sideward scatter signal
sTfR	: soluble transferrin receptor
TBARS	: thiobarbituric acid reactive species
TCA	: trichloric acetic acid
Tf	: transferrin
TfR	: transferrin receptor
v/v	: volume per volume
w/v	: weight per volume

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Curriculum Vitae

De schrijfster van dit proefschrift werd geboren op 9 juli 1961 te Kapelle-Biezelinge. In 1979 haalde zij het gymnasium ß diploma. In 1980 begon zij aan de studie geneeskunde aan de Erasmus Universiteit Rotterdam. Het keuze-onderzoek werd uitgevoerd bij de afdeling Chemische Pathologie van deze universiteit en had als onderwerp "microheterogeniteit van transferrine bij alcoholisten". In het kader van dit onderzoek werkte zij in de zomers van 1984 en 1985 bij Pharmacia in Uppsala, Zweden.

In 1987 begon zij aan de opleiding tot arts klinische chemie (laboratoriumarts) in het Drechtsteden Ziekenhuis in Dordrecht (opleiders: eerst dr. HC Lequin, later dr. RB Dinkelaar). In het kader van haar opleiding haalde zij het diploma stralingsbescherming niveau 3. In 1991 werd zij ingeschreven in het specialisten register van de SRC.

Op 1 april 1991 trad zij toe tot de Maatschap voor Klinische Laboratoriumdiagnostiek in het Sophia Ziekenhuis in Zwolle, met als aandachtsgebied de hemato-oncologie. Hier werd op 1 januari 1994 een begin gemaakt met het hier beschreven onderzoek. Eind november 1996 verwierf zij de SRC erkenning als opleider voor haar specialisme.

Na eerst enkele jaren actief geweest te zijn in de beroepsbelangen commissie van de Vereniging Artsen Laboratoriumdiagnostiek, de Wetenschappelijke Vereniging van de laboratoriumartsen, is zij sinds augustus 1994 secretaris van deze vereniging. Als lid van de wetenschapscommissie is zij mede-verantwoordelijk voor de organisatie van de jaarlijkse wetenschapsdag. Sinds 1 januari 1997 heeft zij zitting in de plenaire visitatie commissie van de VAL en is zij plaatsvervangend SRC lid.

8

De noodzaak tot meer inbreng van de laboratoriumspecialist in het klinische proces rechtvaardigt het bestaan van een eigen register voor de arts klinische chemie.

9

De titel "arts klinische chemie" dient vervangen te worden door "laboratoriumarts".

10

De plaats van een laboratoriumspecialist is in het eigen ziekenhuis.

11

Het bewerken van een proefschrift naast een volledige baan voorkomt fixatie en leidt daardoor tot een bredere vorming.

12

Bij aardappelmoeheid is Seresta de eerste keus.

13

In tegenstelling tot wat Leonardo da Vinci beweert is niet de beeldende kunst maar de muziek de hoogste aller kunsten.

14

Het beperken van de Nocturnen van Chopin tot nachtmuziek doet onvoldoende recht aan de schoonheid van deze muziek.

15

Dieet is meestal die eet niet.