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147

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IRON DEFICIENCY AND IRON DEFICIENCY ANAEMIA IN INFANTS AGED 9 TO 12 MONTHS IN ESTONIA

NEVE VENDT

TARTU UNIVERSITY
PRESS

Department of Paediatrics, University of Tartu

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Supervisors: Heli Grünberg, MD, PhD, Assosiate Professor of Paediatrics, University of Tartu, Estonia
Tiina Talvik, MD, PhD, DrMedSc, Professor Emeritus of Paediatrics, University of Tartu, Estonia
Vallo Tillmann, MD, PhD, Professor of Paediatrics, University of Tartu, Estonia

Reviewers: Heidi-Ingrid Maaroos, MD, PhD, DrMedSc, Professor of Polyclinic and Family Medicine, University of Tartu, Estonia
Agu Tamm, MD, PhD, DrSc, Professor of Laboratory Medicine, Department of Internal Medicine, University of Tartu, Estonia

Opponent: Magnus Domellöf, MD, PhD, Associate Professor. Department of Clinical Sciences, Pediatrics, Umeå University Hospital, Sweden

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To My Family

CONTENTS

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3

ABBREVIATIONS

AUC	area under the curve
BW	birthweight
CDC	Centers for Disease Control and Prevention
CHr	reticulocyte haemoglobin content
CPP	caseinophosphopeptides
CRP	C-reactive protein
Dcyt b	duodenal cytochrome b
DMT	divalent metal transporter
DPG	diphosphoglycerate
EAR	Estimated Average Requirement
EPO	erythropoetin
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
FEP	free erythrocyte protoporphyrin
Hb	haemoglobin
HCP	haem carrier protein
Hct	hematocrit
ID	iron deficiency
IDA	iron deficiency anaemia
IDeA®	immonoturbidimetric method to assay sTfR, Orion Diagnostica
IL	interleukin
LOA	limits of agreement
MCV	mean red cell volume; mean corpuscular volume
NOS	nitrous oxide system
OR	odds ratio
RBC	red blood cell
RDA	Recommended Dietary Allowance
ROC	receiver operating characteristic curve
SGA	small for gestational age
sTfR	serum soluble transferrin receptor
Tf	transferrin
TfR	transferrin receptor
Tf-Sat	transferrin saturation
TIBC	total iron binding capacity
Tina-quant®	immonoturbidimetric method to assay sTfR, Roche Diagnostics
TNF	tumor necrosis factor
UNICEF	United Nations Children's Fund
UNU	United Nations University
US-FNB	US Food and Nutrition Board
WHO	World Health Organisation
ZPP	zinc protoporphyrin

INTRODUCTION

Iron deficiency (ID) is a common nutritional disorder in children. It is promoted by insufficient iron in the diet *(Aggett et al. 2002)*. Iron deficiency develops most commonly in late infancy and during the second year of life because of the high demand for iron during this period of rapid growth *(Oski 1985, Aggett et al. 1989)*. The most obvious result of ID is anaemia.

The terms anaemia, ID and IDA are often used interchangeably, as ID appears to be a common cause of childhood anaemia. However, not all anaemias are due to ID, and ID may occur without anaemia. This means that the prevalence of anaemia and ID varies in different populations, and no consistent relationship between these two disorders has been found *(JWHO and CDCPTC 2005)*.

WHO has postulated that IDA should be considered as a public health problem if the prevalence of low haemoglobin (Hb) concentration exceeds 5.0% of the population *(WHO 2001)*. WHO recommends classifying the public health significance of anaemia based on its prevalence: severe if prevalence ≥ 40.0%; moderate 20.0–39.9%; mild 5.0–19.9%, and normal if the prevalence of anaemia is < 5% *(WHO 2001)*.

There is an urgent need for correct information on the iron status of populations for planning effective interventions to combat ID and anaemia *(JWHO and CDCPTC 2005)*.

REVIEW OF THE LITERATURE

1. The prevalence of iron deficiency and iron deficiency anaemia

Iron deficiency (ID) is widespread in infants and young children, especially in developing countries *(De Maeyer 1989)*. Animal models provide convincing evidence that ID during the brain growth spurt alters metabolism and neurotransmission, myelination, and the profiles of genes and proteins *(Lozoff and Georgieff 2007, Lönnerdal and Kelleher 2007)*.

Low intake of bioavailable iron from complementary foods is the major cause of the high prevalence of IDA among children aged 6 to 24 months in developing countries. Increased dietary diversity and traditional food-processing techniques are generally unsuccessful at completely closing the gap between iron intake and needs. Thus, iron-fortified processed complementary foods or home fortification (using powders, crushable tablets, or fat-based products) will be needed in most populations. However, large-scale studies that include sufficient numbers of iron-sufficient children are lacking *(Dewey 2007)*.

In humans, there is compelling evidence that 6- to 24- month- old infants with IDA are at risk for impared cognitive, motor, social-emotional, and neurophysiologic development in the short- and long-term. In contrast to inconsistent developmental effects of iron therapy for iron deficient infants, recent large, randomized trials of iron supplementation in developing countries uniformly show the benefits of iron, especially on motor development and social-emotional behaviour *(Siddappa et al. 2004, Lozoff and Georgieff 2007)*.

The prevalence of ID varies grately between countries: from 2% in Denmark *(Michaelsen et al. 1995)*, to 20% in Iceland *(Thorsdottir et al. 2003)*. Reasent studies have reported the prevalence of ID was 4% in United Kingdom *(Hopkins et al. 2007*) and 6% in USA *(Brotanek et al. 2007)*. In the multi-centred study of 11 EU countries the average prevalence of ID was 7.2% *(Male et al. 2001)*.

Recent studies in developed countries have shown a small variance in the prevalence of IDA in infants: about 2–3% (*Looker et al. 1997*, *Male et al. 2001, Lind et al. 2003)*. However, IDA is much more frequent in socio-economically deprived areas. For example in United Kingdom 25–40% of infants aged 6–24 months living in socio-economically deprived urban areas have IDA *(Lawson 1995)* and in USA inner-city areas this is around 8% *(Bogen et al. 2000)*. In China the prevalence of IDA has been reported as 7.8% and ID as 36% *(Zhu et al. 2005)*. Very high prevalence figures of IDA has been reported in low-income countries, such as Albania (51%) *(Buonomo et al. 2005)* and Argentina (36%) *(Morasso et al. 2003)*.

The ID prevention programmes have decreased the prevalence of IDA down to 0% in Denmark *(Michaelsen et al. 1995*) and ID to 6% in US *(Brotanek et al. 2007)*.

The previous study in Estonia in 1996 demonstrated that 18% of rural children and 45% of urban 3 to 4 years old children have anaemia *(Ilves-Annunziata et al. 2000)*. However, the authors of this study used only Hb and mean corpuscular volume (MCV) for indices of iron status. Therefore, according to this study the real prevalence of IDA in Estonia is not clear.

2. Regulation of iron metabolism

2.1. Human body iron

Iron is an essential trace element for humans. It altemates between the ferrous (Fe^{2+}) and the ferric (Fe^{3+}) oxidation state *(Schümann et al. 2007)*. Iron in humans is almost exclusively a component of proteins: nonhaem proteins [ferritin, transferrin (Tf), lactoferrins (Lfs), redox-enzymes and Fe-S-proteins)] and haem proteins [nonenzymatic- like Hb and myoglobin and enzymatic- like nitrous oxide system (NOS), cytocromes, catalases and peroxidases]. Iron is in the functional centre of Hb's haem, enabling the transportation of oxygen from lung capillaries to peripheral tissues and iron is in the functional part of the oxidation chain in the cell as the functional part of cytocromes *(Geisser 1998)*.

The total amount of iron in an adult body is about 5 g *(Zilmer et al. 1996)*, but only 0.5 g in a newborn infant *(Smith and Rios 1974, Ohls and Christensen 2004)*. The majority of the iron present in an organism is in use as a component of Hb or myoglobin *(Zilmer et al. 1996)*. In normal circumstances 3 to 4 times less is stored as ferritin and only $1/100^{th}$ is bound to plasma proteins *(Geisser 1998, Arosio and Levi 2002)*.

2.2. Iron metabolism in children

During normal pregnancy iron metabolism should provide a sufficient supply of iron to the placenta and foetus. Therefore only when the mother has severe ID does her iron status affect the newborn infant *(Dallman et al. 1980)*. The iron stores of the newborn are also influenced by the amount of blood transferred from the placenta to the foetus after delivery before the umbilical cord clamped *(Grajeda et al. 1997, Pisacane et al. 1996)*. Therefore, the cord should not be clamped until pulsation has stopped *(Aggett et al. 2002)*. As most of the iron is transferred to the foetus towards the end of pregnancy, preterm infants are born with reduced iron stores and have a greater need for exogenous iron *(Oski 1985)*. Healthy term infants with normal birthweight (BW) have sufficiently

4

stored iron to cover their growth needs during first 6 months of life *(Saarinen et al. 1977, Domellöf et al. 2002a, Aggett et al. 2002)*. The amount of total body iron does not change, although iron stores and iron content per kilogram of body weight decrease during the first 4 to 6 months of life as the infant grows *(Dallman and Yip 1989)*. During this period the infant needs little, if any, exogenous iron. This may explain why neither breast-fed infants nor infants fed with iron-enriched formula (iron up to 2 mg/L) show any signs of depleted iron stores during the first 6 months *(Hernell and Lönnerdal 1996)*.

During the second half of the year the requirement for exogenous iron rapidly increases. The daily requirement of absorbed iron during this time in an infant is about 0.1 mg per body weight (kg), which is three times higher than that of a menstruating pubertal girl *(Dallman and Yip 1989, Verster 1996)*. However, in premature infants ID may already develop at the age of 2 or 3 months *(Oski 1993)*.

The low content of iron in human milk (0.2–1.0 mg/L) limits any iron loss to the mother, which is especially important if the mother is iron deficient and breast-feeding is prolonged. Lactating mothers whose menstruation is suppressed tend to have better iron balance than menstruating women *(Svanberg 1975, Booth and Aukett 1997, Aggett et al. 2002)*.

2.3. Absorption

The main process responsible for modulating iron homeostasis in humans is intestinal absorption of iron. This is influenced by the iron content in the food and by the iron status of the organism itself, iron transport between organs, its uptake and cellular use of iron *(Aggett et al. 2002)*. Iron absorption is increased when iron stores are low such as in ID and when tissue oxygen supply is compromised like in anaemia. Conversely, iron absorption decreases when iron stores are high *(Finch 1994, Schümann et al. 2007)*.

Humans consume iron in two forms: 90% in the nonhaem form (mostly Fe^{3+}) from vegetables and 10% in the haem form (Fe^{2+}) from meat, liver, blood, fish, poultry *etc*. The absorption of haem iron and nonhaem iron from food is different *(Lieu et al. 2001, Rehemaa 2004)*. The extent of what type of iron is absorbed is highly variable and depends both on the diet and individual's iron status. Only 5% of nonhaem iron is absorbed, but in ID this absorption may be doubled *(Oski 1993, Zilmer et al. 1999)*. The molecular mechanisms behind these processes are not fully understood, but many proteins such as duodenal cytochrome (Dcyt) b, divalent metal transporter 1 (DMT-1) and hephaestin are involved in the regulation of iron homeostasis *(Aggett et al. 2002).*The main mechanisms of iron absorption are given in Figure 1. In the duodenal brush border, Fe^{3+} is reduced to Fe^{2+} by Dcyt b and taken up from the lumen to the enterocyte by DMT-1 *(Crichton et al. 2002, Domellöf 2007)*. In the basolateral membrane of duodenal enterocytes hephaestin oxidises Fe^{2+} iron back to Fe^{3+}.

Thereafter, Fe^{3+} is exported to plasma so that it can bind to transferrin *(Schümann et al. 2007)*. The "mucosa block" mechanism reduces iron absorption after a previous high iron exposure, presumably by diminishing the number of DMT-1 receptors *(Frazer et al. 2003)*.

The other 10% of dietary iron is in the haem form, which is derived primarily from the Hb and myoglobin of eaten meat. Haem iron is well absorbed (approximately 20–25%) *(Zilmer et al. 1999)*. The absorption of haem iron is less influenced by diet and body iron stores *(Oski 1993, Engelmann et al. 1998)*. It has been proposed that the haem carrier protein (HCP1) mediates haem's uptake *(Shayeghi et al. 2005)*. Haem is first cleaved by haemoxigenase in enterocytes. The iron released enters the nonhaem iron pool and is transferred to the body via ferroportin *(Schümann et al. 2007)*. Plasma hepcidin concentrations have been shown to regulate intestinal iron absorption in relation to the body's demand. Hepcidin is a hormone produced by the liver and it controls plasma iron levels by regulating the absorption of dietary iron from the intestine, the release of recycled Hb iron by macrophages, and the movement of stored iron from hepatocytes. It binds to ferroportin and inactivates the cellular iron-exporting function of this transport protein *(Nemeth et al. 2004a)*. As ferroportin mediates iron export from duodenal enterocytes and from the reticuloendothelial system into the plasma, this process has been suggested to explain the inhibitory effect of hepcidin on duodenal iron absorption as well as its accumulation in the reticuloendothelial system *(Schümann et al.2007)*. Plasma hepcidin concentration is increased in iron-overload conditions as well as during inflammation, where the increment in hepcidin level is mediated by the IL-6 *(Nemeth et al. 2004b)* and IL-1 cytokines *(Lee et al. 2005)*.

A lactoferrin receptor (LfR) has been found in the small intestine of human foetuses and infants. It may be an important factor for iron uptake because most of the iron in breast-milk is bound to Lf *(Suzuki et al. 2005)*. The functional importance of these LfRs in humans is not fully clear *(Domellöf 2007)*. Although iron is present in breast-milk in low concentrations (0.2–1.0 mg/mL), it is well absorbed, (about 50%) and utilised *(Svanberg 1975, Booth and Aukett 1997)*. A recent study in breast-fed Peruvian infants (*Hicks et al. 2006)* showed that iron absorption was 43% at the age of 5 to 6 months and 52% between 9 to 10 months. The figures for non-anaemic infants in Sweden were 14% and 26% respectively *(Domellöf et al. 2002a)*.

