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Role in detection of iron deficiency

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ISBN 978-951-29-7037-7 (PRINT)
ISBN 978-951-29-7038-4 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama Oy – Turku, Finland 2017

To my beloved Petra, Rasmus and Miska

ABSTRACT

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Soluble transferrin receptor — Role in detection of iron deficiency

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Annales Universitatis Turkuensis, Ser. D, Medica - Odontologica, Painosalama Oy, Turku, Finland 2017

Background and aims: Iron deficiency (ID) is probably the single most common nutrient deficiency in the world. It affects especially elderly persons, menstruating women, adolescent girls and infants. The concentration of the soluble transferrin receptor (sTfR) in the plasma is a relatively new laboratory test to detect ID. The aims of this study were to produce reference intervals (RIs) for sTfR and other hematological laboratory tests, to examine the influence of subclinical ID on the RIs and to establish the appropriate cut-off values for sTfR with regard to subclinical ID in elderly people, adolescent girls and preterm and full-term infants. A further objective was to establish the physiological changes of the hematological laboratory values during the first year of life among preterm and full-term infants.

Results and conclusions: In elderly people and adolescent girls, the upper reference limit of sTfR declined when the RIs were based on the values of an iron-replete reference group compared to a conventional reference group. This indicates that the reference individuals, selected conventionally, may have had subclinical ID which influenced the RIs of sTfR and other markers of iron status. In infants, the RIs were the same when based on the iron-replete reference group as the whole reference group. This suggests that ID was not a marked problem for infants of this study. The iron-replete RIs for sTfR were 1.0–2.4 mg/L and 0.9–2.4 mg/L for elderly people and adolescent girls, respectively. In preterm infants, the RIs of sTfR increased from 1.1–2.2 mg/L to 1.2–2.4 mg/L with advancing age from 20 weeks to 60 weeks. The upper reference limits of the RIs of sTfR may thus be used as decision limits for ID. The values for the preterm and full-term infants provide also valuable information about the kinetics of the hematological laboratory values during the first year of life and may help physicians to interpret the results of these markers for clinical decision-making.

Keywords: transferrin receptor, iron deficiency, iron-deficient erythropoiesis, ferritin, reticulocyte hemoglobin content, reference interval

TIIVISTELMÄ

Timo Takala

Liukoinen transferriinireseptori – Rooli raudanpuutteen toteamisessa

Turun yliopisto, Lääketieteellinen tiedekunta, Kliininen laitos, Kliinisen kemian oppiaine, Turun kliininen tohtoriohjelma (TKT)
Annales Universitatis Turkuensis, Sarja D, Medica - Odontologica, Painosalama Oy, Turku, Suomi 2017

Tausta ja tavoitteet: Raudanpuutteen arvioidaan olevan yleisin yksittäisen ravintoaineen puutos maailmassa. Erityisesti iäkkäät henkilöt, hedelmällisessä iässä olevat naiset, murrosikäiset tytöt ja imeväisikäiset ovat raudanpuutteen riskiryhmiä. Plasman liukoinen transferriinireseptori (sTfR) on verrattain uusi laboratoriotutkimus raudanpuutteen toteamiseksi. Tämän tutkimuksen tavoitteena oli tuottaa viitearvot sTfR:lle ja muille rautataloutta kuvaaville laboratoriotutkimuksille, tutkia mahdollisen piilevän raudanpuutteen vaikutuksia viitearvoihin ja selvittää sTfR:lle päätösrajat raudanpuutteen toteamiseksi vanhuksilla, murrosikäisillä tytöillä sekä keskosina ja täysiaikaisina syntyneillä. Lisäksi pyrkimyksenä oli esittää raudanpuutteen ja verenkuvan laboratoriotutkimuksien normaaleja muutoksia ensimmäisen elinvuoden aikana ja selvittää, miten näiden tutkimuksien arvot eroavat keskosten ja täysiaikaisena syntyneiden välillä.

Tulokset ja loppupäätelmät: Kun viitevälit laskettiin iäkkäille henkilöille ja murrosikäisille tytöille ryhmästä, josta oli poistettu todennäköisestä raudanpuutteesta kärsivät, sTfR:n viitevälin yläraja laski verrattuna normaalista viiteryhmästä määritettyihin viiteväleihin. Tämän perusteella normaalin viitearvoryhmän yksittäisillä henkilöillä oleva piilevä raudanpuute voi vääristää sTfR:n ja myös muiden raudanpuutetta kuvaavien laboratoriotutkimusten viitevälejä. Imeväisikäisillä viitevälit pysyivät samalla tasolla verrattuna koko viitearvoryhmään, kun viitevälit laskettiin ryhmästä, josta oli poistettu ilmeiset raudanpuutteesta kärsivät. Kun raudanpuutteesta kärsivät oli poistettu viitearvoryhmistä, sTfR:n viitearvoiksi saatiin vanhuksille 1,0-2,4 mg/l ja tytöille 0,9-2,4 mg/l. Keskosilla sTfR:n viiteväli nousi 20 viikon ikäisten viitevälistä 1,1-2,2 mg/l tasoon 1,2-2,4 mg/l 60 viikon ikäisillä. Näiden sTfR:n viitevälien ylärajoja esitetään päätösrajoiksi raudanpuutteen toteamiseksi. Esitetyt imeväisikäisten verenkuvan ja rautataloutta kuvaavien laboratoriotulosten muutokset ensimmäisen elinvuoden aikana voivat antaa kliinikoille hyödyllistä tietoa näiden tulosten tulkintaan.

Avainsanat: Transferriinireseptori, raudanpuute, raudanpuutteinen erytropoieesi, ferritiini, retikulosyytin hemoglobiinin määrä, viitearvot

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ABBREVIATIONS

ACD	Anemia of chronic disease
AUC	Area under the curve
CHr	Reticulocyte hemoglobin content
CV	Coefficient of variation
DcytB	Duodenal cytochrome b
DMT1	Divalent metal transporter 1
EPO	Erythropoietin
ERFE	Erythroferrone
Eryt	Erythrocyte count
Fpn	Ferroportin
GI	Gastrointestinal
Hb	Hemoglobin
HCP 1	Heme-carrier protein 1
Hct	Hematocrit (packed cell volume)
HIF-2α	Hypoxia inducible factor-2α
Hmox 1	Heme oxygenase 1
HRG-1	Heme responsive gene-1 protein
ID	Iron deficiency
IDA	Iron deficiency anemia
IDE	Iron-deficient erythropoiesis
Leuk	Leukocyte count
MCH	Mean cell hemoglobin (mean corpuscular hemoglobin)
MCHr	Mean cellular hemoglobin content of reticulocytes
MCV	Mean cell volume (mean corpuscular volume)
NTBI	Non-transferrin bound iron
PCBP1	Poly-r(C)-binding protein 1
PCBP2	Poly-r(C)-binding protein 2
RCT	Red cell transfusion
Ret-He	Reticulocyte hemoglobin equivalent
Retic	Reticulocyte count
RI	Reference interval

sTfR	Soluble transferrin receptor
Tf	Transferrin
TfR	Transferrin receptor (Transferrin receptor 1)
TfR2	Transferrin receptor 2
TfR-F index	Transferrin receptor / log ferritin ratio
ZIP14	A member of the ZIP family of metal transporters
ZnPP	Zinc protoporphyrin

LIST OF ORIGINAL PUBLICATIONS

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II. Takala TI, Suominen P, Lehtonen-Veromaa M, Möttönen T, Viikari J, Rajamäki A, Irjala K. Increased Serum Soluble Transferrin Receptor Concentration Detects Subclinical Iron Deficiency in Healthy Adolescent Girls. Clin Chem Lab Med 2003, 41: 203–208.

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1 INTRODUCTION

Iron is one of the most important micronutrients for the human body. All mammalian cells need iron. Iron has several vital functions in the body, *e.g.*, transporting oxygen to the tissues, participating in cellular energy metabolism and contributing to many enzyme reactions. Probably the best known function of iron is its role in hemoglobin (Hb) synthesis and red blood cell production.

The prevalence of anemia varies greatly in different regions of the world. Globally, the prevalence of anemia is 25–33% in the world's total population, which means that over 2.2 billion people are affected. About half, or even more, of the cases of anemia are caused by iron deficiency (ID), but there is considerable variation by region and population (De Benoist et al. 2008, Kassebaum 2016, Kassebaum et al. 2014). The World Health Organization identifies ID as one of the top ten risk health risk factors globally and regionally in terms of disease burden (World Health Organization 2002).

The progression towards ID begins when the iron requirement of the body exceeds iron supply. Emerging ID may be divided into successive phases: storage iron depletion, iron-deficient erythropoiesis (IDE, also called subclinical ID), and finally iron deficiency anemia (IDA) (Skikne et al. 1990, Suominen et al. 1998). Severe ID and even IDE may impair cognitive, behavioral, sensorial and motor development and performance and the effect may be irreversible (Akman et al. 2004, Algarin et al. 2003, Angulo-Barroso et al. 2016, Bruner et al. 1996, Lozoff et al. 1987, Lozoff et al. 1996, Roncagliolo et al. 1998, Scott and Murray-Kolb 2016, Shafir et al. 2006, Walter et al. 1989). IDA also causes deterioration of physical performance (Haas and Brownlie 2001). In the elderly, anemia is associated with cognitive decline (Andro et al. 2013). IDA may cause general symptoms like fatigue, pallor and palpitations and it may aggravate the symptoms of heart or lung diseases. Once a diagnosis of ID has been made, it is essential to uncover the reason for the ID, one of which is malignant disease, especially in the elderly.

Elderly people, women in fertile age, adolescent girls and infants are groups of people that are susceptible to ID. In the elderly, the reason for ID is usually gastrointestinal hemorrhage (Vannella et al. 2010). Fertile women need more iron because of iron loss with menstrual bleedings and adolescent girls have increased iron needs because of menstrual bleedings together with rapid body growth (Harvey et al. 2005). Infants are at risk of ID due to rapid body growth. The risk is even more pronounced in preterm infants whose iron stores are smaller after birth, growth is faster and blood sampling, which may be frequent, consumes iron. It is important that ID is detected in time in these groups to avoid

the deleterious consequences of ID. Also, a search for the cause of ID must be managed properly.

Assessment of the concentration of soluble transferrin receptor (sTfR) in the plasma is a relatively new laboratory test to detect ID. All mammalian cells have transferrin receptors (TfRs) on their surface where the TfRs are needed for receptor-mediated endocytosis of iron (Huebers and Finch 1987). If the iron supply does not meet the demand for iron, the expression of TfRs on the surfaces of cells is upregulated as a compensatory mechanism to maintain sufficient iron for metabolic needs. sTfR in the plasma is a truncated form of this cell surface receptor (Shih et al. 1993, Shih et al. 1990). Most of the sTfRs in the plasma are detached from maturing erythroid cells (R'Zik and Beguin 2001, R'Zik et al. 2001). The concentration of sTfR increases in ID (Punnonen et al. 1994, Punnonen et al. 1997, Skikne et al. 1990, Suominen et al. 1998), and this takes place even when there is IDE (Skikne et al. 1990, Suominen et al. 1998). The diagnostic use of sTfR has been extensively studied in adults but much less in the elderly, in adolescent girls and in infants. The present study was conducted to clarify the role of sTfR in these groups at risk for ID.

2 REVIEW OF LITERATURE

2.1 Basics of iron metabolism

Adults have a total of 3–5 grams of iron in their body. Up to 80% of the body iron is in the hemoglobin (Hb) of red blood cells in the form of heme. About 10–15% of the iron is present in muscle fibers (in myoglobin) and other tissues (in enzymes and cytochromes). The remaining iron resides in the liver and spleen, where iron is stored in macrophages and, in the liver, also in hepatocytes as ferritin and hemosiderin. The daily loss of iron from the body is normally about 1–2 mg through sloughing of mucosal cells and skin cells, sweating and bleeding; an equivalent amount of iron is absorbed from the intestine (Andrews 1999, Green et al. 1968, Lawen and Lane 2013). Iron absorption into the body can be regulated, but there is no active mechanism that would remove excess iron from the body. Therefore, the iron content in the body is entirely dependent on the regulation of iron absorption from the food. The basics of iron homeostasis are shown in figure 1.

2.1.1 Iron absorption

Dietary iron is present in a heme iron pool and a non-heme iron pool. Heme iron is contained in foods of animal origin as Hb and myoglobin. The heme iron content of various meat types varies considerably even within the meat types of the same animal (Cross et al. 2012). One study found that heme iron accounts for 30–40% of the iron in pork, liver and fish and 50–60% of the iron in beef, lamb and chicken (Cook and Monsen 1976). Non-heme iron is present in a wide variety of foodstuffs. Non-heme iron is the main iron pool in all diets and comprises about 90–95% of daily iron intake (Sharp and Srai 2007).

Iron is absorbed in proximal small intestine. Heme and non-heme iron seem to be absorbed by separate pathways. Heme iron is more efficiently absorbed than non-heme iron. About 20% of the heme iron but only 1–10% of the non-heme iron is absorbed (Martinez-Torres and Layrisse 1971). Non-heme iron absorption is facilitated by ascorbic acid and inhibited by calcium, phytate and phenolic compounds (Conway et al. 2006, Cook and Monsen 1977, Cook and Reddy 2001, Gillooly et al. 1983, Hallberg et al. 1991, Tuntawiroon et al. 1991). There may be still a different pathway for ferritin iron absorption (Theil et al. 2012).