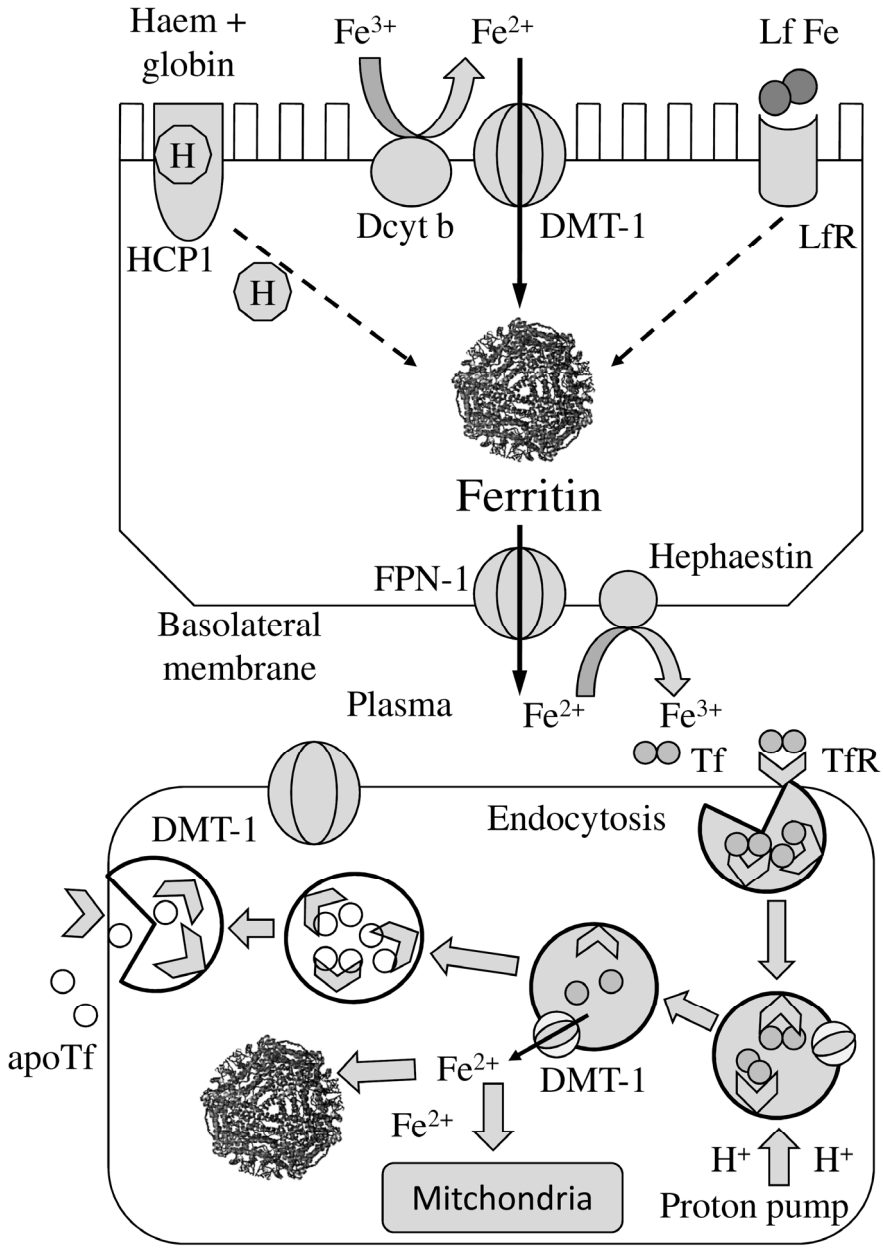

Figure 1. Molecular mechanism of iron absorption and transport (modified from the figures by *Andrews 1999 and Domellöf 2007)*.

H–haem, HCP1–haem carrier protein, Dcyt b–duodenal cytochrome b, DMT–1divalent metal tarnsporter, Lf–lactoferrin, LfR–lactoferrin receptor, FPN-1–ferroportin, Tf–transferrin, TfR–transferrin receptor, apoTf–apotransferrin.

It is important to underline that iron absorption from cow's milk is much lower than from breast-milk. Therefore the high prevalence figures of ID in infants can be explained by the wide use of infant formulas based on cow's milk *(Jackson and Lee 1992, Kibangou et al. 2005)*. Hydrolysis of cow's milk proteins enhances iron absorption *(Hurrell et al. 1989, Domellöf 2007)*. The reasons for poor iron absorption from cow's milk are the high calcium concentration and differences in protein patterns between cow's milk and breast-milk. A recent study *(Kibangou et al. 2005)* suggested that this inhibitory effect on iron absorption is due to α-s-casein and its caseinophosphopeptides (CPP). They confirmed the efficient uptake and absorption of iron bound to the specific CPP of β-casein, which is similar to the pharmaceutical forms of iron (Fe gluconate) and better than the reference salt $FeSO_4$. The caseinophosphopeptide (CPP) complexes inhibiting brush border phosphatise activity had no significant effects on the metabolism of Fe glyconate; it did not modify the absorption of α-s-CPP-bound iron. The significant enhancement of Fe-β-CPP uptake after inhibiting the dephosporylation reaction means that free iron released from this complex is less available than in the bound form *(Pérès et al. 1999, Kibangou et al. 2005)*.

There is very little meat in infant's diet; therefore, most of their dietary iron is nonhaem, and their intake is highly influenced by other dietary factors. Cooking of food increases the availability of iron from the gut. The low gastric pH facilitates the reduction of the Fe^{3+} ion into the Fe^{2+} state *(Zilmer et al. 1999, Rehemaa, 2004)*. Ascorbic acid, citrate and malate enhance the absorption of nonhaem iron, as does haem iron itself *(Derman et al. 1980, Jackson and Lee 1992, Oski 1993)*. Inhibitors of iron absorption include calcium, bran, polyphenols, oxalates, phytates, vegetable fibre, tannins in tea, and phosphates *(Charlton and Bothwell 1983, Jackson and Lee 1992)*.

In contrast to other trace elements there is no regulatory excretion mechanism for iron *(Hallberg et al. 2000, Aggett et al. 2002)*. Iron has high affinity, therefore it bins to many specific and nonspecific macromolecules, leading to the absence of significant iron salt formation. The usual excretory routes enable the loss of only salts *(Zilmer et al. 1999)*. In contrast, the excretion of iron occurs only through the shedding of tissues that are not reutilized (epidermis, hair, mucosal cells of the gastrointestinal and urinary tracts) and physiological/ pathological losses of blood *(Crichton et al 2002, Rehemaa 2004)*. In a healthy adult male the endogenous gastrointestinal iron loss is lower (0.014 mg/kg/day) than in a normal infant or toddler (0.022 mg/kg/day) *(Fomon et al. 2005, Domellöf 2007)*. In a normal human about 40 mg of iron is mobilized each day from different tissues. About 80–95% of this is derived from the catabolism of red blood cells (RBCs), representing the recycling of iron. Most of the remaining 7–8 mg of iron is mobilized from hepatocytes *(Aisen et al. 1999)*. In children only 70% of iron is derived from RBCs and they have to get 30% of their iron from food *(Booth and Aukett 1997, Grünberg et al. 2001)*. In order to achieve this intake of iron, about 0.8 mg of iron has to be absorbed from the diet

every day, of which 0.6 mg is needed for growth and 0.2 mg to replace losses *(Dallman 1989, Booth and Aukett 1997)*.

2.4. Erythropoiesis

Erythropoiesis in the foetus is controlled by erythroid growth factors produced solely by the foetus. Although the mechanism of erythropoietic regulation in adults is known to a significant degree, it is unclear if the same mechanism of erythropoietic regulation exists in foetuses or in premature infants. Production of RBCs is governed by various growth factors produced by accessory cells such as macrophages, lymphocytes, and stromal cells *(Ohls and Christensen 2004)*. These factors stimulate maturation, growth, and differentiation at various stages of RBC production. Of all the factors stimulating erythropoiesis, none has a more important regulatory role than erythropoietin (EPO). Erythropoietin is a 30–39 kD glycoprotein that binds to specific receptors on the surface of erythroid precursors and stimulates their differentiation and clonal maturation into mature RBCs. EPO is produced by the liver during the first and second trimester, principally by cells of monocyte origin. Sometime during the third trimester and the firsts few weeks of life, the anatomic site of EPO production shifts from the liver to the kidneys. This shift may be related to the significant changes in arterial oxygen tension that occur at birth *(Ohls and Christensen 2004)*.

Red bood cells mature for 3–7 days in the bone marrow. The lifespan of RBCs is about 123–127 days and its ends with haemolysis in the spleen *(Ohls and Christensen 2004)*.

The combustion reactions that are essential to life require that tissues receive a constant supply of oxygen. The evolution of oxygen-carrying proteins like Hb increased blood's ability to transport oxygen. Furthermore, the association and disassociation of oxygen with Hb is accomplished without any expenditure of metabolic energy *(Schwartz 2004)*.

2.5. Haemoglobin

Haemoglobin is a complex protein consisting of iron-containing haem groups and the moiety protein is globin. A dynamic interaction between haem and globin gives Hb its unique properties in the reversible transport of oxygen. The Hb molecule is a tetramer made up of two pairs of polypeptide chains, each chain being attached to a haem group. The polypeptide chains of various Hbs are of chemically different types. The major haemoglobin (HbA) of a normal adult is made up of one pair of alfa (α) and one pair of beta (β) polypeptide chains and represented as $\alpha_2\beta_2$. The major Hb in the foetus (HbF) is represented as $\alpha_2\gamma_2$ *(Ohls and Christensen 2004)*.

Six different Hbs may be normally detected within the RBCs of an embryo, foetus, child and adult: Gower-1, Gower-2, and Hb Portland in embryonic Hb, HbF in foetus and HbA and A_2 in adult Hbs *(Ohls and Christensen 2004)*.

The blood of early human embryos contains Gower-1, Gower-2, and Hb Portland. In embryos of 4–8 weeks, Gower Hbs predominate, but by the third month they have disappeared. HbF is the predominant Hb after the eighth gestational week and at 24 weeks gestations it constitutes 90% of the total Hb. A gradual decline occurs during the third trimester, so that at birth the HbF is an average 70% of the total Hb. Synthesis of HbF decreases rapidly postnatally and by the 12^{th} month of age only a trace is present. In older children and adults less than 2.0% of total Hb is present in HbF form. HbA ($\alpha_2\beta_2$) can already be detected in the very early stages of the embryo. Accordingly, it is possible to make a prenatal diagnosis of major β-chain haemoglobinopathies, such as thalassemia major, as early as 16–20 weeks into gestation. By the twenty fourth week of gestation, approximately 5–10% of total Hb is in HbA form. A steady increase follows, so that at term, the HbA accounts for an average of 30% of total Hb (70% is HbF). At birth, less than 1.0% of total Hb is in HbA_2 form which is gradually increased to 2–3.4 % by the age of 12 months. Throughout life the normal ratio of HbA and HbA_2 is about 30:1 *(Ohls and Christensen 2004)*.

The concentration of Hb increases according to gestational age, so that at term, the mean concentration of Hb in cord blood is 168 g/L (147–237) *(Siigur and Oro 2006)*. Mean cord blood Hb concentration in very low birthweight infants is about 10–20 g/L lower. A "physiologic" decrease in Hb concentration is seen at 8–12 weeks in term infants and at about 6 weeks in premature infants (70–100 g/L) *(Ohls and Christensen 2004)*.

3. Iron transport and storage

3.1. Transferrin and lactoferrin

Under physiological conditions most of the cells of the organism acquire iron from a well-characterized class of plasma glycoproteins, transferrins (Tf) *(Suominen 2000)*. Transferrin is a $ß_1$ glycoprotein synthesized in the liver, consisting of a single polypeptide chain of 78 000 Da with two Fe^{3+} binding sites *(Aisen et al. 1999)*. Serum Tf is the main supplier of iron to most body tissues whereas the other Tfs are produced locally and transport iron within sanctuaries that cannot be reached by serum *(Suominen 2000)*. The main mechanism of iron transport is shown in Figure 1. The main determinant of Tf production is the availability of iron. Iron depletion in the bone marrow is associated with the increase and repletion with subsequent decrease of Tf production *(Aisen and Litowsky 1980)*. Inflammation decreases Tf concent-

rations irrespective of the prevailing iron status, whereas pregnancy and the administration of estrogens are associated with increased levels *(Huebers and Finch 1987)*. Assessment of serum iron and serum Tf have largely been disregarded, since they are unpredictably variable and relatively intensive indicators of ID *(Punnonen et al. 1997, Lee 1999)*.

Another group of structurally related compounds are the Lfs, which were first identified in milk and later in tears, saliva, gastric juice, bronchial and gastrointestinal secretions and in neutrophilic leucocytes *(Aisen and Listowsky 1980, Weinberg 1984, Huebers and Finch 1987, Lönnerdal and Iyer 1995)*. Lactoferrin is present in human milk colostrums at about 10 g/L and about 1–2 g/L in mature milk. This suggests that Lf is important for infants, especially during the early stages after birth *(Suzuki et al. 2005)*. Lactoferrin plays an important role in scavenging iron by macrophages and polymorphonuclear neutrophils. It is a major protein component of the specific granules of circulating polymorphonucleocytes which are important in inflammation *(Weinberg 1984)*. After binding iron, the metal saturated protein is ingested by macrophages, probably via receptor mediated endocytosis *(Kawakami and Lönnerdal 1991)*. Macrophages are present in many tissues, including liver (Kupffer cells), lungs (alveolar macrophages), spleen (free and fixed macrophages), nervous tissue (microglia), connective tissue (histiocytes) and the serous cavities (pleural and peritroneal macrophages). These class of cells have two important functions, firstly to orchestrate the host inflammatory response and secondly in the regulation of iron homeostasis. The predominant role of the macrophages in iron homeostasis is to phagocytose senescent erythrocytes in the spleen. Haem oxygenase catabolises haem to Fe^{2+}, the bile pigment biliverdin and the gaseous cellular messenger carbon monoxide. In the macrophage, this free iron is either incorporated into ferritin or released into the circulation to be bound by Tf. The macrophage is able to retain iron during periods of adequate iron nutrition but be able to release it during times of iron paucity *(Arosio and Levi 2002)*. Lactoferrin has high affinity for iron and it can effetely scavenge iron also at septic sites, under local lactic acidosis *(Weinberg 1984, Guillén et al 1998)*. Lactoferrin may also be involved in bactericidal activity within phagocytes by generating hydroxyl radicals through the Haber-Weiss reaction *(Aisen and Litowsky 1980, Ambruso and Johnston 1981)*.

3.2. Transferrin receptors and soluble transferrin receptors

Transferrin binds to specific cell surface receptors- transferrin receptor (TfR) (CD71) that mediate the internalisation of the protein in almost all mammalian cells (Figure 1). Transferrin receptor mediates the flow of Tf-bound iron into the cells by receptor mediated endocytosis *(Bomford and Munro 1985, Huebers and Finch 1987)*. The Tf-TfR complex is internalized in clathrin-coated vesicles. After the vesicles lose their coat they fuse with endosomes. Iron is released

from the Tf-TfR as Fe^{3+} and transported out of the endosome by DMT1. Once the iron has been released it is rapidly bound by intracellular protein ligands, notably the iron storage protein ferritin *(Ponka 1997, Crichton et al. 2002)*.

The synthesis of TfR is tightly regulated by the amount of Tf-bound iron available to the cell. Reduction of Tf-bound iron up-regulates TfR synthesis whereas abundance of iron down-regulates the synthesis. The number of TfRs on the cell surface reflects therefore the dynamic balance between supply and demand of tissues iron, and displays the potential for the cell proliferation *(Crichton et al. 2002)*.

Although it was known that soluble transferrin receptors (sTfR) are crucial for the regulation of iron metabolism, sTfR did not have any clinical interest until Kohgo et al. *(1986)* discovered that small amounts of sTfR are also present in human serum. Circulating sTfR has a molecular mass of 85 kDa and forms a complex with Tf in serum. About 80% of sTfR originates from the erythropoietic cells in bone marrow and from the circulating reticulocytes that eventually shed their receptors during maturation *(Iacopetta et al. 1982, Beguin et al. 1993)*. Serum concentration of sTfR is directly proportional to the total amount of tissues receptors. Serum sTfR has consequently been introduced as an effective and specific measure of functional iron stores, since it correlates directly with the erythropoietic activity and inversely with the amount of iron available for erythropoiesis *(Huebers et al. 1990, Beguin et al. 1993)*. Unlike ferritin, sTfRs retain their specificity to the changes in iron status, particularly in the acute phase *(Huebers et al. 1990, Skikne et al 1990)*. Serum sTfR concentrations are raised in haemolytic anaemia, both in autoimmune anaemia and in hereditary spherocytosis, in megaloblastic anaemia of cobalamin deficiency, α-thalassemia, β-thalassemia and sickle cell anaemia. Serum StFr concentrations are reduced in aplastic anaemia and following marrow ablation prior to bone marrow transplantation *(Kohgo et al 1986, Huebers et al. 1990)*.

3.3. Ferritin

Stored iron works as a buffer between cellular demand and dietary supply of iron *(Aisen and Litowsky 1980)*. Ferritin is the main iron storage protein, widely distributed in many mammalian cell types: in hepatocytes, in liver, spleen and bone marrow macrophages, even in lymphocytes, fibroblasts and reticulocytes. The molecular weight of ferritin is 480 kDa and it consists of 24 iron free subunits – apoferritins – that together form a hollowsphere *(Harrison et al 1986)*. Apoferritin may bind about 4500 iron atoms. In human two types of ferritin subunits are described: H and L. The L subunit is expressed more in liver and spleen, whereas the H subunit is found more in the heart *(Zilmer et al. 1999, Arosio and Levi 2002)*. Two subunit types with specific functionality offers the possibility to regulate the number of ferritin molecules independently, ie., the iron storage capacity, and the number of catalytic sites, ie., the iron

oxidizing capacity. This is particulary evident in tissues with an iron storage function, such as the liver, spleen, or tissues with a high iron load, where the production of a large number of L-chains allows the cell to expand its iron storage capacity without an excess of catalytic sites, which would limit the iron availability. Ferritins are manly cytosolic proteins and keep the stored iron separate from the nucleus and other organelles *(Arosio and Levi 2002)*. Low amounts of ferritin are present in serum and secreted liquids (salivary, urine *etc.)*. In healthy individuals the synthesized and secreted amount of plasma ferritin seems to be proportional to the amount of cellular ferritin produced in the internal iron storage pathway *(Cook et al. 1976)*. Serum ferritin is a clinically important index of body iron stores, even though its functional role has not been fully understood *(Arosio and Levi 2002)*.

The best characterized regulatory system of ferritin expression is the post-transcriptional, iron dependent machinery based on the interaction by the iron regulatory proteins (IRPs) and the iron responsive elements (IRE) located on the target mRNAs. The system is sensitive not only to iron availability, but also to the oxidative status of the cell, and it regulates the L- and H-chains similarity. The synthesis of ferritin is readily upregulated when bioavailable iron is high to produce enough molecules to accommodate the excess iron. Conversely, the storage compartment is reduced when iron is low in order to make iron available for enzyme synthesis. However, the role of ferritin in this mechanism is still not fully understood. It is not clear whether the protein plays an active role in this mechanism of regulating intracellular iron homeostasis *(Arosio and Levi 2002)*.

Ferritin can also be viewed as a member of the group of proteins that respond to stress and inflammation. Inflammatory cytokines, particularly tumour necrosis factor α (TNFα) and IL-2, up regulate ferritin synthesis in mesenchimal cells, hepatocytes and monocyte-macrophages *(Zilmer et al 1999, Arosio and Levi 2002)*. The only condition in which ferritin levels have been shown to be low is ID *(Harju et al. 1984)*.

3.4. Haemosiderin

Degradation of ferritin takes place in the "secondary lysosomes" encapsulated in the membrane. This is supposed to be the source of haemosiderin – the storage form of iron – from which iron can not be taken up any more. Under physiological conditions, haemosiderin is predominantly located in cells of the monocyte-macrophage lineage (erythroid marrow, Kupffer cells of the liver, spleen), but in pathological iron overload conditions haemosiderin may accumulate in large quantities in almost all tissues *(Harrison et al. 1990)*. In such conditions haemosiderin accumulates in the liver (cirrhosis, tumours), in the pancreas (diabetes) and in the heart (heart failure) *(Zilmer et al. 1996)*.

4. Iron deficiency

4.1. Aetiology

The most important factor for developing ID in children is growth spurts *(Provan 1999)*. The risk for ID has been considered to be greatest in late infancy when the growth rate is high and dietary iron intake is usually low *(Dallman et al. 1980, Thorsdottir et al. 2003)*.

Dietary deficiency is the most common cause of iron depletion in infants and children. Breast-feeding for less than 6 months' duration, the use of non-iron-fortified infant formula and a diet deficient of iron are proven risk factors *(Oski 1993, Yager and Hartfield 2002)*. The early (before fist year of age) introduction of unmodified cow's milk is the most common dietary characteristic of infants found to have IDA over their first year *(Fuchs et al.1993, Booth and Aukett 1997)*. Cow's milk is a poor source of iron and also reduces the bioavailability of iron provided by other foods *(Lönnerdal et al. 1984, Male et al. 2001)*. Gastrointestinal blood loss provoked by cow's milk may also contribute to risk of ID *(Jiang et al. 2000, Male et al. 2001)*.

The other risk factors for developing of ID in children are prematurity, male gender, high weight gain, low income poor socio-economical status, living in urban areas, adolescence and menstruations in girls *(Oski 1993, WHO 2001)*.

4.2. Detection and staging of iron deficiency

Before establishing a concept of ID one must first consider the extent of normal physiological variation. IDA was considered as the only clinically relevant stage of ID both for epidemiological as well as for clinical purposes *(Cook et al. 1976, Suominen 2000)*. Most studies have been designed to distinguish patients with IDA from patients with other anaemic conditions with nonanaemic subjects as controls *(Flowers et al. 1989, Punnonen et al. 1994)*. This setting, however, includes subjects with depleted iron stores and subjects already exhibiting iron deficient erythropoiesis in the control group and only patients with fully developed IDA in the iron deficient group. In order to create reference values for a truly iron sufficient population, the subclinical stages of ID, storage depletion and iron deficient erythropoiesis should be excluded. This is difficult to achieve by methods other than bone marrow sampling or iron supplementation trials *(Garby et al. 1969, van Zeben et al. 1990, Suominen 2000)*.

4.3. Depletion of iron storage

Stored iron is the pool of iron in the body that is not being used by tissues. Healthy children and adults (apart from infants' aged 6–11 months) usually have some iron stores to act as a buffer against ID during periods when dietary iron may be temporarily insufficient *(JWHO and CDCPTC 2005)*.

Iron depletion is the state in which stored iron is absent or nearly absent but the tissues that need iron are able to maintain normal physiological functions *(JWHO and CDCPTC 2005)*.

Iron deficiency (ID) is a state in which there is insufficient iron to maintain the normal physiological function of tissues such as the blood, brain, and muscles. Iron deficiency can exist in the absence of anaemia if it has not lasted long enough or if it has not been severe enough to cause the Hb concentration to fall below the threshold for the specific age and sex group *(WHO 2001)*.

The transition from the normal iron sufficient state to the development of IDA has two sequential processes: depletion and exhaustion of stored iron (stage I as prelatent ID) and continued iron loss (stage II, latent or functional ID). There is no additional physiological phenomena associated with the development of IDA itself (stage III), which is therefore merely a sequel to the progressive depletion of the functional iron compartment *(Oski 1993, Suominen 2000, Wick et al. 2003)*.

During the depletion phase (stage I), the consumption or loss of iron exceeds the amount of iron being absorbed from the diet. If this condition is sustained, the bone marrow iron stores become progressively depleted and finally exhausted. Iron depletion is not associated with any clinical symptoms and indicates merely an increased risk of progressive ID resulting from the elimination of the stored iron buffer. Bone marrow aspiration biopsy has been the best test-of-choice to evaluate the levels of stored iron in an individual patient *(Burns et al. 1990)*. Due to its invasive nature, bone marrow aspiration biopsy is rarely used in adults and is not appropriate for use in infants *(Hallberg et al. 1993, Suominen 2000, Domellöf et al. 2002b)*. Serum ferritin concentration decrease in the first stage of ID, which is why it has become the most widely used noninvasive variable for assessing the variations in iron stores *(Cook et al. 1976, Oski 1993; Suominen 2000, Wick et al. 2003)*. The laboratory findings in different stages of ID are seen in Table 1.

Table 1. The laboratory findings in different stages of iron deficiency (adapted from *Tomberg et al. 2001*).

Laboratory test	Laboratory finding	Prelatent iron deficiency	Latent iron deficiency	Iron deficiency anaemia
Ferritin	⇩	→	→	→
sTfR	⇧		→	→
Transferrin	⇧		→	→
Tf-Sat	⇩		→	→
TIBC	⇧		→	→
CHr	⇩		→	→
MCV	⇩			→
Haemoglobin	⇩			→

Tf-Sat- transferring saturation, TIBC- total iron-binding capacity, CHr- reticulocyte Hb content.

4.3.1. Erythropoiesis in iron deficiency

Once the iron stores become depleted beyond a certain threshold, Tf iron saturation (Tf-Sat) becomes reduced to less than what is required to maintain the synthesis of iron compounds *(Suominen 2000)*. Low Tf-Sat indicates a high proportion of vacant iron-binding sites. The Tf-Sat is highest in neonates, decreases by the age of 4 months and increases thorough childhood and adolescence until adulthood *(Dallman et al. 1980)*. The Tf-Sat is based on two laboratory measures, serum iron concentration and total iron-binding capacity (TIBC). In healthy individuals, plasma Tf-Sat is usually 20–35% of maximum, meaning that most Tf molecules have one or two unoccupied iron binding sites and free iron cannot be detected *(Crichton et al. 2002)*. However, Tf-Sat is impractical for screening in the ambulatory setting due to biological variability, such as day-to-day variation, fluctuation with dietary intake, and as acute phase reactant, and therefore quite intensive to minor changes in iron status *(Borel et al. 1991, Punnonen et al. 1997, Ullrich et al. 2005)*.

Serum iron concentration is a measure of the total amount of iron in the serum and is often provided with results from other routine tests evaluated by automated, laboratory chemistry panels. Many factors can affect the results of this test. For example, the concentration of serum iron increases after each meal, infections and inflammations can decrease the concentration, and diurnal variation causes the concentration to rise in the morning and fall at night *(Yip*

and Dallman 1988, CDC 1998). The day-to-day variation of serum iron concentration within individuals is greater than that for Hb concentration and hematocrit (Hct) *(Borel et al. 1991)*.

Currently, the only widely accepted indicator of early ID erythropoiesis is the compensatory elevated sTfR concentration *(Skikne et al. 1990, Oski 1993, Baynes 1996, Wick et al. 2003)*. Serum sTfR reacts more rapidly and specifically to subclinical ID than other variables, and seems to accurately portray depletion of the functional compartment between initial stored iron depletion and the development of anaemia *(Skikne et al. 1990, Cook et al.1994, Suominen 2000)*.

Free erythrocyte protoporphyrin (FEP) and zinc protoporphyrin (ZPP) have also been used to detect the onset of ID erythropoiesis *(Hastka et al. 1996, Suominen 2000)*. FEP refers to the non-ferrous protoporphyrin IX within the erythrocytes which is produced instead of haem, when the iron support to erythropoiesis becomes insufficient *(Marsh et al. 1983*). The normal range of FEP concentration is higher for children aged 1–2 years than for adults, but no consensus exists on the normal range for infants *(Dallman et al. 1989)*. The sensitivity of FEP to diagnose ID (as determined by response to iron therapy) in children and adolescents aged 6 months to 17 years is 42%, and the estimated specificity is 61% *(Margolis et al. 1981)*. ZPP, on the other hand, is produced under similar conditions, when zinc instead of iron is incorporated into protoporphyrin IX *(Hastka et al. 1996, Suominen 2000)*. Values are en-changed in the presence of ID erythropoiesis, but the concentration may also be raised in the presence of lead poisoning, inflammatory and infectious diseases, or in myelodysplastic syndromes *(Hastka et al. 1996)*.