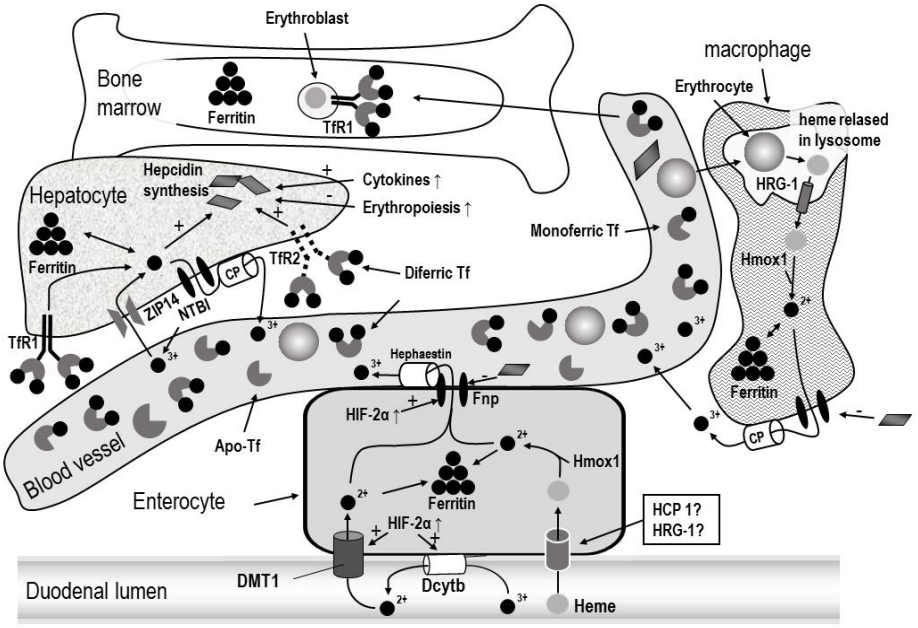


Figure 1. Homeostasis of iron

Non-heme iron is transported from duodenum to enterocyte by DMT1 in the ferrous (Fe^{2+}) form. Before transport, ferric (Fe^{3+}) iron is reduced to ferrous form by DcytB. Heme-iron is transported as an intact molecule by its own transport system. Proposed heme transporters are HCP 1 and HRG-1. In the enterocyte, heme is cleaved probably by Hmox1 to liberate ferrous iron. Ferrous iron is either transported to circulation by Fpn or stored within the enterocyte as ferritin. After transport, ferrous iron is oxidized back to ferric iron with the aid of Hephaestin, which is associated with Fpn. Ferritin bound iron leaves the body with the feces through the sloughing of senescent enterocytes to the gut lumen. In plasma, almost all iron is bound to the iron carrier protein Tf and iron is delivered to tissues. From the circulation, Tf-bound iron enters the target cells through receptor-mediated endocytosis mediated by TfRs. The main part of iron is used for the hemoglobinization of new erythrocytes but iron is needed also by many other kinds of cells. Iron can also be stored within the cells in the iron storage protein ferritin. Liver, spleen and bone marrow are the main iron storage organs. A minor part of iron pool in plasma is not bound to Tf. This NTBI can be uptaken by liver and pancreas by ZIP14.

Senescent erythrocytes are degraded by specialized macrophages mainly in spleen and liver. Erythrocytes are degraded in erythrophagolysosome and heme is liberated. Heme is transported to cytoplasm of macrophage by HRG-1 and is then degraded by Hmox1 to release iron for export or storage. Ferrous iron can be transported to circulation from the macrophages and hepatocytes by Fpn and extracellular iron oxidation is catalyzed by CP.

Hepcidin is a regulator of iron balance. Increase of hepcidin decreases iron absorption and release of iron to circulation by binding to Fpn which causes its internalization into a cell and subsequent gradation. Hepcidin synthesis is positively regulated by the body's iron content and inflammation and negatively by erythropoiesis. Iron absorption seems to be regulated also by HIF-2α, which increases the expression of DMT1 and DcytB in in the apical membrane of enterocyte following iron deprivation and hypoxia. It also directly increases the synthesis of Fpn in enterocytes.

DMT1, Divalent metal transporter 1; DcytB, Duodenal cytochrome b; HCP 1, Heme-carrier protein 1; HRG-1, Heme responsive gene-1 protein; Hmox1, Heme oxygenase 1; Fpn, Ferroportin; Tf, Transferrin; TfR, Transferrin receptor; NTBI, Non-transferrin bound iron; ZIP14, a member of the ZIP family of metal transporters; CP, Ceruloplasmin; HIF-2α, Hypoxia inducible factor-2α; TfR2, Transferrin receptor 2

Heme is liberated from the Hb and myoglobin in the food by proteolytic digestion in stomach. The mechanism of heme iron absorption is not as well known as of non-heme iron absorption (Mackenzie and Garrick 2005). Heme is taken up as an intact molecule at the apical brush border of enterocytes by the heme-binding receptor (Tenhunen et al. 1980). The heme-carrier protein 1 (HCP 1) has been proposed to be the long-sought intestinal heme transporter (Shayeghi et al. 2005). However, a subsequent study has shown that this transporter has a higher affinity for folic acid than for heme and its importance as the heme transporter has been questioned (Qiu et al. 2006). Another possible heme uptake transporter is the heme responsive gene-1 protein (HRG-1) (Rajagopal et al. 2008, White et al. 2013). Inside the cell, heme is probably cleaved by heme oxygenase 1 (Hmox1) which liberates ferrous iron (Fe^{2+}) (Raffin et al. 1974, West and Oates 2008).

Non-heme iron is liberated from its carrier molecules in the acidic environment of the stomach and proximal small intestine. Non-heme iron reaches the duodenum in the ferric (Fe^{3+}) or ferrous (Fe^{2+}) form, mostly ferric. Ferric iron must be reduced to ferrous iron before it can be transported across the apical surface of enterocytes by the divalent metal transporter 1 (DMT1), which is also known as divalent cation transporter 1 (DCT1), natural resistance-associated macrophage protein 2 (Nramp2) and solute carrier family 11, member 2 (SLC11A2) (Canonne-Hergaux et al. 1999, Gunshin et al. 2005a, Gunshin et al. 1997). The reduction reaction is mediated by duodenal cytochrome b (DcytB) and possibly by other reductases in the brush-border membrane of enterocytes (Gunshin et al. 2005b, McKie et al. 2001, Ohgami et al. 2006). The Intestinal brush-border Na+/H+ exchanger (NHE-3) plays an important role in generating a H^+ gradient that drives DMT1-mediated iron uptake at the apical surface of enterocytes (Shawki et al. 2016).

Some of the iron absorbed from the diet is stored as ferritin within the enterocyte. Ferrous iron (Fe^{2+}) in the cytoplasm of enterocytes is delivered to ferritin with the aid of the iron chaperones Poly-r(C)-binding protein 1 and 2 (PCBP1 and PCBP2). They form a complex with ferritin for iron delivery (Leidgens et al. 2013, Shi et al. 2008). Ferritin-bound iron leaves the body with the feces through the sloughing of senescent enterocytes to the gut lumen. This probably plays a role in regulating transport of iron into circulation.

The mechanism of how iron reaches the basolateral membrane of enterocytes is poorly understood, but a mechanism of transcytosis within endocytic vesicles has been proposed (Ma et al. 2006). In the basolateral membrane of enterocytes, iron is transported into the circulation by the only known iron export protein ferroportin (Fpn), which is also known as SLC40A1, metal transporter protein 1

(MTP1) and or iron-regulated transporter 1 (IREG1) (Abboud and Haile 2000, Donovan et al. 2000, McKie et al. 2000). Hephaestin, which is a transmembrane multicopper ferroxidase associated with Fpn, catalyzes the oxidation of the exported ferrous iron back to ferric iron (Vulpe et al. 1999). Then ferric iron is complexed with transferrin (Tf) for transportation within the circulation.

2.1.2 Iron transport and intake into cells

In humans, nearly all iron in plasma is bound to the iron carrier protein Tf. Tf is a glycoprotein mainly synthesized in the liver (Morgan 1981). One Tf molecule can bind two ferric ions (Bailey et al. 1988). Tf maintains iron in a redox-inert state and delivers it to tissues.

Tf-bound iron enters the target cells through receptor-mediated endocytosis mediated by the transferrin receptor (TfR, also transferrin receptor 1, TfR1, CD71) (Bomford and Munro 1985, Karin and Mintz 1981, Trowbridge et al. 1984). The existence of a specific receptor for Tf was first proposed by Jandl and Katz in 1963 (Jandl and Katz 1963), but was not until 1979 that TfR was adequately isolated and characterized (Seligman et al. 1979). Virtually all mammalian cells except mature red cells have TfRs in their surfaces. TfR is coded by a TFRC gene in chromosome 3 (Enns et al. 1982). It consists of two identical disulfide-bonded 760 amino acid glycoprotein subunits which each have molecular mass of ~95 KDa (Huebers and Finch 1987, McClelland et al. 1984, Schneider et al. 1984). Each monomer consists of three subunits: large extracellular portion which binds Tf, a hydrophobic transmembrane segment and cytoplasmic domain (Aisen 2004). The soluble extracellular portion of TfR composes of protease like domain, apical domain and helical domain (Lawrence et al. 1999). In 1999, also another transferrin receptor, namely transferrin receptor 2 (TfR2) was found (Kawabata et al. 1999). However, TfR2 does not play crucial role in iron delivery to cells but regulates hepcidin production on the grounds of circulating iron concentration (Johnson and Enns 2004). Circulating soluble transferrin receptor (sTfR) in plasma is mainly originated from TfR.

On the cell surface, TfR binds diferric Tf in a neutral pH environment with much higher affinity than monoferric Tf or apo-Tf (*i.e.*, Tf without Fe^{3+} ions) (Huebers et al. 1983, Young et al. 1984). The Tf-TfR complex is endocytosed by the cell via clathrin-coated pits. Once internalized, the content of the vesicle becomes acidic (pH 5.5) and iron is released from Tf. Apo-Tf remains attached to TfR in the endosome. Then the endosome is recycled to the cell surface and it rejoins the plasma membrane releasing apo-Tf from TfR in a neutral pH environment (Dautry-Varsat et al. 1983, Klausner et al. 1983).

Non-transferrin bound iron (NTBI) is a minor component of the plasma iron pool in humans with normal iron metabolism. Recently, it was shown that ZIP14, a member of the ZIP family of metal-ion transporters (also called SLC39A14), mediates plasma NTBI uptake by the liver and pancreas (Jenkitkasemwong et al. 2015). ZIP14 is involved in iron loading of the tissues in iron overload disorders, where NTBI is the main source of accumulating iron. Free heme and free Hb in the plasma are bound by haptoglobin and hemopexin to limit their toxic effects. These complexes are scavenged to cells by specific receptors for recycling of iron (Hvidberg et al. 2005, Kristiansen et al. 2001). This cycle plays a major homeostatic role in hemolytic conditions.

2.1.3 Iron storage

The liver and spleen are the main iron storage organs of the human body. In the cells, iron is stored mainly in the cytoplasm in the iron storage protein ferritin. The ferritin molecule is composed of 24 protein subunits that form a shell delimiting a large cavity that can store up to 4500 Fe^{3+} ions (Harrison and Arosio 1996). In addition to iron storage and release, ferritin plays an important role in detoxification reactions by controlling the pro-oxidant activity of iron (Arosio and Levi 2002). Fe^{2+} ions are delivered to ferritin with the aid of iron chaperones, PCBP1 and PCBP2 (Leidgens et al. 2013, Shi et al. 2008). Ferritin binds Fe^{2+} ions to a specific site named the ferroxidase center. Then Fe^{2+} ions interact with oxygen and are oxidized to Fe^{3+} ions which are stored in the ferritin cavity. From there, iron is available for cellular iron needs. The mechanisms involved in iron release from the protein deposit are still unclear (Finazzi and Arosio 2014).

Another protein involved in iron storage is hemosiderin. Hemosiderin is probably a water-insoluble degradation product of ferritin resulting from incomplete lysosomal processing (Richter 1978). Hemosiderin is a smaller iron storage component in healthy subjects compared to ferritin, but it accumulates during iron overload (Selden et al. 1980). The formation of hemosiderin is apparently beneficial in that it prevents iron from participating as a catalyst in iron-catalyzed free radical reactions (O'Connell et al. 1986, Ward et al. 2000). Although the processes leading to the formation of hemosiderin and to mobilization of iron from it are not well known, there have been very little studies on hemosiderin in the recent years.

2.1.4 Iron recycling

Although only 1–2 mg of iron is absorbed from the diet daily, much more iron is needed to cover the daily iron demand of the metabolic reactions in the body. For example, the bone marrow needs approximately 24 mg iron daily to produce over 200 billion erythrocytes (Knutson and Wessling-Resnick 2003). The plasma compartment of iron has a turnover of about two hours and 20–25 mg of iron travels through this compartment per day (Ganz and Nemeth 2012). To secure the daily iron demand, iron must be recycled from the senescent red cells and, to a lesser extent, from other cell types, as well. Human red cells have a life span of about 4 months. Erythrophagocytosis occurs in the reticuloendothelial system (RES) and splenic and hepatic macrophages play a key role in phagocytosis of senescent erythrocytes. Transcription factor Spi-C is required for the development of these specialized phagocytes, at least for the macrophages of the red pulp of the spleen and for efficient phagocytosis of red blood cells (Kohyama et al. 2009). The heme-regulated α-subunit of the eukaryotic translational initiation factor 2 (eIF2α) kinase (HRI) plays a role in maturation of macrophages and for their capacity to phagocytose erythrocytes (Liu et al. 2007). The phagosome of erythrophagocytes is united with lysosomal vesicles forming the erythrophagolysosome where erythrocyte is degraded and heme is liberated. Heme within the erythrophagolysosome is transported to the cytoplasm by HRG-1 (White et al. 2013). In the cytosol, heme can be degraded by Hmox1 to release iron. Subsequently, iron is either stored in ferritin or exported from the macrophage by Fpn (Korolnek and Hamza 2015).

2.1.5 Regulation of systemic iron balance

The human organism controls its iron balance in many ways. These include absorption, storage and recycling of iron. Notably, however, there is no active regulation of iron excretion. Under normal circumstances these iron-controlling mechanisms are stabilized but changes may occur in pathological situations.

The basic iron loss from the body of the adult male is about 1 mg per day; an equivalent amount of iron must be absorbed every day to maintain the iron balance. In fertile women, iron loss is increased by menstruation. During pregnancy, daily iron need increases up to over 6.5 mg / day (Bothwell 2000). Further iron loss may result from chronic bleeding from the gastrointestinal tract. In children, growth increases the iron demands of the body. If more iron is needed for the cells, duodenal absorption of iron and release of iron from iron stores are increased.

During the last fifteen years, research on iron regulation has mainly focused on hepcidin, the discovery of which was published in 2000. At that time hepcidin was named liver-expressed antimicrobial peptide or LEAP-1. (Krause et al. 2000). The following year, a study reported the isolation of the same cysteine-rich peptide, now named hepcidin (Park et al. 2001). The peptide was named hepcidin because of its origin in the liver ("hep-") and because it appeared to have antimicrobial properties ("-cide" for "killing"). In the same issue of the same journal another article appeared which was the first one to connect hepcidin with the regulation of iron (Pigeon et al. 2001). Soon after, hepcidin was identified as a key regulator of iron homeostasis (Nicolas et al. 2001, Fleming and Sly 2001).

Hepcidin is translated as a prepropeptide, 84 amino acid units in size (Park et al. 2001). The hepatic prohormone convertase furin mediates the posttranslational processing of hepcidin (Valore and Ganz 2008). The bioactive, circulating form of hepcidin is a peptide of 25 amino acids (hepcidin-25) and four disulfide bonds, mainly synthesized in the liver (Krause et al. 2000, Park et al. 2001). Hepcidin controls exportation of iron from the cells by regulating the expression of Fpn on the cell membrane. Hepcidin directly binds to Fpn which causes internalization of Fpn into the cell and subsequent degradation (Nemeth et al. 2004b). Reduction of Fpn on the cell membrane reduces export of iron from the cell. This results in a decrease of plasma iron content due to reduced iron absorption (enterocytes of the duodenum) and reduced iron release from the macrophages and iron stores (primarily hepatocytes of the liver).

Hepcidin is regulated by the body's iron content, inflammation and erythropoiesis. Hepcidin synthesis is positively regulated by the iron stores of the body and by the concentration of iron in the plasma. Serum hepcidin appears to have clear diurnal variation, as does serum iron (Corradini et al. 2011, Détivaud et al. 2005, Ganz et al. 2008, Gehrke et al. 2005, Ramos et al. 2011, Schaap et al. 2013). This control is mediated by the Bone morphogenetic protein / sma and mother against decapentaplegic (BMP/SMAD) signaling pathway (Parrow and Fleming 2014). Also, transferrin receptor 2 takes part in regulation of hepcidin production on the grounds of circulating iron concentration (Johnson and Enns 2004). ID reduces hepcidin synthesis, which increases iron absorption. Infections and inflammatory conditions increase the production of hepcidin induced by cytokines, the most important of which is interleukin-6 (Nemeth et al. 2004a, Nemeth et al. 2003). The effect of cytokines is mediated through the Janus Kinase-signal transducer and activator of transcription 3 (JAK-STAT3) pathway (Pietrangelo et al. 2007, Verga Falzacappa et al. 2007). Therefore, hepcidin plays a central role for the progression of anemia of chronic disease (ACD). The increased level of hepcidin causes reduces iron absorption and leads to iron

trapping within the macrophages and hepatocytes. Ultimately, this results in functional ID, *i.e.*, low levels of iron in the plasma and consequent IDE.

Increased erythropoiesis influences negatively hepcidin synthesis and allows a sufficient iron supply for erythropoiesis via increased iron absorption from the gut and mobilization from iron stores. The current view is that erythropoietin (EPO) represses indirectly hepcidin production by inducing secretion of erythroid regulators (Pak et al. 2006, Vokurka et al. 2006). A protein that mediates hepcidin suppression during stress erythropoiesis has also been identified and has been named erythroferrone (ERFE) (Kautz et al. 2014b). ERFE is produced and secreted by erythroblasts in response to EPO. ERFE seems to contribute to hepcidin suppression and iron overload in beta-thalassemia (Kautz et al. 2015) and also to recovery from anemia of inflammation (Kautz et al. 2014a). There is also an association between serum ERFE and administration of erythropoiesis-stimulating agents in hemodialyzed patients, whose ERFE levels correlate negatively with hepcidin and ferritin and positively with sTfR (Honda et al. 2016).

In addition to hepcidin, iron absorption seems to be regulated by Hypoxia inducible factor-2α (HIF-2α). HIF-2α is a regulator of DMT1 and DcytB expression following iron deprivation and hypoxia. A diet poor in iron induces HIF-2α protein expression in the duodenal cells and increases the expression of DMT1 and DcytB in the apical membrane of enterocytes (Shah et al. 2009). This increases iron absorption from the duodenum into enterocytes. HIF-2α signaling is also needed to maintain basal iron absorption, since loss of HIF-2α diminishes DMT1 and DcytB expression (Mastrogiannaki et al. 2009). HIF-2α also directly regulates the synthesis of Fpn in duodenal cells which raises the amount of Fpn in the basolateral membrane of enterocytes, and this, in turn, mobilizes iron into the circulation (Taylor et al. 2011).

2.2 Laboratory tests for detecting iron deficiency (ID)

2.2.1 Plasma iron, transferrin (Tf) and transferrin saturation

Plasma iron, Tf and transferrin saturation constitute the old guard of tests for detecting ID. In ID, plasma iron and transferrin saturation should decrease and Tf should increase. However, the plasma iron concentration varies considerably from day to day, within the same day and also from individual to individual. There is also marked fluctuation in a variety of clinical conditions unrelated to iron stores which makes the concentration of iron in the plasma a non-specific

assay with regard to true ID (Eckfeldt and Witte 1994, Romslo and Talstad 1988, Statland and Winkel 1977, Statland et al. 1976). Tf may react as a negative acute phase protein, which may result in a decrease of plasma Tf during infection, despite an adequate iron status (Szőke and Panteghini 2012). Transferrin saturation combines plasma iron and Tf and is hampered by the same problems as these two tests. Current knowledge does not support the use of plasma iron, Tf and transferrin saturation in the diagnosis of ID (Szőke and Panteghini 2012, World Health Organization 2007).

2.2.2 Plasma ferritin

Ferritin is the main iron storage protein in the body. Plasma ferritin correlates strongly with total body iron stores in healthy subjects and it indirectly reflects total body iron stores (Cook et al. 1974, Walters et al. 1973). A low plasma ferritin is highly specific for poor iron stores, because virtually no other condition causes low ferritin levels. Plasma ferritin is an acute-phase reactant and the levels of ferritin may rise during inflammatory and infectious states regardless of the body's iron status (Harrison and Arosio 1996). In general, a low ferritin indicates poor iron stores, but normal or high values do not rule out ID with certainty. Therefore, scrutiny needs to be applied in the interpretation of normal or high ferritin values and put into relation of the patient's general condition.

There is no consensus as to the optimal cut-off value for ferritin as a marker of ID. The lower reference limit of a reference interval (RI) created in the traditional manner for ferritin is not suitable for assessment of ID, because the apparently healthy reference population, used for determining the reference range the traditional way, probably includes subjects with ID. This yields a lower reference limit that is too low as a cut-off value to detect ID (Rushton and Barth 2010, Rushton et al. 2001, Suominen and Irjala 2001). Cut-off values between 12 and 30 μg/L have been proposed for otherwise healthy adults and higher values in patient populations (Beutler et al. 2003, Cavill 1999, Choi et al. 2005, Ferraro et al. 2012, Hallberg et al. 1993a, Joosten et al. 1991, Mast et al. 1998, Suominen et al. 1998, van Zeben et al. 1990). Recently, a cut-off value of 18 μg/L was proposed for 1–3-year-old children (Abdullah et al. 2017).

2.2.3 Soluble transferrin receptor (sTfR)

Human cells take iron into cells from the circulation via TfRs. TfRs are present in human plasma (Kohgo et al. 1986). These circulating sTfRs are virtually all

truncated forms of cell surface TfRs which lack the first 100 amino acid residues of intact receptor. The cleavage occurs between arginine 100 and leucine 101 in the extracellular domain of the intact receptor just above the cell membrane (Shih et al. 1993, Shih et al. 1990). The levels of these circulating sTfRs correlate with the amount of TfRs on the surfaces of cells and are mainly released from maturing erythroid cells (R'Zik and Beguin 2001, R'Zik et al. 2001). In serum of healthy individuals, the predominant form of sTfR is a dimeric TfR in complex with two molecules of Tf (molar ratio of 2:2) (Hikawa et al. 1996, Kogan et al 2005). It is shown further that in serum of patients with IDA dimeric sTfR with one molecule of Tf (molar ratio of 2:1) predominates (Kato et al. 2002).

Biological day-to-day variation of sTfR in venous plasma is 14% in adults, 11% in elderly women and 4% in iron-deplete young women (Ahluwalia et al. 1993, Belza et al. 2005, Cooper and Zlotkin 1996). Concentrations of sTfR in Children are slightly higher than in adults and there is no significant difference in reference intervals (RIs) between genders (Allen et al. 1998, Danise et al. 2008, Kohgo et al. 1986, Suominen et al. 2001 Vernet and Doyen 2000).

The main determinants of sTfR concentration are body iron status and erytropoietic activity. The concentration of sTfR is increased in ID, even before IDA develops (Skikne et al. 1990, Suominen et al. 1998). The other essential factor of the sTfR concentration, apart from ID, is the degree of erythropoietic activity; thus, the sTfR concentration may be multifold in hemolytic anemia compared to the non-hemolytic state (Huebers et al. 1990, Kohgo et al. 1987). Accordingly, sTfR is not a reliable indicator of ID in patients with high reticulocyte count (Retic). sTfR is not significantly influenced by infection or inflammation and it is superior to ferritin for the differential diagnosis of ID and ACD (Pettersson et al. 1994, Punnonen et al. 1997). sTfR might also be useful for detecting functional ID, regardless of iron storage status (Chang et al. 2007, Ervasti et al. 2004, Suominen et al. 2000).

The usefulness of sTfR in detecting ID in adults and elderly patients has been investigated in several studies which have used the gold standard, bone marrow iron stain for the assessment of iron stores. Most of these studies have concluded that sTfR is a valuable tool to detect ID and it can also be used to make differential diagnosis between ACD and ID (Fitzsimons et al. 2002, Hanif et al. 2005, Lee et al. 2002, Means et al. 1999, Punnonen et al. 1994, Punnonen et al. 1997, Suominen et al. 2000). However, few studies have not found sTfR to be an effective test to detect ID especially in the patients with ACD (Joosten et al. 2002, Junca et al. 1998).

In patients with chronic renal failure, sTfR does not give advantages compared to transferrin saturation or ferritin in the assessment of ID (Fernández-Rodriquez et

al. 1999, Fusaro et al. 2005). It has been proposed that low level of sTfR could have positive predictive value in the response to erythropoietin therapy in chronic renal failure (Ahluwalia et al. 1997, Beguin et al. 1993).

sTfR values are higher in thalassemia patients compared to normal controls and sTfR values correlate with difficulty of the disease (Cazzola et al. 1999, Dedoussis et al. 2002, Rees et al. 1998). The diagnostic accuracy of sTfR in the diagnosis of ID is poor in patients with thalassemia (Ong et al. 2008). However, it has been suggested that sTfR level could be used to predict risk of extramedullary erythropoiesis, therapeutic interventions and iron overloading in patients with thalassemia (Ricchi et al. 2012, Ricchi et al. 2017). Also in sickle-cell disease, sTfR levels reflect rather erythropoietic activity than ID (Al-Saqladi et al. 2012, Lulla et al. 2010, Singhal et al. 1993).

sTfR can be measured with regular, automated clinical chemistry analyzers. Probably the most commonly used automated sTfR assays are manufactured by Roche Diagnostics GmbH (Basel, Switzerland), Siemens Healthcare GmbH (Erlangen, Germany) and Beckman Coulter (Brea, California, United States). A point-of-care test has also been developed (Vikstedt et al. 2004). A drawback of the available commercial sTfR methods is that standardization is lacking and the results are method-dependent (Cotton et al. 2000, Kolbe-Busch et al. 2002, Yeung et al. 1998, Åkesson et al. 1999). The disparity in results between various assays may be caused by differences in TfR preparations used as standards and to raise antibodies (Kogan et al. 2007, Speeckaert et al. 2010). Fortunately, the first WHO reference reagent for sTfR has been established (Thorpe et al. 2010) and the problem might be solved in the future.