The fluctuations in iron supply to the bone marrow leads to decreased Hb production in reticulocytes, resulting in reticulocytes with less Hb and overall reduction in reticulocyte Hb content (CHr) *(Major et al. 1997, Goodnough et al. 2000, Ullrich et al. 2005)*. These studies have shown that CHr to be a better predictor of iron stores than Tf-Sat or MCV, because of reticulocytes lifespan in the circulation is only 24 to 48 hours *(Ullrich et al. 2005)*.

Functional ID develops when the iron stores are almost empty, althrough Hb synthesis is still adequate at this stage- **latent or functional ID**. Functional ID may also develop in impaired iron transport to target tissues. This occurs most commonly in infectious diseases when cytokines are released during inflammation and mediated by hepcidin. Hepcidin causes the characteristic iron decrease in blood (hypoferritaemia of inflammation) during inflammation. The hypoferritaemia is thought to increase host resistance to microbial infection but also leads to anaemia of inflammation (often referred to as anaemia of chronic disease) *(Andrews 2004)*. Iron supplementation or fortification has no benefit in such circumstances. Deficiencies of other nutrients such as vitamin A may also cause a functional ID *(Semba and Bloem 2002)*.

The morphological changes of the RBCs (microcytosis, hypochromia, anisocytosis and poikilocytosis) appear as fairly late manifestation of ID *(Lee 1999)*.

However, there is no agreement on the specific laboratory criteria for ID. The most common criterion for ID is either a low serum ferritin *(Hallberg et al. 1993, Domellöf 2002b)* or a combination of multiple criteria using different iron status variables *(Cook et al. 1976, Mast et al. 1998, Domellöf et al. 2002b, Thorsdottir et al. 2003)***.** The WHO recommends to use ferritin < 12 μg/L as criteria for ID in children up to 5 years *(WHO 2001)*. But these values have been extrapolated from older age groups and may not be appropriate for infants *(Domellöf et al. 2002b)*. The thresholds and ranges for all indicators of iron status need to be defined and validated for children aged 6–24 months *(JWHO and CDCPTC 2005)*.

4.3.2. Iron deficiency anaemia

Iron deficiency anaemia is the most conspicuous results of ID *(Suominen 2000, Yager and Hartfield 2002)*. In clinical terms, **anaemia** is an insufficient mass of RBCs circulating in the blood; in public health term, **anaemia** is defined as Hb concentration below the thresholds determined by WHO, UNICEF and UNU *(WHO 2001)*. These thresholds are set at the 5th percentile of Hb concentration of a normal population of the same sex and age group *(JWHO and CDCPTC 2005)*. The WHO recommendations for diagnosis of IDA is Hb < 110 g/L and ferritin < 12 μg/L in children up to 5 years *(WHO 2001, JWHO and CDCPTC 2005)*. The reference limits for Hb by age groups are shown in Figure 2 *(Siigur and Ora 2006)*.

Mean red cell volume is highest at birth, decreases during the first 6 months of life, and then gradually increases during childhood to adult levels *(Dallman et al. 1980)*.

However, with additional stress or the loss of iron this condition may progress into a manifest ID with hypochromic microcytic anaemia *(Oski 1993, Wick et al. 2003*) (Table 1). Many haematological tests are widely used to screen for ID and IDA. Haemoglobin is the most commonly used haematological screening test, but it is derived from the entire population of RBCs, each with a lifespan of about 120 days, and therefore takes some time to be altered by ID. Consequently, relying on the Hb for screening will delay the detection of ID in infants who are not yet anaemic but for whom adverse neurological consequences may have already begun to occur *(Binkin and Yip 1990, Ullrich et al. 2005)*.

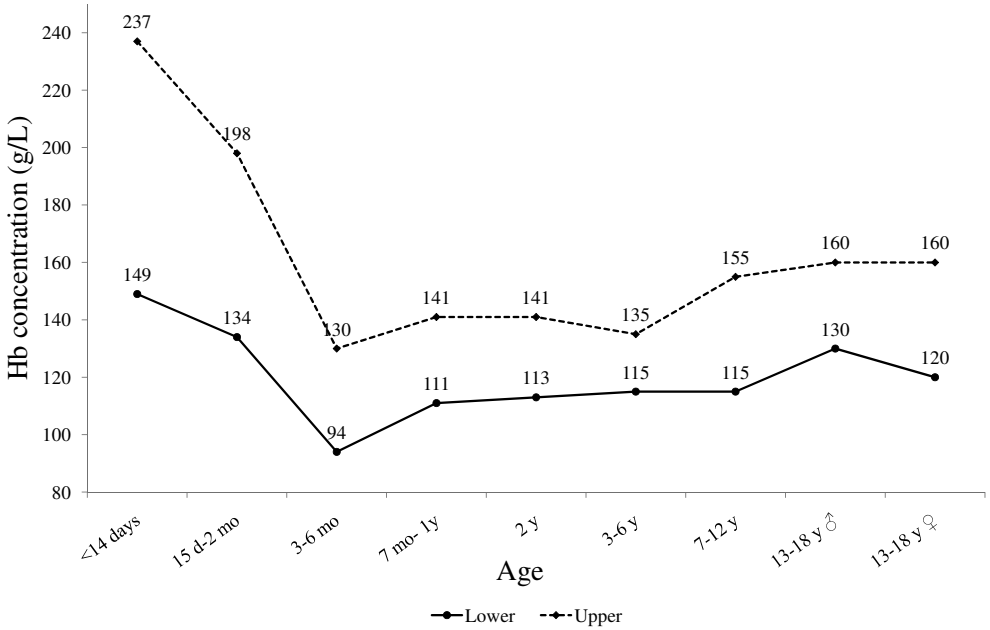

Figure 2. The reference limits of Hb in children in different age groups (adapted from *Siigur and Ora 2006).*

4.4. Manifestations of iron deficiency

4.4.1. Anaemia and symptoms

The signs and symptoms of ID are mainly caused by anaemia, but also by the effect of chronic ID on epithelial tissues. Anaemia is not a specific entity but results from many underlying pathologic processes. A useful classification of the childhood anaemias is to divide them into three groups by their RBCs' MCV: microcytic, macrocytic or normocytic. The classification of the more frequent childhood anaemias by their MCV is given in Table 2.

Iron deficiency anaemia produces many systemic abnormalities. Pallor is the most important clue to ID *(Schwartz 2004)*. In mild to moderate IDA (Hb level 60–100 g/L), the body's compensatory mechanisms, such as increased levels of 2,3 diphosphoglycerate (2,3-DPG) and a shift of the oxygen dissociation curve, may be so effective that the symptoms of anaemia are not noted. Affected children may be irritable and anorectic when Hb level falls below 50 g/L *(Oski 1993, Schwartz 2004)*. Phagophagia, the desire to ingest unusual substances such as ice or dirt, may also be present. In some children the ingestion of lead-containing substances may lead to concomitant plumbism. Tachycardia and cardiac dilatation occur, and systolic murmurs are often present. The spleen is enlarged to palpation in 10–15% of patients *(Schwartz 2004)*. Children with

IDA may be overweight, obese or underweight, with other evidence of poor nutrition *(Schwartz 2004, Brotanek et al. 2007)*.

The typical epithelial lesions encountered in ID are abnormalities of nails (koilinychia), the tongue (absence of filiform papillae and a lesser degrees of papillary atrophy, soreness), the mouth (angular stomatitis), the hypopharynx (dysphagia), and the stomach (achlorhydria and gastritis) *(Lee 1999)*. Iron deficiency may present also as beeturia — a term to describe red or pink urine caused by the beet pigment betanin *(Oski 1993)*.

Table 2. Classification of anaemia by MCV *(Schwartz 2004)*.

Microcytic	**Normocytic**	**Macrocytic**
Iron deficiency	**Decreased production**	Normal newborn
Thalassemias	Aplastic anaemia	Reticulocytosis
Lead poisoning	Pure RBC aplasia	Vitamin B_{12} deficiency
Chronic disease (infection, cancer, renal disease)	Bone marrow replacement	Folate deficiency
Vitamin B_6 responsive	**Blood loss**	Oroticaciduria
Copper deficiency	Internal or external	Myelodysplasia
Sideroblastic (some)	Sequestration	Liver disease
	Haemolysis (Intrinsic RBC abnormalities	Hypothyroidism
	Haemoglobinopathies	Vitamin B6 deficiency (some)
	Enzymopathies	Thiamine deficiency
	Membrane disorders	
	Haemolysis (extrinsic RBC abnormalities)	
	Immunological (haemolytic diseases)	
	Toxins	
	Infections	
	Microangiopathic (Disseminated intravascular coagulation (DIK), *etc.*)	

4.4.2. Iron deficiency and infection

Due to the adverse effect of ID on the immune system, morbidity from infectious disease is increased in iron deficient populations *(Hershko et al. 1970)*. A number of studies have found that several immune parameters are affected during ID, especially cell-mediated immunity and the bactericidal activity of neutrophil granulocytes *(Dallman 1987, Yip and Dallman 1998, Aggett et al. 2002, Ekiz et al. 2003)*, leading to an increased susceptibility to infections *(Booth and Aukett 1997)*. On the other hand, since iron is also an essential nutrient for many pathogens, iron supplementation itself may increase the risk for infections *(Domellöf 2007)*. A meta-analysis by Oppenheimer *(2001)* showed that iron supplementation leads to an increased risk of malaria and other infections in malarious regions.

4.4.3. Iron deficiency and growth

Children with IDA may be underweight *(Schwartz 2004)*. Iron supplementation has improved the growth of children with ID in many developing counties such as Indonesia *(Soewondo et al. 1989)*, Kenya *(Latham et al. 1990)*, and Bangladesh *(Briend et al. 1990)*, as well as in the United Kingdom *(Aukett et al. 1986)* and in the United States *(Judish et al. 1986)*. In the study of Aukett et al. *(1986)* treatment of children with IDA with oral iron for 2 months was associated with increased weight gain when compared to the placebo. The mechanism for this improved growth is unclear, but is most likely due to increased food intake. A possible direct effect of iron on growth has been also suggested by Aukett et al. *(1986)* and by Booth and Aukett *(1997)*. However, studies by Dewey and Brown *(2003)* from Hunduras and Sweden showed that daily low dose iron supplementation for breast-fed infants aged 4–9 months had a negative effect on linear growth.

4.4.4. Iron deficiency and developmental abnormalities

One of the effects of ID and IDA on infants is developmental delay. Oski et al. already reported in *1978* and *1983* that infants with IDA had lower mental developmental scores and these scores improved after short-term iron therapy. Placebo-controlled randomized clinical studies have been conducted in this field since the mid-80s. Lozoff et al. *(1987 and 1991)* studied a group of children starting from infancy to adolescence to determine the effect of early IDA on a child's development. They found that infants with moderate IDA (Hb < 100 g/L) had lower baseline mental and motor scores. The average Bayley scores in infants with persistent ID (despite iron therapy) at 3 months remained significantly lower than in the control group. The children whose ID and anaemia was corrected had no differences in test scores when compared to the control group *(Lozoff et al. 1987)*. At 5 years the children with IDA in infancy had a lower average score on mental and motor functioning tests

compared with their nonanaemic peers *(Lozoff et al. 1991)*. 10 years later they found that those with chronic ID in infancy had particularly lower verbal and full-scale intelligence quotient (IQ) scores and had specific problems in writing and mathematics and motor functioning, when compared with their non-iron-deficient counterparts *(Lozoff et al. 2000)*. Akman et al. *(2004)* showed in their trial that not only IDA (Hb < 110 g/L, ferritin <12 μg/L and MCV < 70 fL) but also ID (ferritin < 12 μg/L and MCV < 70 fL) caused developmental deficits, which were reversed with iron therapy. The other trial by Sherrif et al. *(2001)* showed that developmental deficits can occur when Hb is below 95 g/L or above 150 g/L.

4.4.5. Other effect of iron deficiency

Stroke has been associated with ID in adults and children (*Perloff et al. 1993, Hartfield et al. 1997, Swann and Kendra 2000, Maguire et al. 2007)*. Three hypotheses have been proposed to explain the association between stroke and ID: a) thrombocytosis secondary to ID; b) ID causes a hypercoagulable state; c) anaemic hypoxia *(Ready and Lowry 1989, Yager and Hartfield 2002)*.

Breath-holding episodes have also been associated with ID. Episodes occur in up to 27% of children *(Colina and Abelson 1995, Daoud et al. 1997, Yager and Hartfield 2002)*. Anaemia has been suggested to exacerbate the likelihood of breath-holding episodes because the lower Hb level reduces the blood's decreased oxygen-carrying capacity and results in more rapid secondary cerebral anoxia *(Colina and Abelson 1995, Daoud et al. 1997, Yager and Hartfield 2002)*. Some researches have demonstrated that iron treatment significantly reduced the frequency of breath-holding episodes *(Bhatia et al. 1990, Daoud et al. 1997, Mocan et al. 1999)*.

5. Prevention

Preventing ID and IDA are important parts of paediatric health-care. The increased iron requirements due to the rapid growth, replacement of foetal Hb and the decline in Hb concentration during the first 6 months of life will make this age group of children at partuclar risk. The US Food and Nutrition Board (US-FNB) has stated that an infant's iron requirements are met exclusively by breast-feeding, which provides an average of 0.27 mg Fe per day (0.78 L milk/ day x 0.35 mg Fe/L) *(Food and Nutrition Board and Institute of Medicine 2001)*.

Taking an average body weight of 9 kg of an infant at the age of 6–12 months and an iron bioavailability of 10%, the US-FNB Established Average Requirement (EAR) and Recommended Dietary Allowance (RDA) of iron is 6.9 mg and 11 mg Fe per day respectively *(Food and Nutrition Board and Institute of Medicine 2001)*. For children aged 1–3 years the US-FNB

recommended EAR and RDA of 3.0 mg Fe per day and 7.0 mg Fe per day and for children aged 4–8 years 4.1 mg and 10.0 mg Fe per day respectively *(Food and Nutrition Board and Institute of Medicine 2001)*.

Since cereals are widely used as early complementary foods, they should be fortified with iron during their commercial preparation, by extrusion, cooking or mixing processes. Centrally processed milk-based foods designed for infants should also be iron-fortified. Adding small-particle-size metallic iron is the most widely used form for enriching formulars with iron. An iron complex with ammonium-orthophosphate which is less reactive and has better absorbability is also recommended, whereas iron pyrophosphate and orthophosphate should not be used due to their poor bioavailability *(Davidsson et al. 2000, WHO 2001)*.