2.2.4 Transferrin receptor / log ferritin ratio (TfR-F index)

In 1990, the sTfR:ferritin ratio, which takes into account the reciprocal relationship of the two variables in ID, was studied for the first time as a single variable for assessment of body iron (Skikne et al. 1990). The study was carried out on repeatedly phlebotomized, healthy subjects and no major benefit from the use of this ratio was noted. However, in 1997 Punnonen and co-workers presented that combining sTfR and ferritin in the ratio between TfR and log ferritin gives a high specificity and sensitivity when IDA and ACD need to be differentiated (Punnonen et al. 1997). They named this ratio the TfR-F index. The usefulness of the TfR-F index in differentiating between of IDA and ACD has subsequently been ascertained (Bultink et al. 2001, Lee et al. 2002, Rimon et al. 2002, Skikne et al. 2011, Suominen et al. 2000). On the other hand, a recent meta-analysis concludes that sTfR may be of greater clinical value for detecting

IDA than the TfR-F index (Infusino et al. 2012). An inherent problem is that the criteria for separating between IDA, ACD and other groups has varied — and has occasionally been even poorly defined — in the studies included in the meta-analysis (Margetic et al. 2005, Marković et al. 2005, Matsuda et al. 2002, Skikne et al. 2011). Probably the main barriers against increased clinical use of the TfR-F index are method-dependent variation of the sTfR results and the lack of well-established RIs and decision limits for the different methods that are in use.

2.2.5 Reticulocyte hemoglobin content (CHr)

Reticulocytes are immature erythrocytes that have lost their nucleus but still contain ribosomal RNA. Maturation of the reticulocyte to a mature erythrocyte takes 3 to 4 days. Maturation takes place at first in the bone marrow and the last day or two in the circulation. Circulating reticulocytes do not synthesize Hb, while the younger reticulocytes in the bone marrow do. This raises the potential of measuring blood reticulocyte hemoglobin as a marker of the availability of iron early in erythropoiesis.

Measurement of reticulocyte cellular indices (including mean cell volume (MCV), mean cell hemoglobin concentration and CHr) from blood with automated blood cell analyzers were launched in the early part of 1990's for H*3 blood analyzer (Brugnara et al. 1994a). Of these, CHr is calculated from the reticulocyte mean cell volume and mean hemoglobin concentration which are directly measured. CHr gives an estimation of how much functional iron has been available for erythropoiesis during previous 3–4 days and reflects the effectiveness of iron supplementation (Brugnara et al. 1994b). CHr is closely associated with the hemoglobin content of mature erythrocytes (d'Onofrio et al. 1995). In adult patients who had undergone bone marrow aspiration, CHr less than 28 pg had highest overall sensitivity and specificity compared to ferritin, transferrin saturation and MCV for detecting absent iron stores by receiver operator curve analysis in patients with MCV < 100 fL (Mast et al. 2002). Values of CHr between 25 and 27.5 pg have been proposed as a cut-off limit for ID in infants and toddlers (Brugnara et al. 1999, Mateos et al. 2008, Torsvik et al. 2013, Ullrich et al. 2005). In hemodialysis patients, CHr < 29 pg is recommended for assessment of IDE and as a guide for the need for intravenous iron administration (Fishbane et al. 2001, Thomas et al. 2013). In genetic forms of microcytosis, *e.g.*, thalassemia and in megaloblastic anemia, CHr is not a reliable indicator of ID since the mean cellular volume is a component in calculation of CHr and these conditions introduce error (Mast et al. 2002, Skarmoutsou et al. 2003).

CHr is available only in ADVIA analyzers (Siemens Healthcare GmbH, Erlangen, Germany) which are successors of the H*3 blood analyzer. Similar parameters based on a different methodology are, nevertheless, available also in a some other blood cell analyzers (like the reticulocyte hemoglobin equivalent (Ret-He) in Sysmex analyzers from Roche, Basel, Switzerland and the mean cellular hemoglobin content of reticulocytes (MCHr), in the CELL-DYN Saphire analyzer from Abbott, Illinois, USA) (Piva et al. 2015). There is good agreement between CHr by Advia and Ret-He by the Sysmex analyzer (Brugnara et al. 2006, Buttarello et al. 2010). The results of CHr by Advia and MCHr by the CELL-DYN Saphire analyzer correlate well, but there is a significant absolute bias since CHr gives slightly higher results (Ermens et al. 2012).

2.2.6 Zinc protoporphyrin (ZnPP)

In the last step of heme synthesis, ferrous iron is incorporated into protoporphyrin IX. When the iron supply is limited, as in ID, the Zn^{2+} ion instead of the Fe^{2+} ion is linked to protoporphyrin IX leading to accumulation of ZnPP in erythrocytes (Lamola and Yamane 1974). ZnPP reflects inversely the iron stores in the bone marrow (McLaren et al. 1975). ZnPP may increase already during IDE (Hastka et al. 1994) and it may be a useful screening test for ID (Magge et al. 2013). In addition to ID, increased concentrations of ZnPP are due to ACD and lead poisoning (Hastka et al. 1993, Labbe et al. 1999). ZnPP is usually determined with hematofluorometer which makes it a potential test for point-of-care testing as well as for field studies (Labbe and Dewanji 2004). Recently, a non-invasive measurement of ZnPP (a fluorescence method from the lower lip) was introduced (Hennig et al. 2016). However, there are no automated methods for measuring ZnPP, which hampers the routine use of ZnPP in bigger laboratories and hospitals and limits significantly its general use.

2.3 Iron deficiency (ID)

ID is the most common nutritional deficiency in the world. In 2013, the global burden of anemia was estimated to be 27% of the world population which means almost 2 billion people, albeit the prevalence of anemia seems to be decreasing (Kassebaum 2016). ID accounted for about 60% of these cases. The prevalence of ID varies considerably between different areas of the world (Kassebaum 2016) and also among different age- and sex-groups (Cogswell et al. 2009, Looker et al. 1997).

2.3.1 Etiology of ID

ID may develop because of insufficient iron intake and absorption, excessive iron losses or increased physiological iron demands (Table 1). Insufficient iron intake is rarely the cause for ID in developed countries but more so in developing countries. Iron malabsorption may be a consequence of celiac disease, *Helicobacter pylori* infection, atrophic gastritis (gastritis-induced achlorhydria), gastrectomy or proton-pump inhibitor medication (Betesh et al. 2015). Excessive iron losses are usually due to chronic hemorrhage from the gastrointestinal (GI) tract (ulceration in the upper GI tract or GI cancer) or to menorrhagia in premenopausal women. Pregnancy and rapid growth in infancy and puberty increase the physiological demand of iron (Camaschella 2015, Lopez et al. 2016).

Table 1 Causes of iron deficiency (ID)

Insufficient iron supply
Iron-poor diet
Malabsorption of iron
Celiac disease
Helicobacter pylori infection
Atrophic gastritis
Gastrectomy
Proton-pump inhibitor medication
Excessive iron losses
Chronic hemorrhage from the gastrointestinal tract
Peptic ulceration
Gastrointestinal cancer
Inflammatory bowel disease
Hemorrhoids
Menorrhagia
Regular blood donation
Chronic hematuria
Increased physiological iron demand
Pregnancy
Rapid body growth
Infancy
Puberty

2.3.2 Stages of ID

The process leading to ID is fairly straight-forward, and may be divided into three successive phases (Suominen et al. 1998): storage iron depletion, IDE and IDA. These phases are associated with a characteristic pattern of changes of the hematological and biochemical iron status markers (Clark 2008). Typical findings in the hematological and biochemical iron status analytes in the different stages of ID are shown in Table 2. The understanding of the changes characteristic to each phase becomes relevant not only for the detection of pure subclinical ID or IDA, but also for interpreting laboratory results when ID may be suspected to concur with other anemic conditions (ACD, pregnancy, masking signs of megaloblastic anemia) or where ID may be caused by some chronic hematologic condition (sustained polycythemia vera) or therapeutic intervention (EPO treatment). The dynamics of the different analytes vary according to the phase of ID and any coexisting complicating conditions. This may become important in the search for an optimal marker of iron status for particular populations or conditions.

Table 2 Typical findings in the hematological and biochemical iron status analytes in the different stages of iron deficiency (ID)

Stage	Ferritin	sTfR	Tf	CHr	ZnPP	Hb
Normal iron stores	normal	normal	normal	normal	normal	normal
Storage iron depletion	low	normal	normal	normal	normal	normal
Iron-deficient erythropoiesis (IDE)	low	high	high	low	high	normal
Iron deficiency anemia (IDA)	low	high	high	low	high	low

sTfR, Soluble transferrin receptor; Tf, Transferrin; CHr, Reticulocyte hemoglobin content; ZnPP, Zinc protoporphyrin; Hb, Hemoglobin

2.3.2.1 Storage iron depletion

Storage iron depletion is the first sign of incipient progressive ID. It is caused by a negative iron balance sustained for a sufficiently long period of time. The main causes for this perturbation are related to accelerated loss (bleeding, hemolysis), impaired absorption (dietary restrictions, gastritis, celiac disease and other forms of malabsorption, cow-milk anemia in children) or increased demand (growth, pregnancy, malignancy) of iron. The iron stores function as a buffer against short-term physiological variations in iron supply and demand. Depletion of the iron stores is initiated when the amount of iron garnered by the storage system is

no longer able to replenish the amount mobilized from the stores. Serum ferritin is the most common test to indirectly evaluate bone marrow storage iron status and to detect depletion of iron stores. A low concentration of ferritin reflects accurately storage ID and is a reliable marker of storage ID in otherwise healthy subjects (Cook et al. 1974, Hallberg et al. 1993a, Harju et al. 1984, Jacobs et al. 1972). Another but invasive way to examine iron stores is to stain a bone marrow aspirate for iron, and this has been considered to be the gold standard for assessing the bone marrow iron status (Burns et al. 1990). Patient discomfort and costly and time-consuming laboratory proceedings restrict the use of bone marrow analysis to situations where anemia is profound or there is a clinical suspicion of some serious underlying condition requiring bone marrow aspiration for diagnosis. Bone marrow aspiration is rarely indicated for assessment of isolated storage iron depletion or subclinical ID.

2.3.2.2 Iron-deficient erythropoiesis (IDE)

When storage ID has progressed to the phase of depletion, the mechanisms to compensate for the poor availability of iron are activated. Upregulation of Tf has the teleological function of maintaining the bulk of the iron transport pool to satisfy the iron needs of different tissues and, especially, erythropoiesis. The circulating transferrin-borne iron is transported into cells mainly by the action of TfR, the expression of which is also compensatorily upregulated by ID (Huebers and Finch 1987, Skikne et al. 1990, Suominen et al. 1998). These mechanisms are, however, able to maintain an adequate flux of iron to tissues only to a limited degree, and as the iron availability of iron decreases, IDE results, since hemoglobinization of evolving erythrocyte progenitors is restricted (declining reticulocyte indices) (Brugnara et al. 1994b).

IDE may be detected by measuring components involved in the compensatory mechanisms (Tf, sTfR) (Skikne et al. 1990, Suominen et al. 1998) or by measuring the products of iron-deficient hemoglobin synthesis (CHr, ZnPP) (Brugnara et al. 1994b, Hastka et al. 1994). Clinically, the detection of IDE is important since it indicates the presence of the process, which, if sustained, will lead to IDA. In populations at risk for IDA (infants, adolescents, pregnant women) this may indicate a need to institute iron supplementation or dietary counselling to prevent progression into anemia. On the other hand, in populations where serious underlying conditions should be considered (elderly), suspicion of IDE may prompt further investigations to uncover the cause of ID.

2.3.2.3 Iron deficiency anemia (IDA)

IDA develops when IDE has continued long enough leading to a fall of the Hb concentration below the lower reference limit. Typically, IDA is hypochromic and microcytic (*i.e.*, the mean cell hemoglobin (MCH) and MCV are below the lower reference limits) (Auerbach and Adamson 2016). In uncomplicated IDA, ferritin and CHr are low and sTfR and ZnPP are high. A diagnosis of IDA should lead to further studies to identify the culprit of ID (Polin et al. 2013). Iron supplementation, and in serious cases also red cell transfusions (RCTs), are needed to treat anemia and ID.

2.3.3 Consequences of ID

The clinical presentation of ID may vary considerably. It may be asymptomatic, but can also cause a number of clinical symptoms. The best known consequences of IDA are pallor, weakness, fatigue, palpitation, dyspnea, irritability, headache and poor exercise and work performance (Liu and Kaffes 2012). In the elderly, anemia is associated with cognitive decline (Andro et al. 2013). Other symptoms of ID include pica-sign, koilonychia, cheilosis, decreased papillation of the tongue, dysphagia, restless legs syndrome and hair loss (Auerbach and Adamson 2016). Also, the symptoms of heart and lung diseases may worsen. In infants and older children, a major concern of severe ID and even IDE is that they may impair the child's cognitive, behavioral, sensorial and motor development and performance and the effect may be irreversible (Akman et al. 2004, Algarin et al. 2003, Angulo-Barroso et al. 2016, Bruner et al. 1996, Lozoff et al. 1987, Lozoff et al. 1996, Roncagliolo et al. 1998, Scott and Murray-Kolb 2016, Shafir et al. 2006, Walter et al. 1989).

3 AIMS OF THE STUDY

- to produce reference intervals (RIs) for soluble transferrin receptor (sTfR) and other hematological laboratory tests for elderly individuals and preterm and full-term infants
- to examine how subclinical iron deficiency (ID) affects the RIs of sTfR and other hematological laboratory tests in elderly individuals, adolescent girls and infants
- to establish the decision limits for sTfR to detect subclinical ID in elderly individuals, adolescent girls and infants
- to investigate how physical activity, puberty and advancing age influence the iron status of adolescent girls
- to present the physiological changes of hematological laboratory test values during the first year of life
- to clarify how hematological laboratory test values differ between preterm and full-term infants during their first year of life

4 MATERIALS AND METHODS

4.1 Patients, subjects and study design

4.1.1 Elderly (I)

This study was a part of the larger Lieto study which was carried out in Lieto, a semi-industrialized rural municipality in southwestern Finland. All residents born in or before 1933 living in Lieto on February 16, 1998 were invited to participate. The number of invitees was 1596 (666 male and 930 female). Of those eligible, 1260 subjects (82%) participated in the study (727 women and 533 men, 58% and 42% respectively). The participants of the Lieto study were interviewed by a trained nurse and clinically examined by a research physician. Their diseases were recorded with the diagnosis codes of the Tenth Revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10), and their medication was recorded. Venous blood samples were taken after an overnight fast.

The reference group of this study was selected retrospectively from the Lieto study population. Subjects who had diseases considered to interfere with their iron status laboratory tests were excluded. In addition to certain diagnoses, increased C-reactive protein ($\geq$ 10 mg/L) and creatinine ($\geq$ 125 μmol/L in women and $\geq$ 135 μmol/L in men) concentrations were also exclusion criteria for the general reference group. The only medication that excluded subjects from the general reference group was iron supplementation. The detailed list of exclusion criteria is shown in supplemental Table 1A of paper I. Altogether 547 subjects (220 male and 327 female) were included in the general reference group. We also selected an iron-replete subgroup from the general reference group by including 465 subjects (207 male and 258 female) with serum ferritin values $\geq$ 22 μg/L (Suominen et al. 1998). RIs were calculated for blood Hb, hematocrit (Hct), erythrocyte count (Eryt), MCV, MCH, leukocyte count (Leuk), platelet count, serum sTfR, ferritin, iron, Tf, transferrin saturation and the TfR-F index for the general reference group and the iron-replete subgroup; in the latter ferritin and the TfR-F index were not included.

4.1.2 Adolescent girls (II)

The study population consisted of 191 healthy Caucasian girls aged 9–15 years (66 competing gymnasts [34.6%], 65 competing runners [34.0%] and 60 control subjects with normal physical activity [31.4%]) who participated in a long-term health study in 1997–2000. Altogether 210 girls volunteered for the study; 13 subjects were excluded because of diseases and six subjects withdrew at the beginning of the study. The participants were recruited as volunteers from local sports clubs and schools in the city of Turku and its vicinity.

The subjects were followed up for 1 year. Daily dietary iron intake was estimated at baseline and every 6 months thereafter on the basis of a 4-day food diary. Every 6 months the stage of pubertal development was evaluated according to the method of Tanner (stages 1–5) (Tanner 1962). Greater weight was given to the degree of breast development when there were discrepancies between breast stage and pubic hair stage. Physical activity was recorded by a detailed questionnaire on every research visit.

Venous blood samples for the analysis of blood Hb, red cell indices, serum ferritin and sTfR were drawn at 6 and 12 months. After the visit at 6 months, the participants started 3 months of oral iron supplementation at a daily dose of 50 mg or 100 mg of elemental iron as ferrous sulphate (Retafer®, Orion, Finland). The purpose was to see how the laboratory analytes respond to iron supplementation. Girls who weighed less than 50 kg received 50 mg of elemental iron daily and those who weighed 50 kg or more received 100 mg. Concurrently, daily multivitamin supplementation was administered (Optivit®, Leiras, Finland) which contained 75 mg of ascorbic acid and 2 mg of elemental copper (Cu^{2+}) to improve the bioavailability of ingested iron (Eisenstein 2000, Lynch 1997).

The study population was divided into quartiles by their mean daily iron intake (which included both supplemented and dietary iron) per body surface area during the last 6 months of the study (iron intake groups 1–4). This made it possible to study the effect of iron intake on the laboratory analytes for detecting ID. For statistical analysis, the subjects were also divided into three groups by exercise habits (gymnasts, runners and controls) and into 5 groups by Tanner stage.

4.1.3 Preterm and full-term infants (III, IV)

Altogether 100 preterm infants and 50 full-term infants were recruited at the Department of Pediatrics of the Turku University Hospital during 2002–2004.

The inclusion criterion for the group of preterm infants was gestational age ≤ 34 weeks or birth weight < 2000 g. The preterm infants were treated according to the routines of the hospital. Red cell transfusions (RCTs) were given, if needed, to treat anemia. No EPO was used. The control group consisted of clinically healthy, full-term infants born after 37 weeks of gestation. The infants were followed up until they were 12 months old (corrected age in preterm infants). The preterm infants were further divided into small and large preterm infants by birth weight (< 1500 g and ≥ 1500 g) to allow a comparison of how birth weight affects the hematological laboratory values.

Venous blood samples were taken of the preterm infants for analysis of Hb, Hct, Eryt, MCV, MCH, Leuk, platelet count and reticulocyte parameters (reticulocyte count (Retic) and CHr) after birth and then weekly until discharge, at term and at corrected ages 4, 8 and 12 months and for analysis of sTfR and ferritin after birth and then every second week until discharge, at term and corrected ages 4, 8 and 12 months. Venous blood samples were taken of the full-term infants after birth and then at ages 4, 8 and 12 months. Serum for the measurement of sTfR and ferritin was stored frozen at -70°C until analysis. The infants underwent a clinical examination by a physician after birth and during the visits at corrected ages 4, 8 and 12 months.

4.2 Methods

4.2.1 Laboratory analyses

Serum sTfR assays were performed with a commercial automated immunoturbidimetric method (IDeA sTfR-IT, Orion Diagnostica, Espoo, Finland) on a Hitachi 917 (Studies I-II) or Modular P analyzer (Studies III–IV) (Roche Diacnostics, Basel, Switzerland). The method uses polyclonal anti-human TfR $F(ab)_2$ antibodies bound to SVBC-latex particles. The intra-assay coefficient of variation (CV) of this method is shown to be 0.3–1.8% for four serum samples with concentrations of sTfR between 0.6–7.7 mg/L with 10 replicates. The inter-assay CV of this method is shown to be 2.4-3.5% for four serum samples in 10 subsequent measurements over two weeks. The method is linear for the entire reportable range (0.3–8.5 mg/L). (Suominen et al. 1999) This method has been evaluated and compared to other method also in another study (Cotton et al. 2000). The same sTfR assay has been used for example in studies of Bultink et al., Danise et al., Ervasti et al., Lee et al., Punnonen et al., Suominen et al and

Vendt et al (Bultink et al. 2001, Danise et al. 2008, Ervasti et al. 2004, Lee et al. 2002, Punnonen et al. 2000, Suominen et al 2001, Vendt et al 2007).

Serum ferritin was measured with an automated time-resolved immunofluorometric assay on an Autodelfia analyzer (Wallac, Turku, Finland) (Studies I–II) or with the Elecsys ferritin electrochemiluminescence immunoassay on a Modular E analyzer (studies III–IV) (Roche Diagnostics, Basel, Switzerland). Complete blood cell counts and reticulocyte parameters were analyzed with an automated hematology analyzer (Technicon H*2 in Study II and Advia 120 hematology system in Studies I, III and IV, Bayer Diagnostics, Tarrytown, NY, USA). Serum Tf was measured on a Behring Nephelometer (Behringwerke AG, Marburg, Germany) (Study I). Serum iron was measured with a ferrozine reaction (Roche Diagnostics GmbH) (Study I). The transferrin iron saturation was calculated according to the formula [serum iron / (25 x serum Tf)] x 100% (Study I), while the TfR-F index was calculated according to the formula sTfR / log serum ferritin (Study I).

4.2.2 Estimation of dietary iron intake (Study II)

The daily intake of iron with the diet by adolescent girls was estimated at baseline and every 6 months thereafter on the basis of a 4-day food diary. The calculations were made with the Micro-Nutrica software (Social Insurance Institution, Turku, Finland) by a trained dietitian.

4.2.3 Statistical methods

Statistical analyses were mainly performed with the SAS® system for windows (version 8.0 in Study I, version 8.2 in Study II and version 9.1 in studies III-IV; SAS Institute, Cary, NC, USA). However, SPSS 10.0 for windows software (SPSS Inc., Chicago, IL, USA) was used to perform the Wilcoxon signed rank-sum test in Study II. Differences were considered statistically significant if the p-value was < 0.05. RIs in Study I and II were calculated by nonparametric bootstrap estimation of the reference limits and their confidence intervals with version 3.43 of the RefVal software (Solberg H.E., Department of Clinical Chemistry, Rikshospitalet, Oslo, Norway) (Solberg 1995). Traditional methods for calculation of RIs were not suitable for data of preterm and full-term infants, because the observations were not independent: subjects were serially sampled, and the analytes studied are age-dependent during the first months of life. Thus, unconditional RIs were calculated using individual quadratic curves (Owen and

Ogston 1998). For more detailed information on the calculation of unconditional RIs, see the original publications (Studies III and IV). The RIs (2.5th and 97.5th percentiles) and medians of full-term infants aged 1–6 days were calculated using the nonparametric Harrell-Davis method (Study IV).

The existence of gender differences in Study I were analyzed with Wilcoxon's rank-sum test. The same test was used in Study II to perform pairwise comparisons between 6-month and 12-month values. In Study II, differences in laboratory test values between the iron intake groups, exercise groups and the groups of different Tanner stages were tested with the Kruskal-Wallis test. When a significant difference was identified, Mann-Whitney's U-test with Bonferroni's correction was performed. The association of growth rate or age with laboratory values or changes in laboratory values was evaluated with Spearman's linear correlation. Linear regression was used to examine how the initial serum sTfR value predicts the change in the sTfR value following iron supplementation.

In Study III, the time since RCT was categorized into several levels (one week, two weeks, etc.), and laboratory test values before RCT were compared, using mixed models, with the corresponding values after transfusion and with the values of patients who did not require RCT. The relationships between the other analytes and Hct were analyzed by comparing the differences between the mean pre-transfusion analyte levels of the two groups dichotomized by a pre-transfusion Hct level 0.30; this was done for neonates from age seven days who had received RCTs. For this analysis, a mixed model analysis of variance was used. The subject was considered to be a random effect and the Hct classification (< 0.30, ≥ 0.30) a fixed effect.

In Study IV, we used the area under curve (AUC) as a summary measure of the level of the analytes during the study period. The influence of birth weight classification ($<$ 1500 g and $\geq$ 1500 g) on the AUCs of the laboratory analytes (except Leuk and platelet count) in preterm infants was studied using the independent samples t-test. Associations between gender and the AUCs and between the amount of RCTs (no RCTs, 1–3 RCTs or $>$ 3 RCTs) and the AUCs were studied with general linear models. These analyses were controlled for birth weight classification. If significant differences occurred between the three groups of RCTs, pairwise comparisons between each group was done with the Tukey-Kramer method.

4.3 Ethics

The protocols of these studies were approved by the Ethics Committee of the Hospital District of Southwest Finland (formerly Joint Committee of Ethics of the Turku University Hospital and the University of Turku). All study subjects were volunteers and written informed consent was obtained from all the participants and /or their parents.

5 RESULTS

5.1 Reference intervals (RIs) of soluble transferrin receptor (sTfR) and other hematological laboratory variables

Table 3 presents the medians or estimated mean values and RIs of the most important laboratory tests used to detect ID or IDA (*i.e.*, Hb, sTfR and ferritin) in elderly, adolescent girls and preterm and full-term infants. The so-called iron-replete RIs are presented, when applicable, in addition to the RIs calculated from general reference groups. These RIs are calculated from the reference groups which include only individuals who should not have ID of any degree, not even subclinical ID. Exclusion of iron deficient control individuals relied on low ferritin values: elderly had a cut-off value of 22 µg/L (Suominen et al 1998) and a cut-off value of 10 µg/L was selected for infants. In the group of adolescent girls, ID was excluded by provision of oral iron supplementation for three months preceding the testing. For the calculation of RIs for preterm infants, we used values that were measured from serial blood samples drawn from the infants. All other laboratory test results were included in the calculation of RIs, except the results which were received within two weeks after a blood transfusion to 1–16-week-old infants and data points with a studentized residual > 3 for infants aged 16 weeks or more.

5.1.1 Effect of gender on RIs

In the elderly, there was a statistically significant difference ($p < 0.01$) between males and females in all hematological laboratory tests except sTfR ($p = 0.32$). Therefore, the RIs for all tests except sTfR were calculated separately for males and females. This gender difference was not present among infants.

5.1.2 RIs of sTfR

The medians or estimated means and RIs of sTfR are shown in Table 3. In the elderly, the median age of the general reference group was 71.6 years and of the iron-replete subgroup 71.1 years. The age range was 65–100 years in both reference groups. The upper reference limit of sTfR calculated from the general reference group sank substantially when the RI was calculated from the iron-replete subgroup. The RI changed from 1.0–3.0 mg/L to 1.0–2.4 mg/L (when

serum ferritin was ≥ 22 µg/L). The RI was virtually unchanged when the RI of sTfR was calculated for the subjects whose serum ferritin was ≥ 100 µg/L.

Of the 191 adolescent girls who entered the study, 156 were included in the final analysis (58 competing gymnasts [37.2%], 51 competing runners [32.7%] and 47 non-athletic control subjects [30.1%]). There were five dropouts (2.6%) during the year and one participant was excluded due to obvious IDA and the remaining 29 (15.2%) exclusions were due to missing laboratory values or food diary information. Compliance with the use of iron supplementation varied among the subjects, as shown in Table 1 of Study II. Calculation of the RI for sTfR was based on the post-supplementation values of iron intake groups 3–4 (*i.e.*, the girls whose mean daily iron intake — including both supplemental and dietary iron — had been the greatest during the last 6 months of the study). This was considered to be the iron-replete reference group and the calculated RI for sTfR was 0.9–2.4 mg/L. The upper reference limit of 2.4 mg/L coincided with the sTfR concentration above which iron supplementation induced a consistent decrease in sTfR concentration with a probability of 97.5% (Figure 1, Study II).