These recommendations are intended to guide primary health-care providers in preventing and controlling ID in infants *(DeMaeyer 1989)*. Primary prevention through appropriate dietary intake and secondary prevention through detecting and treating IDA are discussed below.

5.1. Primary prevention

The primary prevention of ID in infants should be achieved through diet. The most important recommendation is to encourage breast-feeding, especially exclusive breast-feeding (without supplementary liquid, formula, or food) for 4–6 months after birth. For infants aged less than 12 months who are not breast-fed or who are partly breast-fed, iron-fortified infant formula is recommended only as a substitute for breast-milk. When exclusive breast-feeding is stopped, it is encouraged to use an additional source of iron (daily approximately 1 mg/kg of iron) preferably from supplementary foods. For breast-fed infants who receive insufficient iron from supplementary foods by the age of 6 months (i.e., less than 1 mg/kg per day) daily 1 mg/kg of iron drops have been suggested *(CDC 1989, Committee of Nutrition 1992, CDC 1998)*. In Denmark a daily iron supplementation of 15 mg is recommended for infants between 6 and 12 months of age who are not receiving a daily minimum of 400 ml of iron-fortified formula *(Michaelsen et al. 1995)*.

For breast-fed infants who are preterm or have low BW, daily 2–4 mg/kg of iron drops (to a maximum of 15 mg/day) are recommended starting 1 month after birth and continuing until 12 months after birth *(Committee of Nutrition 1992)*. It is encouraged to use only breast-milk or iron-fortified infant formula for any milk-based diet (e.g., in infant cereal). Low-iron milks such as cow's milk, goat's milk and soy milk are not recommended to use until the age of 12 months *(CDC 1998)*. At 4–6 months iron-fortified infant cereals are recommended. Two or more servings per day of iron-fortified infant cereal meet an infant’s requirement for iron at this age. Plain pureed meat is recommended for infants after the age of 6 months or when the infant is ready to consume such food *(CDC 1989, CDC 1998)*. At the age of 6 months foods rich in vitamin C

(eg., fruits, vegetables, or juice) are recommended to improve iron absorption *(CDC 1989, CDC 1998)*. However, some authors warn the extra use of co-supplementation of vitamin C and iron-rich food. Vitamin C is known to increase the gastrointestinal absorption of non-haem iron by reducing it to a form that is more easily absorbed *(Bendich and Cohen 1990)*. Nevertheless, low dietary levels of vitamin C may be advantageous in cases of iron overload, such as hemochromatosis and treatment of β-thalassemia, due to potential iron induced tissue damage *(Livrea et al. 1996)*. Furthermore, in vitro ascorbic acid reducing iron and other transition metals may be able to promote the production of hydroxyl radicals and lipid alkoxyl radicals *(Winterbourn 1981, Buettner and Jurkewicz 1996, Rehman et al. 1998)*. Whether ascorbic acid supplementation has a prooxidant effect in vivo continues to be debated *(Podmore et al. 1998, Carr and Frei 1999, Premkumar and Bowlus 2004)*.

5.2. Secondary prevention

In populations at high risk of IDA, all children should be screened for ID and IDA between the ages 9 and 12 months, 6 months thereafter, and annually from the ages of 2 to 5 years. The criteria has been recommended as Hb < 110 g/L for anaemia and ferritin concentration < 12 μg/L for ID in children up to 5 years *(WHO 2001)*. In populations where IDA is not common, children with following risk factors should be screened: preterm or low-BW infants; infants fed with non-iron-fortified infant formula for more than 2 months; infants introduced to cow's milk before the age of 12 months; breast-fed infants who do not consume iron enriched foods after the age of 6 months (ie. those who receive insufficient iron from complementary foods); children who consume more than 500 ml cow's milk per day; children who have special health-care needs (eg., children who use medications that interfere with iron absorption or children who have chronic infection, inflammatory disorders, restricted diets, or have extensive blood loss from wounds or surgery *(CDC 1989, CDC 1998*).

6. Excess iron intake

Young children are especially at risk of overdoses of oral preparations of iron as they may accidentally ingest high doses of iron in relation to their body weight *(Anderson 1994)*. In mice it has been shown that in acute iron intoxication high quantities of absorbed iron will cause mucosal erosions in stomach and intestines. Oral intake of 180–300 mg Fe/kg body weight caused symptoms of shock by arteriolar dilatation, capillary leakage and finally heart failure *(Anderson 1994, Schümann et al. 2007)*.

Iron overload is a well-known problem, but it can be still underestimated as well as overemphasized *(Crichton et al. 2002, Rehemaa 2004)*. Generally iron

9

overload may be caused by increased genetic haemocromatosis haemolysis, transfusions and alimentary intake, especially when combined to vitamin C and alcohol *(Zilmer and Zilmer 1994, Huang 2003, Rehemaa 2004*). Since there is no evidence that large iron stores confer a benefit to the individual, a high daily intake of iron may have negative consequences. These include competition with respect to absorption of other minerals and prooxidant effects *(Solomons 1986, Schneider and Leibold 2003)*. Iron overload has been also shown to aggravate the symptoms of diseases where iron absorption is increased, such as cardiovascular disease, diabetes mellitus and thalassemia *(Lynch 1995, Aggett et al. 2002)*.

Iron has a potential to cause direct erosion and irritation to gastrointestinal mucosa and to also cause oxidative damage to lipid membranes, proteins or DNA. In addition, iron may stimulate inflammation or, as an essential nutrient, fertilise the growth of different pathogens *(Hallberg 2002, Schümann et al. 2007)*.

Infant formulas with high iron content may have negative effects on absorption of copper and zinc *(Hawkins 1964)*. In a study of healthy term infants, infant formula with an iron content of 7 mg/L resulted in a significant decrease in serum copper *(Lönnerdal and Hernell 1994)*. However, to our knowledge no prooxidative effects of iron have been demonstrated in term healthy infants. However, there is no proof of causality of such an effect, and its relevance for infants and young children is unknown *(Danesh and Appleby 1999, Aggett et al. 2002)*.

AIMS OF THE PRESENT STUDY

1. To estimate the prevalence of iron deficiency and iron deficiency anaemia in infants aged 9–12 months in Estonia (Study I).
2. To estimate the associated background factors of iron deficiency in children aged 9–12 months (Study I).
3. To establish reference values in infants aged 9–12 months for soluble transferrin receptors measured by two commercially available reagents and to evaluate and compare their diagnostic characteristics in the diagnosis of iron deficiency (Study II).
4. To establish reference values for haemoglobin, MCV, serum ferritin and to evaluate their diagnostic characteristics in the diagnosis of ID in infants aged 9–12 months living in Estonia (Study III).

SUBJECTS AND METHODS

1. Subjects (Studies I–III)

The population based study was carried out in 2 periods: from July 2002 to February 2003 and from October 2004 to March 2005. Family doctors in 7 different counties (Harju and Tallinn, Tartu County and city, Põlva, Võru, Viljandi, Pärnu, Kohtla-Järve and Narva) from all over Estonia were asked to make a list of all infants aged 9 to 12 months under their care. The letters and questionnaires (n = 330) were sent to every second parent on the list. The questionnaire contained 20 questions about living conditions, parents' education, previous illnesses and the infant's feeding habits (Appendix I).

Thirty (30/330) children were listed with incorrect address (letters were returned with note "the person is not known"); therefore the final list consisted of 300 infants. We received 225 filled questionnaires. The study group consisted 114/225 (51%) boys and 111/225 (49%) girls. The infants participating in the study were living in the following counties: Tartu (n = 60), Tallinn (n = 62), Põlva (n = 18), Võru (n = 23), Pärnu (n = 17), Viljandi (n = 12) and Kohtla-Järve and Narva (n = 33). Thirty (30/225) parents refused blood tests. Thus the final study group consisted of 195/300 infants who's parents gave their consent to participate in the study and permitted blood tests.

2. Methods (Studies I–III)

2.1. Clinical examination and data collection

All children who participated in the study were clinically examined. The child's weight and length were measured and his/ her developmental abilities were assessed. Apgar's scores, weight, length, head circumference at birth, 3 and 6 months were collected from the patients' files.

For blood tests the skin was anaesthetised with EMLA 5% cream *(Astra-Zeneca, Bedfordshire, UK)*. EMLA 5% cream is a local anaesthetic and contains 2.5% lidocaine and 2.5% prilocaine. Blood samples were taken from veins punctured only once, maximally 4 ml. In the county of Tartu the blood was taken in the Children's Clinic of Tartu University Hospital. For the other counties the same investigators took blood at respective primary care centres.

Blood samples were collected from all 195 infants. However, sufficient blood for all the repeat tests was avaiable from 174 infants. In 21 cases the blood was divided for only one or two measurements: Hb and MCV were measured in 183 infants, ferritin in 188 infants and sTfR in 190 infants.

Parents and family doctors were informed about the test results. If any abnormalities in the blood tests were found, the infant was referred to the family

doctor or pediatrician and written recommendations about feeding were given if necessary.

The Ethics Review Committee on Human Research at the University of Tartu approved the study. Informed consent was obtained from the parents before blood tests.

2.2. Laboratory analyses

Blood counts were measured using an automated analyzer *(Sysmex K-1000 and Sysmex XE 2100, Kobe, Japan)* on the admission day. Serum was separated by centrifugation and frozen at –20ºC (maximal frozen time was 90 days) until analysed at the United Laboratories of Tartu University Hospital, Tartu, Estonia. Serum ferritin concentration was measured using a chemiluminescence assay *(Immulite ® 2000, DPC, California, LA, US) (Kühnel 2000)*. Serum sTfR assays were performed using two different methods. An immunoturbidimetric method IDeA® sTfR-IT *(Orion Diagnostica, Espoo, Finland)* on analyser Cobas Mira *(ABX Diagnostics, Basel, Switzerland)* and a Tina-quant® sTfR *(Roche diagnostics GmbH, Mannheim, Germany)* on analyser Cobas Integra 400 *(Roche Diagnostics GmbH, Mannheim, Germany)*. C-reactive protein (S-CRP) was measured using a latexturbidimetric method *(Cobas Integra 400, Roche Diagnostics GmbH, Mannheim, Germany)*. C-reactive protein > 5 g/L was used as a criterion to exclude cases with possible increase in ferritin due to infection.

The coefficients of variations for Hb and MCV were 1.0%, 2.4–2.6% for ferritin, 4.5–5.7% for sTfR by the IDeA® sTfR-IT method and 2.2% for sTfR by the Tina-quant® method.

2.3. Diagnostic Criteria

2.3.1. Epidemiological study (Study I)

In the epidemiological study the criteria for ID was ferritin < 12 μg/L and MCV < 74 fL. The criteria for IDA was ferritin < 12 μg/L and MCV < 74 fL and Hb < 105 g/L. The cut-off value of 105 g/L for Hb has been used previously and deemed to be appropriate for this age group *(Siimes et al. 1984, Michaelsen et al. 1995, Lind et al. 2003, Thorsdottir et al. 2003)*, as well as the cut-off value of 74 fL for MCV *(Fuchs et al. 1993, Gill et al. 1997, Thorsdottir et al. 2003)*. The value of 12 μg/L for serum ferritin is according to WHO criteria *(INACG et al. 1998, WHO 2001)*. Three infants (3/174) were excluded from the study because their CRP was elevated. Therefore, the final study group comprised of 171 infants. Infants who had either ferritin or MCV or both above these cut-off points were classified as iron sufficient. Infants were categorized

into 4 groups: 1) iron sufficient, not anaemic; 2) iron sufficient, anaemic; 3) iron deficient, anaemic and 4) iron deficient, not anaemic as ID (Table 3).

Table 3. Results of blood tests in the study and control groups.

Study	Total	Heal-thy	ID	ID criteria	IDA	IDA criteria	Anaemia without ID	Exc-luded
I	171	117	40	Ferritin < 12 µg/L and MCV < 74 fL	16	Hb < 105 g/L Ferritin < 12 µg/L and MCV < 74 fL	14	3
II	179	146 (80)*	33 (10)*	Ferritin < 10 µg/L	10 (1)*			11
III	175	150	25	sTfR > 2.4 mg/L	7			20

* the number of subjects who had their sTfR measured by the two methods is given in the brackets. An explanation of the differences in numbers of infants between the studies is given in Methods pg. 37.

The families who agreed to participate in the study and to give blood (n = 195) and those who did not (n = 30) did not differ in their living conditions, parents' education, past illnesses or feeding habits (Table 4). Only the proportion of girls was higher in the group of non-participants (20/30) compared to participants (91/195).

Table 4. Background (clinical, socio-economic) characteristics of infants and their families who participated in the study (n = 195) and those who did not (n = 30).

Factors	Participated infants Mean (95% CI)	Non-participated infant Mean (95% CI)	p
Age (months)	10.6 (10.3–11.0)	10.0 (9.0–11.0)	1.0
Male gender	54%	34%	0.04*
BW (g)	3577 (3500–3654)	3603 (3346–3860)	0.98
Birth length (mm)	510 (505–515)	500 (484–516)	0.97
Gestational age (months)	39.9 (39.7–40.1)	38.0 (36.0–40.0)	0.12
Anaemia in pregnancy	33%	26%	0.08
Mother's education	Secondary	Secondary	1.0
Living in urban area	58%	70%	0.08
Duration of exclusive breast-feeding (months)	3.6 (2.9–4.7)	3.4 (2.7–4.2)	0.12
Total duration of breast-feeding (months)	7.0 (5.5–9.2)	6.1 (4.7–7.5)	0.08
Introduction of solid food (months)	4.7 (4.5–4.9)	4.8 (4.5–5.1)	0.65
Formula feeding	63%	68%	0.97
Cow's milk before 9 months	33%	45%	0.08
Numbers of infections	4.0 (3.6–4.4)	3.6 (2.4–4.8)	0.09

*difference between groups is statistically significant, $p < 0.05$.

2.3.2. Reference limits and cut-off values for serum ferritin, mean red cell volume (MCV), soluble transferrin receptors (sTfR) and haemoglobin (Hb) (Studies II–III)

Different methods have been used to establish the cut-off values for iron status variables. The most common approach is the normative population method in which the reference range is calculated for a "healthy" population sample. The basic approach is to sample population likely to have low prevalence of ID, but without further selection of iron sufficient subjects within the population sample.