In preterm infants, the RI of sTfR made a slight drop at age 3.5–15 weeks after which it remained quite stable until the age of one year (Figure 1F in Study III, Figure 1F in Study IV). The RIs of iron-replete 16-week-olds and older infants (calculated from the values of infants who had serum ferritin ≥ 10 µg/L) did not change to any great extent. The cutoff value of 10 µg/L was selected, because the RIs of Hb, sTfR and CHr were also calculated from the values of preterm infants whose ferritin was > 20 µg/L and > 30 µg/L and there were no significant differences in the RIs. The RIs of full-term infants were similar with the RIs of preterm infants, but the upper reference limit of sTfR increased slightly when the subject approached the age of one year. In iron-replete full-term infants the upper reference limit of sTfR was minimally lower than the upper reference limit of all full-term infants.

5.1.3 RIs of ferritin

The medians or estimated means and RIs of ferritin are shown in Table 3. Both the lower and the upper reference limit of ferritin was higher in elderly male than female (7 vs. 15 and 238 vs. 342 µg/L, respectively).

Table 3 Estimated mean or median values and reference intervals (RIs) of hemoglobin (Hb), soluble transferrin receptor (sTfR) and ferritin for preterm and full-term infants, adolescent girls and elderly.

patient group	Hb (g/L)		sTfR (mg/L)		ferritin (µg/L)	
age	mean / median	RI	mean / median	RI	mean / median	RI
preterm infants[a]						
0.5 wk	150	102-222				
2.5 wk	129	94-176	1.3	0.8-2.3	200	66-609
4.5 wk	114	86-150	1.2	0.7-2.0	163	41-640
7.0 wk	102	78-134	1.1	0.7-1.8	126	23-702
11 wk	95	72-126	1.1	0.7-1.9	84	11-659
14 wk	101	76-133	1.3	0.8-2.2	55	7-445
20 wk	112	92-137	1.5	1.1-2.2	38	7-194
30 wk	121	104-141	1.6	1.1-2.2	25	7-87
40 wk	127	109-149	1.6	1.2-2.2	19	6-60
50 wk	130	110-154	1.6	1.2-2.3	16	5-50
preterm infants[a] (iron-replete)[c]						
20 wk	112	93-135	1.5	1.1-2.2		
30 wk	122	106-140	1.6	1.1-2.1		
40 wk	129	111-149	1.6	1.2-2.1		
50 wk	131	113-154	1.6	1.2-2.2		
full-term infants[a]						
0.5 wk	193	154-259	2.0	1.3-3.3	377	190-711
20 wk	120	102-141	1.5	1.1-2.1	72	20-258
30 wk	118	102-138	1.5	1.1-2.2	41	9-175
40 wk	118	102-137	1.6	1.2-2.4	27	6-118
50 wk	120	104-138	1.7	1.1-2.6	22	7-73
full-term infants[a] (iron-replete)[c]						
20 wk	121	103-141	1.5	1.1-1.9		
30 wk	119	104-137	1.5	1.1-2.0		
40 wk	119	104-136	1.6	1.1-2.2		
50 wk	120	105-138	1.6	1.1-2.5		
female[b]						
10–16 y				1.1-2.7[d]		
>65 y	133	113-150	1.5	1.0-3.0	49	7-238
female[b] (iron-replete)[c]						
10–16 y				0.9-2.4[c]		
≥ 65 y	133	115-151	1.4	1.0-2.4		
male[b]						
≥ 65 y	145	121-165	1.5	1.0-3.0	92	15-342
male[b] (iron-replete)[c]						
≥ 65 y	145	124-165	1.4	1.0-2.4		

a estimated mean
b median
c Iron replete: ferritin ≥ 10 µg/L for infants, ferritin ≥ 22 µg/L for elderly and adolescent girls. Iron deficiency (ID) was excluded by the oral iron supplementation for three months.
d RI calculated from the same study population and published in the study of Suominen et al. (Suominen et al. 2001)

The between-individual variation of the serum ferritin concentration of preterm infants during the first weeks of life was considerable, which yielded wide RIs. The ferritin concentrations of infants decreased progressively during the first year of life. When the RI for ferritin was calculated in the group of preterm infants without RCTs, the mean value as well as the lower and, especially, the upper reference limits were lower than in the whole preterm infants group (Study III, Table 3). The estimated mean serum ferritin values of preterm infants were lower than those of full-term infants for the whole follow-up time from 20 weeks onwards. However, the lower reference limit of full-term infants reached the level of preterm infants around the age of 40 weeks (Study IV, Figure 1E).

5.1.4 RIs of hemoglobin (Hb)

The medians or estimated means and RIs of Hb are presented in Table 3. In the elderly, median and, the lower and the higher reference limit of males were higher than females. There was a marginal increase of the lower reference limit of iron-replete males and females.

In preterm infants, there was decrease in the Hb concentrations during the first weeks of life. The estimated mean value (and RI) declined from 151 g/L (102–222 g/L) during the first postnatal week to a nadir of 95 g/L (72–126 g/L) by the 11th postnatal week. Thereafter, Hb concentrations increased. By age 20 weeks, preterm infants had a lower estimated mean Hb concentration and RI than full-term infants. However, the difference levelled off near age 30 weeks, after which the values of the preterm infants overshot the values of the full-term infants. Calculation of iron-replete RIs raised the lower reference limits of Hb only marginally.

5.1.5 RIs of other hematological laboratory tests among elderly (Study I)

The RIs of the other hematological laboratory tests (*i.e.*, others than sTfR, ferritin and Hb) of elderly subjects are shown in Table 1 of Study I. The RIs for Eryt, Hct, MCV, MCH, serum iron and transferrin saturation were slightly higher among elderly males than in females. The upper reference limit of the TfR-F index was higher in females than males. Calculation of iron-replete RIs raised slightly the lower reference limits of MCV, serum iron and transferrin saturation and decreased the upper reference limit of serum Tf in both females and males.

5.1.6 RIs of other hematological laboratory tests among preterm and full-term infants (Studies III and IV)

The RIs of the other hematological laboratory tests among infants (*i.e.*, others than sTfR, ferritin and Hb) are shown in Table 2 of Study III and Tables 1 and 2 of Study IV. Hct followed the trends of Hb, as expected. Retic of the preterm infants made a nadir soon after birth after which it rose and peaked at age 11 weeks. Thereafter, Retic started to decline again. The RI for Retic among the preterm infants was on a higher level than among the full-term infants until the end of the first year of life, but they converged each other. Among the full-term infants, the RI of Retic was quite stable between ages 20–55 weeks. CHr was highest after birth and then started to decline. Immediately after birth, CHr was higher among the preterm infants than full-term infants. CHr reached a stable level among preterm infants by age around 15 weeks. In the group of full-term infants, there was a slight decline in CHr values near age 35 weeks. MCV declined markedly after birth. This decline continued until age 40 to 45 weeks in both groups and then increased slightly. The trend of MCH was similar. Leuk and platelet counts were, on average, slightly higher in preterm infants at age 20 to 55 weeks. The Leuk value tended to increase in preterm infants and to decrease in full-term infants. The platelet count decreased in both groups as of age 20 weeks.

5.2 Prevalence of iron deficiency (ID)

Fifteen percent of the elderly Lieto study population was storage iron deficient judged by a serum ferritin concentration of less than 22 µg/L; 7.0% had a serum sTfR concentration > 2.4 mg/L compatible with ID and 1.8% had frank IDA (anemia and sTfR > 2.4 mg/L).

Of the adolescent girls, 10% (n = 16) had serum sTfR concentration > 2.4 mg/L before iron supplementation indicating ID.

5.3 Correlation between age and hematological laboratory tests among elderly (Study I)

The correlations between age and laboratory tests to measure the iron status among the elderly are shown in Table 4. There was a slight statistically significant positive correlation between age and the sTfR concentration and a slight statistically significant negative correlation between, on the one hand, age

and, on the other hand, Eryt, Hct, TfR-F index, serum ferritin (only males), and serum iron (only males). These correlations became weaker as the population was narrowed down to the iron-replete subgroup: now the trends could be partly attributable to subclinical ID. Figure 1 in Study I shows how the median Hb values among males and females and the median sTfR values change with age in the general reference group.

5.4 Effect of iron intake on iron status laboratory tests among adolescent girls (Study II)

After iron supplementation, the serum ferritin concentrations were significantly lower in iron intake group 1 (daily total iron intake 3.9–13.6 mg/m^2) than in iron intake groups 3 and 4 ($p < 0.001$, daily iron intake 21.9–27.2 mg/m^2 and $p = 0.017$, 27.3–39.6 mg/m^2, respectively). The serum ferritin levels were not significantly different between these groups before iron supplementation. Also, the supplementation-induced changes were markedly smaller in iron intake group 1 than in groups 2, 3 and 4 ($p = 0.014$, $p < 0.001$, and $p = 0.010$ respectively). There were no significant differences in Hb and sTfR values between the iron intake groups neither before nor after iron supplementation. However, iron supplementation did induce a significant reduction in sTfR concentrations in all iron intake groups.

5.5 Effect of exercise and puberty on iron status of adolescent girls (Study II)

There were no significant differences in sTfR, ferritin or Hb concentrations between gymnasts, runners and controls before or after iron supplementation nor in Hb or ferritin concentrations between the different Tanner stages before or after iron supplementation. There was a significant difference in sTfR between Tanner stages 2 and 4 ($p = 0.019$) before iron supplementation and between Tanner stages 2 and 4 ($p = 0.028$) and stages 1 and 4 ($p = 0.009$) after iron supplementation.

5.6 Effect of red cell transfusions (RCTs) on hematological laboratory values in preterm infants (Study III)

47 preterm infants received RCTs during their first 16 weeks of life. No significant post-transfusion effects were seen at any point in Retic, CHr or sTfR.

Table 4 Elderly subjects: Correlations between laboratory test values and age in the general reference group and in the iron-replete group (serum ferritin ≥ 22 µg/L)

	General reference group (220 men and 327 women)		Iron-replete group (207 men and 258 women)	
Variable	Spearman's correlation coefficient (r)	p-value	Spearman's correlation coefficient (r)	p-value
Hb				
Male	- 0.168	0.0126	-0.135	0.0527
Female	-0.199	0.0003	-0.161	0.0098
Eryt				
Male	-0.136	0.0438	-0.136	0.0512
Female	-0.179	0.0012	-0.139	0.0252
Hct				
Male	-0.143	0.0343	-0.108	0.1206
Female	-0.198	0.0003	-0.162	0.0092
MCV				
Male	-0.009	0.8955	0.061	0.3817
Female	0.017	0.7612	-0.010	0.8722
MCH				
Male	-0.022	0.7429	0.047	0.5019
Female	0.004	0.9403	-0.011	0.8520
sTfR				
All	0.198	< 0.0001	0.159	0.0006
Serum ferritin				
Male	-0.159	0.0184	-0.090	0.1960
Female	-0.078	0.1617	0.041	0.5148
TfR-F index				
Male	0.209	0.0018	0.149	0.0326
Female	0.188	0.0006	0.114	0.067
Serum iron				
Male	-0.139	0.0390	-0.105	0.1309
Female	-0.085	0.1251	-0.031	0.6200
Serum Tf				
Male	-0.016	0.8166	-0.060	0.3916
Female	0.005	0.9333	-0.107	0.0863
Transferrin saturation				
Male	-0.086	0.2075	-0.047	0.4989
Female	-0.073	0.1891	0.020	0.7534
Leuk				
Male	-0.032	0.6358	-0.028	0.6983
Female	0.050	0.3663	0.060	0.3409
Platelet				
Male	0.040	0.5572	0.044	0.5320
Female	-0.028	0.6168	-0.084	0.1807

Hb, hemoglobin; Eryt, erythtrocyte count; Hct, hematocrit; MCV, mean cell volume; MCH, mean cell hemoglobin; sTfR, soluble transferrin receptor; TfR-F index, transferrin receptor / log ferritin ratio; Tf, transferrin; Leuk, Leukocyte count; Platelet, platelet count

However, RCTs had a significant effect on Hb, Hct and serum ferritin which lasted for longer than 2 weeks. The effect of RCTs on ferritin lasted for several months. When preterm infants were followed until age 12 months of corrected age, the AUCs of ferritin were significantly higher in preterm infants who had received more than three RCTs during their first months of life compared with preterm infants who had received 1–3 or no RCTs ($p < 0.0001$) when controlled for birth weights.

5.7 Evaluation of need for RCTs by hematological laboratory tests in preterm infants (Study III)

Hct is traditionally used to determine the need for RCTs in preterm infants. The differences of hematological laboratory test values other than Hct were analyzed in two dichotomous groups divided by a Hct value of 0.30, which is the usual level triggering RCT. The values are shown in Table 4 in Study III. The median Hb at Hct 0.30 was 102 g/L. Mean sTfR and ferritin levels did not differ significantly between these groups ($p = 0.52$ and $p = 0.12$, respectively), but the mean values of Hb, Retic and CHr did ($p < 0.001$, $p = 0.02$ and $p < 0.001$, respectively). We also examined the levels of Retic and CHr at the time of transfusion decision. CHr was below the lower reference limit (calculated from non-transfused preterm infants) in seven out of 82 (8.5%) subjects and Retic in four out of 82 (4.9%).

5.8 Effects of birth weight, gender or amount of RCTs on follow-up levels of the hematological laboratory tests until age one year (Study IV)

Preterm infants with a birth weight ≥ 1500 g had statistically lower Retic AUCs after age 16 weeks than preterm infants with a birth weight < 1500 g ($p < 0.0001$). There were no other significant differences in the average laboratory test values between these birth weight groups. When controlled for birth weight, the AUCs of the laboratory tests did not differ statistically significantly between the genders. The number of RCTs affected the AUCs of Eryt ($p = 0.0283$) and ferritin ($p < 0.0001$) when controlled for birth weights. Eryt were lower in preterm infants who received more than three RCTs compared to preterm infants who received 1 to 3 RCTs. Ferritin was higher in preterm infants who received over three RCTs compared with preterm infants who received 1 to 3 or no RCTs.