Only healthy (without any inflammatory diseases, with normal BW, single born and healthy term) infants were included in the study. Altogether 20 (10%) infants were excluded from the study for various reasons (Table 5).

Table 5. The main characteristics of infants (n = 20) for exclusion from the study group (n = 195).

Parameters	Exclusion criteria	Excluded infants (n)
Gestational age (weeks)	< 38	7
BW (g)	< 2500	3
Twins	Yes	2
Iron supplementation	Yes	0
CRP	> 5 mg/L	8
Total excluded		20

The reference limits (the 5th and 95th percentiles) were calculated using all the infants in the study group. Serum ferritin concentrations < 10 µg/L were used as the "golden standard" to classify patients either into the ID group (n = 33) or the healthy (iron sufficient) group (n = 146) *(Looker et al. 1997, INACG et al. 1998)* for calculating the optimal cut-off value for sTfR (the maximum point of efficiency curve).

Optimal cut-off values for serum ferritin, MCV and Hb to diagnose ID were determined by ROC curves, where the diagnosis of ID was defined by sTfR > 2.4 mg/L (n = 25 in ID and n = 150 in the healthy group).

2.4. Statistical analyses

Statistical software package SAS Version 8.02 *(SAS Institute Inc., Cary, North Carolina, US),* StatsDirect Version 2.5.6 *(StatsDirect Ltd., Cheshire, UK)* and R Development Core Team *(Version 1.7.0, 2003)* were used in statistical analyses. Continuous variables are presented as mean values with 95% confidence intervals (95% CI), while discrete variables are presented as relative frequencies of absolutes. The mean, reference limits (5th and 95th percentiles)

and 95% CI for the mean and reference limits for the study population were calculated. The Kolmogorov-Smirnov criterion was used for the assessment of normality. Statistical comparisons between normally distributed continuous variables and discrete variables were performed with the Student's test. In the case of asymmetric continuous variables, the tested hypotheses were based on the calculations of nonparametric tests, such as Mann-Whitney U-test. To compare proportions (qualitative variables) the Chi-square test and the Fisher's Exact test (when expected values were < 5%) were used. Odds ratios (OR) and 95% CI were used to estimate relative risk for ID. Stepwise-regression analysis was used to define the determinants of iron status and the associated background factors for ID.

Receiver operating characteristic (ROC) curves were constituted and the corresponding areas under the curves (AUC^{ROC}) were derived with the StatsDirect statistical software. The optimal cut-off values of serum ferritin, sTfR, MCV and Hb for ID were determined *(Lee and Hsiao 1996)*.

The measuring agreement analysis was used in the comparison of IDeA® sTfR-ITs to Tina-quant® sTfRs of measurement *(Bland and Altman 1999)*. A p-value < 0.05 was considered to be statistically significant.

RESULTS

1. Prevalence of iron deficiency and iron deficiency anaemia (Study I)

A total 171 infants (88 boys/ 83 girls) were included in the final analysis. 117 infants (68.4%) composed the healthy (iron sufficient, non anaemic) control group, with Hb and either ferritin or MCV or both above the cut-off points (Group I) (Table 3). Fourteen infants (8.2%) had anaemia without ID (Group II), 16 infants (9.4%) had IDA (Group III). Twenty four infants (14.0%) had ID without anaemia (Group IV). Seven (7/171; 4%) babies were preterm, all with BW above 2000 g: one was born at the 34^{th}, one at the 36^{th} and five at the 37^{th} gestational week.

2. Causes of iron deficiency (Study I)

To find the associated background factors for ID and IDA in infants, groups III and IV (ie. iron deficient groups) were analysed together. The mean BW in the infants of iron deficient group was significantly lower than in the control group [(3393 g (3227–3559) *versus* 3627 g (3534–3720), $p < 0.01$]. Weight gain since birth was significantly higher in the ID group (mean age 10.8 months) than in the control group (mean age 10.4 months) [6825 g (6349–7301) *versus* 6288 g (6059–6516), $p < 0.05$]. The weight gain in infants with BW < 3000 g was not different within the ID and control group as well as from those with BW > 3000 g. The identified risk factors for ID are given in Table 6. Birthweight less than 3000 g was a significant risk factor for ID: odds ratio (OR) 9.4 (95% CI 2.7–33.0) ($p < 0.0005$). Twenty five out of 92 (27.2%) boys were iron deficient while only 10 (12.6%) of the 79 girls had ID. The boys had a tendency to have a lower ferritin than girls but the difference was statistically not significant [21 µg/L (17–25) *versus* 27 µg/L (22–32); $p < 1.0$]. Socio-economic variables such as age and educational level of the mother, living in urban or rural area did not have an effect on iron status.

Table 6. Different factors associated with the risk of ID (n=171).

Determinants	OR	95% CI	p
Birthweight < 3000 g	9.4	2.7–33.0	0.0003*
Male gender	2.2	0.9–5.3	0.058
Preterm	8.5	0.6–457.9	0.060
Urban area	1.1	0.4–3.2	0.80
Maternal education	1.4	0.7–3.2	1.00
Anaemia in pregnancy	0.8	0.3–1.9	0.68
Exclusive breast-feeding	1.5	0.7–3.2	0.09
Partly breast-feeding	3.8	1.7–8.8	0.005*
Introduction of solid food	0.9	0.4–2.1	0.16
Formula-feeding	0.7	0.6–0.9	0.01*
Cow's milk	1.6	0.7–3.7	0.24

*statistically significant factor, $p < 0.05$.
Exclusive breast-feeding: only breast-milk up to six months; partly breast-feeding: breast-feeding and solid food at the time of the study; introduction of solid food: before or at 6 months of age; formula-feeding: have received formula before the study; cow's milk: received cow's milk before and/or at 9 months.

In the analysis of feeding habits from questionnaires we found that 21% (36/171) of infants were exclusively breast-fed up to six months and 39.8% (68/171) were partly breast-fed ie. were fed with breast-milk and solid food at the time of the study. Duration of exclusive breast-feeding [3.6 months (2.9–4.7)] or duration of total breast-feeding [7.0 months (5.5–9.2)] was similar in the iron deficient group and in the control group. However, infants who were exclusively breast-fed until the age of 6 months had significantly lower Hb [113 g/L (111–115)] and ferritin [19 μg/L (15–22)] levels than infants who were exclusive breast-fed only until the age of 3 months [Hb 117 g (115–119)] and ferritin [28 μg/L (23–33)] (both $p < 0.05$). The same tendency was seen in Hb values between groups defined by the age of introducing solid food: the infants fed with solid food before 6 months had statistically higher Hb values than infants whom the solid food was introduced after 6 months of life [118 g/L (115–120) *versus* 114 g/L (110–117), respectively, $p < 0.05$)]. Infants fed with breast-milk and solid food had lower ferritin [18 μg/L (14–23)] than infants fed with formula and solid food [33 μg/L (27–39) ($p < 0.005$)]. Infants in whom meat was introduced before or at 7 months of age had the tendency for higher ferritin values than infants who were introduced to meat after 7 months [25 μg/L (21–28) *versus* 18 μg/L (12–25) respectively, $p < 0.1$]. Their MCV was statistically higher than in infants who were introduced to meat after 7 months of age [74 fL (72–76) versus 67 fL (64–68), $p < 0.05$]. Infants fed with formula had statistically higher ferritin value than infants without any formula-feeding [29 μg/L (22–34) *versus* 17 μg/L (14–20) respectively, $p < 0.005$].

Feeding infants with formula protected against developing ID [OR 0.7 (0.6–0.9), $p < 0.05$]. The ORs are seen in Table 6.

Nearly every third infant (56/171) was fed with cow's milk before 9 months of age, and 16 of those had ID. Their ferritin was lower compared to the infants who had not received cow's milk [19 µg/L (14–24) *versus* 26 µg/L (20–40), ($p < 0.05$)].

We suspected secondary IDA in 2 infants with poor weight gain, but no underlying cause was found.

3. The reference limits and cut-off values for soluble transferrin receptor (sTfR) (Study II)

The IDeA® method was used in 179 infants (92 boys/ 87 girls) to measure sTfR levels. Half of these children (90/ 179) also had it measured by the Tina-quant® method. Serum sTfR concentration was not normally distributed in either method. The mean, 5th and 95th percentiles, the 95% CI for mean and reference limits for sTfR in the IDeA® and in the Tina-quant® study groups are given in Table 7. There were no statistical differences in sTfR values between boys and girls ($p < 1.0$).

Table 7. The mean and 5th and 95th percentiles with 95% CI in brackets for Hb, MCV, serum ferritin, sTfR concentration measured by the IDeA® sTfR-IT and by the Tina-quant® sTfR methods in infants aged from 9 to 12 months.

Marker	Mean	5th and 95th percentiles
Hb (g/L) (n = 173)	114 (112–115)	101 (99–103) – 128 (127–130)
Ferritin (µg/L) (n = 175)	25 (21–27)	5 (3–7) – 55 (50–60)
MCV (fL) (n = 173)	73 (73–74)	68 (65–71) – 80 (79–82)
IDeA® sTfR-IT (mg/L) (n = 179)	2.2 (2.1–2.3)	1.5 (1.2–1.4) – 2.7 (2.5–2.7)
Tina-quant® sTfR (mg/L) (n = 90)	6.1 (5.8–6.4)	4.1 (3.2–4.1) – 7.8 (7.5–8.4)

Mean serum sTfR concentration was significantly lower in the healthy infant group than in the group of infants with ID and IDA measured either by the IDeA® method [2.0 mg/L (1.9–2.0) *versus* 3.0 mg/L (2.5–3.4), $p < 0.0001$] or by the Tina-quant® method [5.8 mg/L (5.5–6.1) and 7.0 mg/L (6.4–7.7), $p < 0.005$]. Mean serum sTfR concentrations measured by IDeA® was significantly lower 2.3 mg/L (2.1–2.4) ($p < 0.0001$) in the ID-only group compared

to the IDA group 4.4 mg/L (3.2–5.6) by the IDeA® method or 6.9 mg/L (6.3–7.5) *versus* 9.7 mg/L in the Tina-quant® method respectively.

We used an ROC curve to find the optimal cut-off value for the level of sTfR that could diagnose ID. There were 146 healthy infants and 33 children with ID for the IDeA® method and 80 healthy infants and 10 children with ID for the Tina-quant® method. We found that the optimal cut-off value to diagnose ID was 2.4 mg/L [sensitivity 84% (95% CI 64–94) and the specificity 94% (88–97)] for IDeA® sTfR-IT, and 7.4 mg/L [sensitivity 80% (44–97) and specificity 92% (83–96)] for Tina-quant® sTfR. There were no statistically significant differences in the corresponding areas under the curves (AUC^{ROC}) between the two methods (Paper II, Figure 1).

The mean MCV (95% CI) was significantly higher in infants with an sTfR level < 2.4 mg/L than those > 2.4 mg/L [75 fL (74–75) *versus* 68 fL (65–70), $p < 0.0001$] in IDeA® method.

The comparison of the two methods is given in Paper II, Figure 2. It is seen that the two methods give systematically different results, and that all the observations lie under the line of equality (Paper II, Figure 2a). The mean ratio with 95% limits of agreement (LOA) was 2.9 (2.4–3.6), showing the poor agreement of the two methods (Paper II, Figure 2b) *(Bland and Altman 1999)*.

4. Reference values and diagnostic characteristics for serum ferritin, mean red cell volume (MCV) and haemoglobin (Hb) to diagnose iron deficiency in infants (Study III)

The mean and reference limits of Hb, MCV and ferritin are given in Table 7. There were no significant differences in Hb values or iron indices between genders ($p < 1.0$). According to the ROC curve the optimal cut-off value to diagnose ID (defined as IDeA® sTfR-IT value > 2.4 mg/L) the Hb was less than 107 g/L, serum ferritin less than 10.9 µg/L and MCV less than 71 fL (Figure 3). The test with the highest efficacy was MCV < 71 fL which provided sensitivity of 86% (95% CI 80–92) and specificity of 83% (65–94). Serum ferritin < 10.9 µg/L provided sensitivity of 83% (76–88) and specificity of 80% (62–92). Haemoglobin less than 107 g/L showed the best specificity of 87% (80–91), but the sensitivity was only 67% (47–83). The difference of area under the ROC curves between Hb and ferritin was 0.13 ($p < 0.0001$) and between Hb and MCV 0.17 ($p < 0.0001$) indicating that Hb is less efficient than ferritin and MCV in the diagnosis of ID (Figure 3).

12

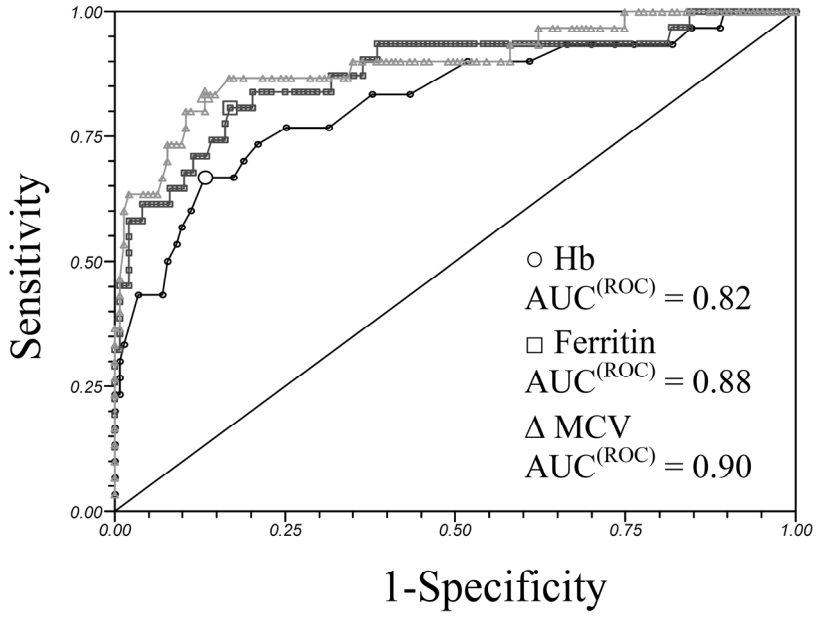

Figure 3. Receiver operating characteristic (ROC) curves for Hb, MCV and ferritin.

DISCUSSION

1. Prevalence of iron deficiency and iron deficiency anaemia (Study I)

This is the first population based epidemiological study to estimate the prevalence of ID and IDA in 9–12 month old Estonian infants. Iron deficiency, and specifically IDA, remains one of the most severe and important nutritional deficiencies in the world today *(WHO 2001)*. The terms anaemia, ID, and IDA are often used interchangeably, as ID is such a common cause of childhood anaemia *(JWHO and CDCPTC 2005)*. Therefore, the prevalence of ID and IDA is very different and often the differentiation between ID and IDA in some papers is not clear *(Morasso et al. 2003, Bounomo et al. 2005, Hopkins et al. 2007)*.

We found that the prevalence of ID was 23%, ID without anaemia 14% and IDA 9% in infants aged 9–12 months, which is disturbingly high.

The prevalence of ID (23%) in our study was higher than the average 7% of the 11 developed EU countries *(Male et al. 2001)*, in Sweden (18%) *(Lind et al. 2003)* and in Iceland (20%) *(Thorsdottir et al. 2003)*. The average prevalence of IDA has also been reported lower in the EU countries than in Estonia: in EU at around 2% *(Gregory et al. 1995, Male et al. 2001, Thorsdottir et al. 2003, Hopkins et al. 2007)*, in Scandinavian countries between 0–3% *(Michaelsen et al. 1995, Lind et al. 2003, Hay et al. 2004)* and 3% in US *(Looker et al. 1997)*. Even China has reported lower prevalence of IDA (7.8%) *(Zhu et al. 2005)* than our study group. However, the prevalence of IDA in Estonia was lower than in low-income countries such as Albania (51%) *(Buonomo et al. 2005),* Argentina (36%) *(Morasso et al. 2003)* and also lower than some socio-economically deprived areas of the United Kingdom *(Gregory et al. 1995, Booth and Aukett 1997, Hopkins et al. 2007)*. One reason for such a high prevalence of IDA in Estonia may be the fact that feeding with iron-fortified cereals is not common in Estonia. Feeding infants with cow's milk, particularly in rural areas, is still common practice. The second reason might be that Estonia does not have a direct programme of ID and IDA prevention in infants. Our results have shown that a prevention programme of ID and IDA is needed particularly in the primary health-care system. The value of such a programme for preventing IDA was shown in Denmark. In Danish infants aged 9 months the prevalence of ID and IDA was near to zero *(Michaelsen et al. 1995)* and ID in US was 6% *(Life Science Research Office 1984, Brotanek et al 2007)*.

The prevalence of iron sufficient anaemia in our study was 8.2%. It is known that the second most common cause of anaemia at this age is infection *(WHO 2001)*. A comparative study of the development of the immune response to allergens in Estonian which is lower than in Swedish children, showed that the frequency of respiratory illnesses during the first 2 years of life was

significantly higher in Estonian children (median range 6.2) than in Swedish children (3.6) *(Voor et al. 2005)*. This may explain why the prevalence iron sufficient anaemia in Estonia was quite high.

2. Causes of iron deficiency (Study I)

According to the results of our study infants with BW < 3000 g had a nearly 10 fold increased risk for developing ID. It is known that the majority of children born small for their gestational age (SGA) do catch up in weight and length during the first 2 years of life *(Fewtrell et al. 2001)*. Thus, the increased requirement for iron during this rapid period of growth may lead to ID *(Oski 1993)*. Indeed we found that infants with ID had lower BW, but gained significantly more weight than control babies. Morasso *et al. (2003)* also found relationship between low BW and ID in infants. In addition, a positive correlation between BW and serum ferritin concentration has been previously reported *(Male et al. 2001, Thorsdottir et al. 2003)*. Therefore, infants with small BW need primary prevention of ID. Gender apparently also plays an important role in the infant's iron status (*Domellöf et al. 2002c, Thorsdottir et al. 2003*). We found that boys had a tendency to have lower ferritin values than girls, but the difference was not statistically significant. Other authors have reported similar sex difference *(Male et al. 2001, Domellöf et al. 2002c, Thorsdottir et al. 2003)*.

Nearly 40% of our infants were partly breast-fed at the time of the study. Infants fed exclusively with breast-milk until the age of 6 months had lower Hb and serum ferritin levels than infants fed with breast-milk for a shorter period. Our study revealed interestingly that breast-fed infants with additional solid food at the age of 9–12 months had nearly 4 fold increased risk for developing ID. This appears to be contrary to the study carried out in Iceland, where infants with ID had shorter duration of only breast-feeding (5.3 months *versus* 7.9 in the iron sufficient infants) *(Thorsdottir et al. 2003)*. Introducting meat before 7 months of age had tendency to better the iron condition, especially for MCV at the age of 9–12 months. Therefore infants at least at the age of 6 months should be introduced to meat, which is traditional and implemented in Estonia. However, breast-fed infants with additional solid food had poor protections against developing ID, therefore changes may be required in composition of the complementary foods. According to our study formula-feeding have a positive effect on iron indices and is slightly protective against ID (OR 0.7, $p < 0.05$). Therefore, even for partly breast-fed infants iron rich formula or iron-fortified food should be recommended at least the age of 6 months.

It is known that early introduction of cow's milk is the main dietary risk factor for ID in infancy *(Oski 1993, Michaelsen et al. 1995, Booth and Aukett 1997, Male et al. 2001, Thorsdottir et al. 2003)*. We found that nearly every third child in out study was fed with cow's milk and not with formula before the

age of 9 months. We did not find that feeding with cow's milk is a risk factor for ID. However, infants who received cow's milk had significantly lower serum ferritin levels than those who had not received it.

3. The reference limits and cut-off values for soluble transferrin receptor (sTfR) (Study II)

We aimed to evaluate and compare sTfR normal reference values and cut-off values between the IDeA® and Tina-quant® reagents. There is no agreement for a specific cut-off value for serum ferritin for the diagnosis of ID. Several countries use the 5th centile of ferritin level as diagnostic value for ID (in GB <16 µg/L, in Finland <14 µg/L, WHO suggests <12 µg/L) *(Saarinen and Siimes 1978, Sherrif et al. 1999, WHO 2001)*. Most of the studies have established the reference values of ferritin in infants and young children by extrapolating it from children of older ages *(Domellöf et al. 2002b)*, or they have studied it in infants fed with infant formula or supplemented with iron *(Saarinen and Siimes 1978, Sherrif et al. 1999)*. Only Domellöf et al. *(2002b)* studied the diagnostic tests for IDA in exclusively breast-fed infants, and they suggested that the cut-off value for ferritin should be < 5 µg/L in 9-month-old infants. In our study a serum ferritin value < 10 µg/L was used as the cut-off value for the diagnosis of ID as published earlier *(Looker et al. 1997, INACG et al. 1998)*.

Although the diagnostic characteristics of the two kits in the diagnosis of ID were similar, the mean values were quite different: 2.2 mg/L in the IDeA® sTfR-IT method and 6.1 mg/L in the Tina-quant® sTfR method. The two methods had poor agreement and the Tina-quant® method differed 2.9 times with the IDeA® method. This difference might be due to the lack of a standardization of methods. The study results of Kolbe-Busch and their colleagues *(2002)* in healthy adults also showed that the numeric values of these methods differed 2.5 times. Therefore, it was necessary to determine the reference limits for both methods in children.

We also looked at the sTfR value between the ID and IDA groups and found that the mean value of sTfR was significantly higher in the IDA group than in the ID group, as expected. In a negative iron balance, functional ID is an intermediate dynamic stage restricted to the time it takes to develop anaemia, which appears when the majority of the normal RBCs have been replaced by hypochrome microcytes *(Oski 1993)*. It has been known that increased serum sTfR concentration is an early marker of functional ID and may help to prevent its development into *IDA (Cook et al.1996, Suominen et al. 2001, Angeles Vazquez Lopez et al. 2006)*. However, sTfR has consequently been introduced as an effective and specific measure of functional iron stores, since it correlates directly with the erythropoietic activity and inversely with the amount of iron

available for erythropoiesis *(Huebers et al. 1990, Beguin et al. 1993, Angeles Vazquez Lopez et al. 2006)*. Unlike ferritin, sTfR retains its specificity to changes in iron status and is thus responsive to acute-phase reactions *(Huebers et al. 1990, Skikne et al. 1990, Punnonen et al. 1997, Suominen et al. 2001)*. Our data confirms that serum sTfR concentration is a useful and sensitive test to diagnose ID. However, serum sTfR concentration is not specifically raised in functional ID, but also in haemolytic anaemia in both hereditary spherocytosis and in autoimmune anaemia or megaloblastic anaemia of cobalamin deficiency, α-thalassemia, β-thalassemia, and sickle cell anaemia. Serum sTfR is reduced in aplastic anaemia and following marrow ablation prior to bone marrow transplantation *(Kohgo et al. 1986, Huebers et al. 1990)*.

It is particularly important to prevent the development of IDA during brain growth and maturation which is fast during the first years of life. Sherriff et al. *(2001)* showed that infants with an Hb concentration below 90 g/L had significantly lower locomotor scores than those with a normal Hb value. Moreover, Akman et al. *(2004)* showed that not only IDA but also iron depletion in infancy may cause developmental delay in children. Therefore it is important to already diagnose functional ID. The turning point from prelatent ID to functional ID might be the sTfR value of 2.4 mg/L in the IDeA® sTfR method. We found that in infants with a sTfR level below 2.4 mg/L the MCV value was in normal ranges, but that if the sTfR level is higher the tendency of an abnormal MCV value was more common.

The mean serum sTfR concentration in IDeA® was lower in our infants than in previously published values for infants (mean 2.2 mg/L *versus* 2.7–4.5 mg/L) *(Reeves et al. 1984, Choi et al. 1999, Yeung and Zlotkin 1997)*. These differences can be explained by the different methodology used: our study included a more-restricted age group (9–12 months) compared to other authors (9–15 months, 4–24 months, 6 months–4 years) *(Reeves et al. 1984, Virtanen et al. 1999, Choi et al. 1999)*. However, some differences in values might be the result of national differences between populations (Estonian, Korean and Canadian) and food recommendations in different countries *(Choi et al. 1999, Yeung and Zlotkin 1997)*

To our knowledge, there have been no published reference values for sTfR in children measured by the Tina-quant® method. Available data published by the manufacturer *(Heil et al. 2004)* contain sTfR reference values for children (2.2–6.3 mg/L) obtained by Oriola IDeA sTfR kits *(Oriola OY, Espoo, Finland)*. The reference values for sTfR using Tina-quant® method in males and females in Estonia were similar to those published by the manufacturer – 1.9–4.4 mg/L *(Kolbe-Busch et al. 2002, Heil et al. 2004, Rohtla et al. 2006)*. Our data is in good correlation with the results of other authors *(Choi et al. 1999, Virtanen et al. 1999)*, who found that the sTfR values in children are higher than in adults. There were no statistical differences in sTfR concentration between boys and girls in our study, as was reported earlier *(Choi et al. 1999, Suominen et al. 2001,)*.

The weakness of our study was that it was a cross-sectional study design and not a prospective study. But on the other hand infants aged 9 to 12 months represent an age at a high risk of developing ID and IDA *(Aggett et al. 1989)*. This design was also selected for an ethical reason – to not take blood samples from "healthy" infants more than once; also, parents do not accept superfluous invasive procedures on their infants.

On the basis of our study we can conclude that the serum sTfR concentration is a good marker in the diagnosis of functional ID before IDA develops. The reference limits for sTfR concentrations as presented in Table 7 can recommend for infants aged 9–12 months in Estonia. The standardisation of sTfR assays when different methods are used is very important. Further studies are necessary to clarify the cut-off and reference values in different age groups of children.

4. The reference values for serum ferritin, mean cell volume (MCV) and haemoglobin (Hb) in infants (Study III)

This is the first study investigating iron status indices in 9–12 months old Estonian infants. WHO have produced the most widely used guidelines to diagnoses IDA *(WHO 2001)*. According to these data, the criteria for IDA in children up to 5 years of age must have Hb less than 110 g/L and ferritin less than 12 μg/L *(WHO 2001)*.

As there is no "golden standard" for ID, and ID itself is a continuously changing condition. It is very difficult to define the normal population and to establish reference values for Hb, ferritin, MCV or sTfR. In this study we decided to use sTfR concentrations above 2.4 mg/L to diagnose ID. In the remaining 150 healthy children we establish optimal cut-off values for Hb, MCV and serum ferritin concentrations.

Serum sTfR has been introduced as an effective and specific measure of functional iron stores, since it correlates directly with erythropoietic activity and inversely with the amount of iron available for erythropoiesis *(Huebers et al. 1990, Beguin et al. 1993, Angeles Vazquez Lopez et al. 2006)*. Unlike ferritin, sTfR retains its specificity to changes in iron status and is thus responsive to acute-phase reactions *(Huebers et al. 1990, Skikne et al. 1990, Punnonen et al. 1997, Suominen et al. 2001)*. Therefore, sTfR should be useful in the diagnostic criteria for ID. Unfortunately, our 95th percentile of sTfR in the IDeA® method was lower than the previously published cut-off value (3.3 mg/L) *(Suominen et al. 2001)*. Therefore reference limits for our own study group were needed. We calculated that in infants with non-hypoferritinaemia, a cut-off value of > 2.4 mg/L sTfR showed the best diagnostic efficacy in diagnosing ID (sensitivity 84% and specificity 94%).

We used the fifth and ninety-fifth percentiles to define appropriate reference limits as suggested by the WHO *(JWHO and CDCPTC 2004)*. The reference limits for serum ferritin, MCV and Hb as presented in Table 7 can be recommend for infants aged 9–12 months in Estonia. In our study group the 5th percentiles of ferritin, MCV, and Hb were lower than those mentioned above. The iron indices were similar in boys and girls. Several authors have suggested alternative cut-off values for Hb and ferritin in infants and young children *(Michaelsen et al. 1995, Milman 1996, Emond et al. 1996, Freeman et al. 1998, Domellöf et al. 2002b)*.