6 DISCUSSION

Elderly people, fertile women, adolescent girls and preterm and full-term infants are susceptible to ID. The detection of ID in these groups is important if the deleterious consequences and causes (possibly malignant) of ID are to be avoided and managed. In the elderly, detection of ID or IDA is essential, because ID is increasingly a marker of malignant disease, *e.g.*, colorectal cancer (Coban et al. 2003, Cook et al. 1986, Joosten et al. 1999, Sari et al. 2002). Incipient and emerging anemia and ID may provoke symptoms of other diseases, especially heart or lung diseases, increase mortality and impair the person's health-related quality of life (Anand et al. 2005, Comin-Colet et al. 2013, Enjuanes et al. 2014, Hsu et al. 2013, Zakai et al. 2013). Pre-operative anemia is associated with surgical morbidity and mortality and diagnosis and management of anemia is essential before surgery (Clevenger and Richards 2015, Musallam et al. 2011). Improved clinical detection of ID among the elderly is particularly important, since the early stages of ID may be difficult to separate from ACD, which, together with ID, is the leading cause of normocytic and microcytic anemias in the elderly.

In adolescent girls, ID may impair learning skills, cognitive function and physical endurance (Halterman et al. 2001, More et al. 2013, Murray-Kolb and Beard 2007, Scott and Murray-Kolb 2016). Timely detection of ID in infants is particularly important, because ID may impair cognitive and behavioral development. These adverse effects may be non-reversible (Akman et al. 2004, Angulo-Barroso et al. 2016, Bruner et al. 1996, Lozoff et al. 1987, Lozoff et al. 1996, Roncagliolo et al. 1998, Shafir et al. 2006, Walter et al. 1989).

6.1 Reference intervals (RIs) of soluble transferrin receptor (sTfR) and other hematological laboratory tests

sTfR is a relatively new laboratory test to detect ID and its use has not previously been extensively studied in the groups of people discussed above. sTfR detects subclinical ID. The problem with sTfR as a laboratory value is that there is no agreement on standardization. This means that sTfR methods of different manufacturers give variable results when the one and the same sample is assessed (Cotton et al. 2000, Kolbe-Busch et al. 2002, Yeung et al. 1998, Åkesson et al. 1999). Hopefully, however, this problem might become obsolete, since the 1st WHO reference reagent for sTfR has been agreed upon and established in 2010 (Thorpe et al. 2010). In the present study RIs for sTfR for elderly individuals (Study I) and infants (Studies III and IV) were derived

concomitantly with other laboratory tests that can be used to detect ID or IDA. There was no significant difference in the RIs of sTfR between genders, which is consistent with previous studies (Allen et al. 1998, Kohgo et al. 1986, Vernet and Doyen 2000).

It is recommended that a minimum of 120 reference values should be used for the reference-interval determination (CLSI 2008). This was not quite fulfilled in the present study in reference sample groups of adolescent girls (Study II) and infants (Studies III and IV) which may impair the confidence of produced RIs.

The RIs of laboratory tests are typically calculated from values that are measured from blood samples of apparently healthy individuals (*i.e.*, excluding individuals with known systemic diseases and pathophysiological disorders) (PetitClerc and Solberg 1987, Van Den Bosch et al. 2001). Because subclinical ID or mild IDA do not usually cause any detectable symptoms, it is very likely that the reference groups include, in fact, individuals with subclinical ID or mild IDA. It is known that sTfR concentrations together with other variables of iron status may be affected by subclinical ID (Skikne et al. 1990, Suominen et al. 1998). Therefore, the reference values of the reference individuals with subclinical ID or mild IDA may widen inappropriately the RIs of the laboratory tests describing the iron status. This will impair the sensitivity of the laboratory tests compared with RIs derived from iron-replete individuals (Rushton and Barth 2010, Rushton et al. 2001, Suominen and Irjala 2001), a problem especially in populations where ID is a risk. To overcome this hurdle, iron-replete RIs for sTfR and other laboratory tests defining iron status were derived. In elderly individuals and adolescent girls, there were clear reductions in the upper reference limits of sTfR in iron-replete RIs compared with the respective limits of the general reference groups (2.4 mg/L vs. 3.0 mg/L in the elderly and 2.4 mg/L vs. 2.7 mg/L in adolescent girls, Table 3). In the elderly, similar but less prominent changes in the upper reference limit of serum Tf and in the lower reference limit of MCV, serum iron and transferrin saturation were recorded. These findings support the idea that the general reference group did, in fact, include patients with subclinical ID and that the upper reference limit of iron-replete RI could be used as a decision limit to establish ID in elderly individuals and adolescent girls. In the RIs of full-term and preterm infants there were no similar changes when RIs were calculated from the iron-replete subgroup (ferritin $\geq$ 10 µg/L). In addition to using ferritin value of < 10 µg/L as an exclusion criterion, the RIs of Hb, sTfR and CHr were calculated from values of preterm infants with ferritin > 20 µg/L and > 30 µg/L and there were no significant changes in RIs in these calculations, either. Thus, ID was not present in the full-term or preterm infants of this study population, which implies that routine iron supplementation of the preterm infants was

sufficient in this study. The RIs of sTfR in infants and iron-replete RIs of sTfR in adolescent girls and elderly were similar.

In this study (Study II), adolescent girls were asked to take a specified dose of elemental iron (50 or 100 mg) daily for three months, but compliance was not adequate. However, since the amount of ingested iron was documented and was found to vary widely, it became possible to estimate how the amount of ingested iron influences the values of iron status laboratory tests. The steps correcting ID were visible (Suominen et al. 1998, Wheby 1980). In the lowest iron intake group (daily intake $\leq$ 13.6 mg/m^2), only the daily requirements of iron were met and this led to a slight decrease in sTfR but the ferritin value was unchanged. In the other iron intake groups, the increment of iron intake replenished the iron stores to some extent and was recorded as a significant increase of the serum ferritin concentration. Iron supplementation did not affect the Hb concentrations. This indicates that the iron supply for erythropoiesis had been sufficient even before iron supplementation, probably owing to increased sTfR concentration in some subjects with subclinical ID. The values of the upper half of study subjects by iron intake were chosen to calculate the iron-replete health-related RIs associated with an adequate iron supply.

Unfortunately, manufacturing of IDeA sTfR-IT assay of Orion diagnostic (Espoo, Finland) which was used in this study is finished. This compromises the use of the presented RIs. In general, based on the findings of this study, it can be suggested that the upper reference limit of sTfR calculated from the results of carefully selected, iron-replete reference group may be used as a cut-off limit to detect ID.

Serum ferritin cut-off values between 22 and 75 µg/L have been presented as decision limits for ID in the elderly (Choi et al. 2005, Guyatt et al. 1990, Holyoake et al. 1993, Suominen et al. 1998). A ferritin concentration of 22 µg/L can be used as a cut-off value to exclude patients with depleted iron stores (Suominen et al. 1998, Choi et al. 2005). In this study (Study I), the RIs of ferritin were 7–238 µg/L for elderly females and 15–342 µg/L for elderly males. This shows that the RIs are influenced by ID and the lower reference limits of these RIs do not reliably rule out ID.

The serum ferritin concentration decreased in preterm and full-term infants during the one year of follow-up. Preterm infants had lower mean ferritin concentration during the whole study period than full-term infants. There are additional explanations to this finding than iron supplementation. It is known that preterm infants are born with small iron stores because most of the body iron of the neonate is received during the last trimester (Rao and Georgieff 2007).Frequent blood sampling and rapid body growth increase the need of iron

proportionately more in preterm infants than full-term infants. Although iron supplementation did not prevent the decline of ferritin in pre-term infants, it seemed to delay it, since the ferritin concentrations of preterm and full-term infants converged during follow-up. The RCTs given to some preterm infants during their first weeks of life affected the iron status of preterm infants to some degree. The lower reference limit of serum ferritin of preterm infants was < 10 µg/L from age 13 weeks and of full-term infants from age 35 weeks. This means that infants whose ferritin value is near lower reference limit have very poor iron stores. Altogether, by one year of age both preterm and full-term infants have small iron stores.

In the elderly, the RIs of Hb (males 121–165 g/L, females 113–150 g/L) were slightly lower than RIs of Hb of adults in the Nordic countries (males 134–170 g/L, females 117–153 g/L) (Nordin et al. 2004). However, the RIs of Hb derived in Study I are quite similar to the RIs of Hb previously determined for elderly in Finland (males 122–171 g/L, females 113–161 g/L) (Mattila et al. 1986). The Hb RIs of iron-replete subjects influences only slightly the RIs (males 124–165 g/L, females 115–151 g/L). It is known that there is a significant age-related decline of Hb from age 70 to 88 among healthy males and less so among healthy females, which could explain the difference between the whole adult population and elderly population (Nilsson-Ehle et al. 2000). Nevertheless, in the elderly of this study (Study I) the decline of Hb with advancing age was minimal. The trend seemed to be partly associated with subclinical ID, because the correlation became weaker when calculated for the iron-replete group.

The reason for the lower RI of Hb among elderly compared to younger adults is not known. There is data suggesting that the basal hematopoietic potential is well preserved with aging but the hematopoietic cytokine network undergoes complex remodeling with age (Bagnara et al. 2000). The question is: Does the decline of Hb relate to “normal” aging or is it a consequence of increased morbidity (although reference populations do include apparently healthy individuals)?

The estimated mean Hb concentration of the full-term infants was comparable with the median value of 12-month-old healthy European infants in the Euro-Growth study and slightly higher than the mean values of Norwegian, Swedish and Estonian infants (Domellöf et al. 2001, Hay et al. 2004, Male et al. 2001, Vendt et al. 2007). The estimated mean Hb concentration of preterm infants exceeded the concentration of full-term infants by age one year. These observations indicate that ID did ninfluence notably Hb concentrations in this study, which is supported by the finding that exclusion of infants with low ferritin values had virtually no influence on the RIs of infants.

Reticulocytes are erythrocyte precursors and are the youngest circulating red cells in the blood of healthy persons. They mature to erythrocytes in the circulation in 1–2 days. Therefore, the reticulocyte-derived variable CHr is a potential test for early detection of IDE. In adults, decreased CHr has been shown to detect IDE and an early response to iron therapy (Brugnara et al. 1994b, Mast et al. 2002). In preterm infants, CHr was highest after birth and then declined until age around 15 weeks after which the value remained stable. The decrease in CHr during first weeks of life does not seem to reflect the iron supply available for erythropoiesis, as judged by the spectrum of laboratory findings available. The resurgence of Hb and the decrease of sTfR speak against ID. An explanation for the decrease of CHr could be the normal physiological maturation process. The lower reference limit of CHr remained steadily at a level of 26 pg and a reduction of CHr under this level could indicate IDE. A cut-off value of 26 pg for CHr is reportedly optimal for the diagnosis of ID in children (mean age 2.9 years, SD 2.0) (Brugnara et al. 1999), but a value of 27.5 pg has also been reported concerning infants aged 9 to 12 months (Ullrich et al. 2005). The first study used a transferrin saturation level < 20% to define ID and the latter a level < 10%, and clearly transferrin saturation is not an explanation for the different cut-off values. The reason for this difference remains open.

6.2 Prevalence of iron deficiency (ID)

ID is common worldwide, especially in developing countries. In African refugee camps, the prevalence of ID ranged between 23% and 75% among 6–59-month-old children (Seal et al. 2005). The National Health and Nutrition Examination survey (NHANES) run in the United States in 1999–2000 reported a prevalence of ID of 7% in 1–2-year-old children, 9% in 12–15-year-old females, 3% in ≥ 70 year-old males, 9% in 50–69-year-old females and 6% in ≥ 70 year-old females (CDC 2002). In the same study, prevalence of IDA was 2% in 1–2-year-old children, 2% in 12–19-year-old females, 3% in 50–69-year-old females and 1% in ≥ 70-year-old females. In the present study (Study I), 7.0% of the elderly had ID (serum sTfR concentration > 2.4 mg/L) and 1.8% had IDA (anemia and sTfR > 2.4 mg/L), rates which are of the same magnitude as in the reports from the United States. Before iron supplementation 10% of the adolescent girls had ID (serum sTfR concentration > 2.4 mg/L) which corresponds to the US data. It is noteworthy that the definition of ID varies between different studies which influences the prevalence of ID (Cogswell et al. 2009, Engle-Stone et al. 2013). In a study from Cameroon the prevalence of ID ranged from 14% to 68% among 12–59-month-old children and from 12 to 32% among 15–49-year-old females, depending on which indicator of iron status was used (Engle-Stone et al. 2013).

6.3 Effects of physical activity and advancing puberty on iron status

The risk of ID is increased in adolescent girls because incipient menstrual cycling and rapid body growth (Hallberg et al. 1993b, Hallberg and Rossander-Hultén 1991). Intensive physical training is also considered to be a risk factor for ID, but the results addressing this question are divergent (Balaban et al. 1989, Fogelholm et al. 2000, Sandström et al. 2012, Schumacher et al. 2002, Spodaryk 2002). A reduction of iron stores by advancing puberty and growth is supported by this study but not by intensive physical training. Similar findings have been reported previously (Fogelholm et al. 2000, Sandström et al. 2012).

6.4 Changes of hematological laboratory tests in preterm and full-term infants during the first year of life

Very little is known about the kinetics of sTfR, ferritin and reticulocyte-derived parameters in preterm and full-term infants. The present study (Studies III and IV) gives valuable information about the changes in sTfR and other hematological laboratory tests during the first year of life of preterm and full-term infants. The age-related RIs and scatter diagrams in Studies III and IV provide clinicians with an insight as to how the values of these laboratory tests behave during health and when there are signs of abnormal changes. This will help to assess the cause of anemia, ID or ineffective erythropoiesis. Decreased reticulocyte counts with concurrent normal values for sTfR and CHr may indicate ineffective erythropoiesis as a cause for anemia, and these infants might benefit from EPO treatment. Reticulocytosis in connection with high sTfR and low CHr values implies functional ID. These neonates would need iron supplementation and, if necessary, RCTs. RCTs could be needed at least for the patients with low retic, low CHr and increased sTfR. In this study (Study III), CHr was below the lower RI only in 7 out of 82 infants and retic only in 4 out of 82 infants when the decision of RCT was made. The use of all of these variables for decision making could reduce the number of inappropriate RCTs, especially in infants.

The plasma concentration of sTfR reflects, on the one hand, the iron supply available for the cells and, on the other hand, the rate of erythropoiesis (Huebers et al. 1990, Kohgo et al. 1987). Consequently, high values of sTfR are seen in ID and in conditions with increased erythropoiesis. In this study (Study III), the reticulocyte count of the preterm infants was high for a few days following birth but sank remarkably already by age one week. After birth, the sTfR

concentration seemed to follow the decline of the reticulocyte count, and higher values of sTfR after birth and a rapid decline subsequently can be explained by the degree of erythropoiesis. After that, the sTfR concentrations remained quite stable and rose only very slightly until age one year. The reticulocyte count remained quite stable after 20 weeks of life. So far, there has not been compelling evidence that the specificity of sTfR would vary by age. Therefore, a sTfR concentration above the upper RI in infants indicates ID, if the reticulocyte count is normal. In this study, there was one infant with clear IDA at age four months, as evidenced by anemia, microcytosis, hypochromia, low ferritin concentration, low CHr and high sTfR, while reticulocytes were normal (Hb 79 g/L, MCV 72 fL, MCH 23 pg, ferritin 6 µg/L, CHr 19.9 pg, sTfR 3.7 mg/L, retic 68 x 10^9/L).

6.5 Future perspectives

Assessment of sTfR and ferritin have a recognized place in the detection of ID and they have replaced the older diagnostic test of ID, *i.e.*, serum iron, Tf and transferrin saturation. The use of CHr may also increase, if CHr becomes more readily available and the experience of its clinical value grows. In the future, serum hepcidin and serum ERFE may bring additional information on how to solve problems of iron metabolism disorders. There are several mass spectrometric assays and classical immunoassays to measure hepcidin, but the use of hepcidin has been hampered by challenges like differences in hepcidin levels in various assays, absence of standardization and a lack of specificity for hepcidin-25 in immunoassays (Girelli et al. 2016, Kroot et al. 2009b). There is also marked diurnal variation in serum and especially urine hepcidin levels, and this complicates the clinical use of hepcidin (Kroot et al. 2009a). Nevertheless, hepcidin may provide additional value to the tests mentioned for the differential diagnosis of ID and ACD, and may help to assess the benefit of iron supplementation. ERFE has been discovered recently and the its clinical role remains to be seen.

7 SUMMARY AND CONCLUSIONS

Identification of iron deficiency (ID) is essential, because it may have deleterious consequences and it may be due to malignant disease. This study was conducted to clarify the use of the soluble transferrin receptor (sTfR) and other hematological laboratory tests to investigate the iron status in elderly individuals, adolescent girls and infants — groups of people who are exceptionally susceptible to ID. Reference intervals (RIs) for sTfR and other hematological laboratory tests were established for a conventionally selected reference group and also for iron-replete subjects. It turned out that elderly individuals and adolescent girls had a lower upper reference limit of sTfR when the RIs were based on an iron-replete reference group. This indicates that subclinical ID or mild iron deficiency anemia (IDA) of the reference individuals may have influenced the RIs of sTfR and also of the other markers of iron status when calculated from a conventionally selected reference group. This may distort the cut-off values for ID. In infants, the RIs remained unchanged when calculated from the iron-replete reference group compared with whole reference group, suggesting that ID was not a marked problem in infants of this study.

The iron-replete RIs for sTfR were 1.0–2.4 mg/L (Study I) and 0.9–2.4 mg/L (Study II) for elderly individuals and adolescent girls, respectively. The upper reference limits of these RIs of sTfR may be used as cut-off limits for ID. With these cut-off limits, the prevalence of ID was estimated to be 7% among the elderly and 10% among the adolescent girls. In preterm infants (Studies III and IV), the RI of sTfR increased from 1.1–2.2 mg/L to 1.2–2.4 mg/L with advancing age from 20 weeks to 60 weeks. The lower reference limit of the reticulocyte hemoglobin content (CHr) can also be used in addition to sTfR in the detection of ID in preterm infants. When interpreting sTfR values, it should be noted that there is no standardization of the different methods measuring sTfR, and method-specific RIs are needed. In general, based on the findings of this study, it can be suggested that the upper reference limit of sTfR calculated from the results of carefully selected, iron-replete reference group can be used as a cut-off limit to detect ID.

Intensive physical activity did not have any significant effect on the iron status of adolescent girls. There was a slight but consistent decrease in median sTfR concentrations with increasing age from 10 to 16 years with a concurrent slight decrease in the median ferritin concentration. The decrease in sTfR is explained by the known age-related decrease in sTfR and the decline of ferritin due to reduced iron stores in connection with the advancing puberty and the menarche.

This study (Studies III and IV) has provided valuable information about the kinetics of hematological laboratory tests of preterm infants. Age-related RIs should help physicians to interpret the results of these markers for clinical decision making. The RIs should be helpful for physicians to evaluate any problems in erythropoiesis or the iron status of anemic infants. The results also show how these values differ between preterm and full-term infants and that the values of preterm and full-term infants converge over time.

ACKNOWLEDGEMENTS

The present study was carried out in the Department of Clinical Chemistry, University of Turku, during years 2000-2017.

I would like to thank Professor Ilkka Mononen, retired Head of the Department of Clinical Chemistry for providing me support and working facilities to make this challenge possible. I would also like to thank Professor Kale Juva, retired Head of the department of Clinical Chemistry who about twenty years ago enabled my first steps in the field of science. I deeply thank Adjunct Professor, Director Paula Grönroos for the possibility to work and to make research in Tykslab, the Laboratory of Turku University Hospital.

I owe my deepest respect and gratitude to my supervisors, Adjunct Professor Kerttu Irjala and Adjunct Professor Allan Rajamäki, for they guidance, encouragement and patience throughout these years. Kerttu Irjala as a highly respected authority and long-term family friend has provided me advices and support whenever I have needed them. Allan Rajamäki has deepen my knowledge in the field of hematology and given a secure working atmosphere with his restful behaviour and exemplary attitude towards life.

PhD Pauli Suominen deserves also my deep thankfulness. He was like my third supervisor during the first years of this study and has many times served me hand to hand guidance. His never-ending and extensive train of thoughts, now and then difficult to follow with my capacity, has given many new ideas to our articles.

I am indebted to Adjunct Professor Lotta Joutsi-Korhonen and Adjunct Professor Kari Punnonen, the official reviewers of this thesis, for their constructive criticism and comments that accomplished the final improvements in this thesis.

I am grateful that I had a chance to make collaboration with many skilful and appreciated clinicians and other co-workers. Professor Raimo Isoaho, Professor Sirkka-Liisa Kivelä and PhD Minna Löppönen gave me access to the wide data of Lieto Study. Your positive attitude and constructive comments were crucial for the completion of the manuscript of study I. I am grateful to MSc Olli Peltola, who made me familiar with sTfR measurement on Hitachi-analyser. He was always available when I needed aid with determinations or analytical issues. PhD Marjo Lehtonen-Veromaa, Professor Timo Möttönen and Professor Jorma Viikari gave me opportunity to study iron balance in adolescent girls and provided me valuable advices throughout the analysing and writing process of study II. I am especially pleased that my highly respected godfather, Jorma Viikari, was one of the co-authors in my thesis. I thank my co-authors from the department of pediatrics, Adjunct Professor Päivi Lähteenmäki, Professor Liisa Lehtonen, Adjunct Professor Helena Lapinleimu, Professor Toivo T. Salmi, and MD

Enni Mäkelä who let me in to the interesting and also problematic field of iron balance in the preterm and full-term infants. Päivi Lähteenmäki deserves special thanks for making remarkable effort in doing the clinical examinations for the infants and she made the study go forward with her diligence. Special thanks goes also to Enni Mäkelä who had essential role in the data collection and writing the manuscript of study III. MSc Jaakko Matomäki gave essential help by making all the statistical analyses for the data of the infants. I thank you all for the smooth and fruitful collaboration.

All the voluntary study subjects, aged people in Lieto, pubertal girls, preterm and full-term infants and their parents, deserves my heartfelt gratefulness.

I wish to thank Adjunct Professor Robert Paul for revising the language of this thesis.

I am appreciative to Adjunct Professor Tarja-Terttu Pelliniemi of all the advices, support and hematological information she has given me. I am grateful to MSc Riitta Vanharanta for teaching me the basics of Advia-analyzer and helping me with organizing the reticulocyte analyses. I also thank Adjunct Professor Aila Leino for the assistance with the measurements of ferritin and sTfR on Modular-analyzer.

All the past and present academic staff in TYKSLAB has been kind and helpful to me and I want to thank them all. I am grateful to my present colleagues in the laboratory of hematology, i.e., my immediate superior PhD Anri Tienhaara and PhD Tarja-Leena Penttilä, PhD Saila Loikas and PhD Anna Linko-Parvinen for fair teamwork. I am especially grateful to Saila Loikas for supportive conversations and advices in the field of research. I thank my former roommate and collaborator during the first years of my research career, PhD Marita Kailajärvi, for relaxing conversations during the working days. Also Maija Aalto, Heidi Berghäll, Marja Berlin, Mikhail Fomichev, Inka Harju, Elisa Heikkilä, Inga Hjelm, Päivi Holm, Mauri Hämäläinen, Kari Ivaska, Vesa Juvonen, Hanna-Leena Kaihola, Janita Johansson, Veli Kairisto, Taina Katajamäki, Katri Kivinen, Marja-Kaisa Koivula, Pertti Koskinen, Annika Kouki, Diana Kujala, Riikka Kurkijärvi, Kaisa Kurvinen, Rutta Kuusela, Pia Leino, Olli-Pekka Lehtonen, Britt-Marie Loo, Malin Lövberg, Tuija Lundán, Irma Matinlauri, Kari Mattila, Olli Meurman, Hanna-Mari Pallari, Ritva Palo, Max Parkki, Laura Pihlgren, Kari Pulkki, Maria Puustinen, Maria Raitakari, Kaisu Rantakokko-Jalava, Anne Renvall, Maaret Ridanpää, Jukka Saarimies, Anne Tolonen, Heidi Turkia, and Ari Törmä are all acknowledged. I hope that I did not forget anyone and if I did, I thank them also.

I remember warmly all friendly and skilled personnel in TYKSLAB. Especially I thank my workmates in my present work community, laboratory of hematology, for comfortable working atmosphere. Tuula Laukkanen deserves my gratitude for managing the archive of blood samples and giving me help to find correct boxes and tubes.

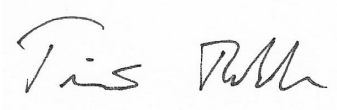
I warmly thank all my friends and relatives for the time spent together during these years. Especially I wish to thank my nearest friends Jussi and Riitta, Sami and Kati and their families who have given me many joyful moments in leisure time and taking my mind away from work. With Jussi and Sami I have had many unforgettable moments in the jogging tracks and hikes. I also thank my neighbourhood, Huistuppula, for your friendship, careless living and many memorable, planned and unplanned events. Furthermore, football team LiePa 04 deserves to be mentioned for team spirit and exciting moments in substitutes' bench. Also, players in “pyhä sunnuntaisähly” are acknowledged.

This work would not have been possible without the support and love of my family. I am deeply grateful to my dear parents, Irma and Ismo, who have given me love and stable and secure ground for my life. My brother Teemu gets my gratitude for being stand-by all my life. I also thank his wife Outi and children Tuomas, Kaapo, Iida, and Risto for sharing many memorable moments with me and my family. I am very thankful to my mother-in-law Iiris who has unselfishly given assistance for me and my family whenever we have needed any help. Also, my father-in-law Harri is acknowledged. I thank my sister-in-law or “little sister” Mira for understanding and sharing the problems of research and for being the best aunt for my boys. I am grateful to you and your family, Jonkka and Max, for the friendship and shared leisure time.

My warmest and deepest gratitude and love goes to my wife Petra and my boys Rasmus and Miska. You have shared my many good and a few bad moments. Petra, thank you for your love. You have walked by my side over half of my life and given me carefree living. And when needed, you have tried to cultivate me with supportive criticism. Rasmus and Miska have given me their sincere love and many and many happy memories. You have given me required counterbalance to my work. You are the best things I have ever done. Rasmus deserves a special thank for being a full-term control in the study of infants.

This study was financially supported by Finnish Association of Haematology, Finnish Medical Foundation, Finnish Society of Clinical Chemistry, the Laboratory Medicine Foundation, Orion Research Foundation, Turku University Foundation, University of Turku, and Turku University Hospital Research fund.

Lieto, November 2017



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Annales Universitatis Turkuensis

Turun yliopisto
University of Turku

Painosalama Oy, Turku, Finland 2017

ISBN 978-951-29-7037-7 (PRINT)
ISBN 978-951-29-7038-4 (PDF)
ISSN 0355-9483 (Print) | ISSN 2343-3213 (Online)