A study of Estonian adults *(Tamm et al. 2003)* has shown that women have lower Hb reference values than those suggested by WHO *(WHO 2001)*. The same study also showed that the extent of reference range for Hb in both men and women were smaller suggested by WHO *(WHO 2001)*.

The 5th percentile of serum ferritin value (5 µg/L) in our study was lower than that recommended by WHO (< 12 µg/L) *(WHO 2001)* or seen in other studies: in GB it is < 16 µg/L *(Sherrif et al. 1999)*, in Finland < 14 µg/L *(Saarinen and Siimes 1978)*. One of the reasons for lower ferritin values in our study may the fact 21% of our subjects were exclusively breast-fed up to 6 months and 40% were still partly breast-fed during the study. The 5th percentile for ferritin in our study (5 µg/L) is similar to the result of Domellöf et al. They have also shown that the cut-off value for ferritin to diagnose ID should be < 5 µg/L in a 9-month-old up to 6 months exclusively breast-fed infants. *(Domellöf et al. 2002b)*.

During the period of rapid growth the demand of systemic iron is increased. This makes the interpretation of ferritin concentration difficult in infants and young children *(Aggett et al. 2002)*. It is important to highlight that to diagnose ID appropriate reference values are needed separately for every age group *(JWHO and CDCPTC 2005)*. According to our results diagnosing ID based on serum ferritin and MCV levels would have shown better sensitivity and specificity than using Hb. The low efficacy of Hb was also demonstrated by Ullrich, et al. *(2005)* who preffered CHr. They concluded that using solely Hb as preventive screening tool for ID is less useful than CHr because the decline in the Hb level will develop during the relatively late stage of ID *(Oski 1993, Lee 1999, Wick et al. 2003, Ullrich et al. 2005)*.

Further studies are necessary to clarify the cut-off and reference values in different age groups of children.

5. Suggestions for preventing iron deficiency and iron deficiency anaemia in infants aged 9 to 12 months in Estonia

WHO has postulated that IDA should be considered as a public health problem if the prevalence of IDA exceeds 5.0% in the population *(WHO 2001)*. Using the now established cut-off values from our study of serum ferritin < 10.9 µg/L and MCV < 71 fL or sTfR > 2.4 mg/L to diagnose ID and Hb < 101 g/L to diagnose anaemia, the prevalence of ID and IDA are: using only ferritin 19% (33/175) of 9–12 months old infants in Estonia have ID and 6% (10/175) have IDA. Using MCV the figures are 18% (30/173) and 5% (10/173); using ferritin and MCV together gives 12% (22/173) and 5% (10/173). Using sTfR, the prevalence of ID is 13% (25/173) and IDA 5% 10/173). These methods show that the prevalence of ID is comparable using different tests for the diagnosis of ID.

Our data underlines that a prevention programme for ID and IDA in infants is needed in Estonia, particularly in the primary health-care system.

In primary prevention we should follow the well known recommendation of CDC and WHO *(CDC 1989, CDC 1998, WHO 2001)*. The results of our study show that we need to reappraise infants' feeding recommendation, especially the time of introducing solid foods and the supplementary diet in breast-fed infants.

One possible way to prevent ID and IDA is to start with an additional source of iron from complementary foods. For infants aged less than 12 months who are not breast-fed or who are partly breast-fed, it is essential that only iron-fortified infant formula be used as a substitute for breast-milk. When exclusive breast-feeding is stopped, the use of an additional source of iron (approximately 1 mg/kg per day) should be encouraged, preferably from complimentary foods. For breast-fed infants whose BW is less than 3000 g and who receive insufficient iron from supplementary foods by the age of 6 months (less than 1 mg/kg per day), we suggest 1 mg/kg of iron drops per day. We should encourage the introduction of solid foods i.e. pureed meats at the age of 6 months or when the infant is developmentally ready to consume such food. By around 6 months, encourage feeding one meal a day of foods rich in iron (eg., meat, iron-fortified cereals or formula) and it may be reasonable to also add iron-fortified cereals to breast-fed infants at 6 months, 200 ml infant formula or make the porridge with formula.

In secondary prevention the screening ID and IDA the infants at aged 9-12 months is needed in Estonia. We found that to diagnose ID serum ferritin and MCV should be used as they both had good sensitivity and specificity. As we use for diagnosis of ID MCV and ferritin together the sensitivity is 88% and specificity 85%.

14

For screening ID the serum ferritin < 10.9 μg/L and MCV < 71 fL may use for diagnosis of ID.

Our study found that Hb measures for diagnosing IDA had poor sensitivity and specificity, therefore using only Hb is not reasonable for screening IDA. It is important to underline IDA can lead to preventable developmental delays in infants.

CONCLUSION

1. The prevalence of iron deficiency without anaemia and iron deficiency anaemia in infants aged 9–12 months are 14% and 9% respectively. According to WHO criteria (IDA > 5%) iron deficiency anaemia is considered as public health problem in Estonia therefore the prevention of iron deficiency and iron deficiency anaemia is needed in Estonia.
2. The most important associated background factor for iron deficiency is birthweight less than 3000 g.
3. The reference values for infants aged 9 to 12 months in Estonia for sTfR are 1.5–2.7 mg/L in the IDeA® method and 4.1–7.8 mg/L in Tina-quant® sTfR method. The serum sTfR concentrations above 2.4 mg/L in the IDeA® method or 7.4 mg/L in the Tina-quant® sTfR method can be recommend for diagnosis iron deficiency in infants aged 9–12 months in Estonia.
4. The reference values in 9- to 12-months-old infants in Estonia for serum ferritin are 5–55 µg/L and for MCV are 68–80 fL. Serum ferritin value less than 10.9 µg/L and MCV less than 71 fL can be recommend for diagnosis iron deficiency in infants aged 9–12 months in Estonia.
5. The reference values for Hb are 101–128 g/L in infants aged 9–12 months living in Estonia. Hb concentrations are less sensitive for the diagnosis of iron deficiency and its use as single paramether for screening of iron deficiency should be avoided.

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15

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16

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17

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SUMMARY IN ESTONIAN

Rauapuudus ja rauapuudusaneemia 9–12 kuu vanustel imikutel Eestis

Kokkuvõte

Rauapuudusaneemia on lapseeas kõige sagedasem toitumisega seotud haigus kogu maailmas *(Aggett et al. 2002)*. Imikutel ja väikelastel on rauapuudusaneemia sagedaim põhjus kiire kasvuperioodi vajadusi mittekattev hemoglobiini (Hb) sünteesiks vajaliku raua vähesus või halb biosaadavus *(Aggett et al 1989, Oski 1993)*. Uuringud on näidanud, et rauavaegus põhjustab häireid erinevates organsüsteemides, pärsib rakulist immuunsust ning suurendab infektsioonhaigustesse jäämise riski *(Oski 1993, Booth and Aukett 1997)*. Rauapuudus koos väljakujunenud aneemiaga on kognitiivse funktsioonihäire riskiteguriks *(Lozoff et al. 1991, Yager and Hartfield 2002)*.

Rauapuudusaneemia kujuneb kolmes faasis. Esmalt väheneb depooraua hulk maksas, põrnas ja luuüdis. Teine faas on latentne rauapuudus ilma aneemiata, kuid transportraua hulk väheneb ja tekib rauadefitsiitne erütropoees. Kolmandas faasis kujuneb Hb vähenenud sünteesist tingituna rauapuudusaneemia *(Oski 1993, Wick et al. 2003)*.

Uuringud on näidanud, et arenenud riikides on rauapuudusaneemia levimus väga erinev (0–8%) (*Michaelsen et al. 1995, Male et al. 2001, Lind et al. 2003, Thorsdottir et al. 2003, Hay et al. 2004, Zhu et al. 2005, Hopkins et al. 2007, Brotanek et al. 2007*). Arengumaades on rauapuuduse levimus veelgi suurem (30–51%) (*Morasso et al. 2003, Buonomo et al. 2005*). Suur erinevus rauapuudusaneemia levimuses võib olla seotud diagnostiliste erinevustega. 1996. aastal Viljandi maakonnas ja Tallinnas teostatud uuringus saadi 3–4 aastaste laste hulgas aneemia levimuseks 18–45% *(Ilves-Annunziata et al. 2000)*, kuid uuringu andmetest ei saa teha mingit järeldust rauapuudusaneemia esinemise kohta.

Rauapuudusaneemia diagnostilisteks laboratoorseteks kriteeriumiteks on vähenenud Hb kontsentratsioon koos vähenenud erütrotsüüdi keskmise mahu (MCV), vähenenud seerumi ferritiini või suurenenud seerumis lahustuvate transferriini retseptorite (sTfR) kontsentratsiooniga *(Mast et al. 1998)*. Kahjuks puuduvad 9–12 kuu vanuste imikute rauapuuduse ja rauapuudusaneemia diagnoosimiseks kindlad kriteeriumid. „Kuldne standard" on invasiivne raua hulga määramine luuüdis, mis on harva kasutatav meetod täiskasvanutel ning veelgi raskemini kasutatav lastel *(Domellöf et al. 2002)*.

Eestis puuduvad andmed laste kõige levinuma aneemia sageduse ja põhjuste kohta, samuti pole ühtseid soovitusi, kas ja millal teha imikule ning väikelapsele vereanalüüs rauapuuduse ja rauapuudusaneemia avastamiseks. Samuti puuduvad meil Eestis lastel raua markerite ja Hb referentsvahemikud. Rauapuudus-

aneemia levimus on aga üks oluline laste tervise hinnangu marker, eriti hetkel, mil laste tervise hindamise on esmatasandi lastearstidelt üle võtnud perearstid.

Töö eesmärgid

1. Rauapuuduse ja rauapuudusaneemia levimuse hindamine 9–12 kuu vanustel imikutel Eestis.
2. Rauapuuduse ja rauapuudusaneemia riskitegurite leidmine 9–12 kuu vanustel imikutel Eestis.
3. Leida Eestis sobivad referentsvahemikud ja parimad diagnostilised väärtused (*cut-off value)* rauapuuduse diagnoosimiseks seerumi ferritiinile, MCV-le, Hb-le ja sTfR-le 9–12 kuu vanustel imikutel.

Uuritavad ja metoodika

Juhusliku valiku meetodil valiti üle kogu Eesti 7 maakonna perearstide nimistust iga teine 9 kuni 12 elukuu vanune imik (kokku 330 last). Lapsevanematele saadeti infokiri uuringu eesmärkidest ja ankeet imiku arengu-, kasvu-, tervisening toitmisandmete kohta. Kokku nõustus uuringus osalema 195 (65%) uuritavat. Imikult võeti vereanalüüs aneemia ja rauapuuduse markerite määramiseks: Hb, MCV, seerumi ferritiin, sTfR ja C– reaktiivne valk (CRV). Kokku osales epidemioloogilises uuringus 171 uuritavat. Aneemia kriteeriumiks oli Hb < 105 g/L ja rauapuuduse kriteeriumiks üheaegselt ferritiin < 12 µg/L ja MCV < 74 fL.

Tervetel ajalistel imikutel määrasime referentsvahemike piirid (5. ja 95. protsentiil). Parima diagnostilise väärtuse (*cut-off value*- väärtus, mis on parima tundlikkuse ja spetsiifilisusega) leidmiseks kasutasime ROC analüüsi, milles seerumi ferritiini, MCV ja Hb hindamisel oli rauapuuduse kriteeriumiks sTfR väärus > 2.4 mg/L (n = 25). Seerumi sTfR kontsentratsiooni määrasime 179 imikul vanuses 9–12 kuud, kasutades *IDeA*® ja *Tina-quant*® meetodeid. Rauapuuduse kriteeriumiks võtsime seerumi ferritiini väärtuse < 10 µg/L (n = 33).

Peamised tulemused

(I) Rauapuudus ilma aneemiata oli 14.0%-l (24/171) imikutest ja rauapuudusaneemia 9.4%-l (16/171) imikutest.

Peamiseks rauapuuduse põhjuseks oli sünnimass väiksem kui 3000 g (OR=9.4; p < 0.0005).

Toitumistavasid uurides leidsime, et 21% (36/171) imikutest olid kuni kuuenda elukuuni ainult rinnapiima toidul ja 40% (68/171) imikutest said uuringu ajal veel rinnapiima. Nendel lastel, kes uuringu ajal said lisaks lisatoidule rinnapiima, oli statistiliselt oluliselt väiksem ferritiini väärtus 18 µg/L (95% CI 14–23) kui imikutel, kes said piimasegu ja lisatoitu [ferritiini väärtus 33 µg/L (27–39) (p < 0.005)]. Meie uuringus selgus, et imiku toitmine

piimaseguga avaldas positiivset mõju raua markeritele, šansside suhe OR 0.7 (0.6-0.9). Liha lisamine lapse menüüsse enne 7. elukuud näitas küll tendentsi kõrgemale ferritiini väärtusele 25 µg/L (21-28), kuid võrdluses pärast 7. elukuud liha saanud lastega 18 µg/L (12–25), jäi tulemus statistiliselt mitteoluliseks ($p < 0.1$). Ferritiini väärtus enne 9. elukuud lehmapiima saavatel imikutel oli statistiliselt oluliselt väiksem kui samas vanuses lehmapiima mittesaanud lastel [19 µg/L (14–24) *versus* 26 µg/L (20–40) ($p < 0.05$)].

Eestis esineb rauapuudusaneemiat 9–12 kuu vanustel imikutel üsna sagedasti. Seega on Eestis vajalik rauapuuduse ja rauapuudusaneemia ennetus ja varase diagnoosimise programm esmatasandi arstidele. Rauapuuduse ennetamiseks on vajalik lapsevanemate nõustamine imiku õigest toitmisest: soovitada liha lisamist püreedesse, rauaga rikastatud putrusid või piimasegu k.a rinnapiima saavatele lastele alates 6. kuust ning vältida lehmapiima kasutamist esimesel eluaastal. Erilist tähelepanu tuleb rauapuuduse ja rauapuudusaneemia ennetamisel pöörata väikese sünnimassiga imikutele.

(II) Seerumi sTfR kontsentratsiooni referentsvahemik oli 1.5–2.7 mg/L määratuna *IDeA*® ja 4.1–7.8 mg/L *Tina-quant*® meetodil. Kahe meetodi vahel puudus kooskõla *(agreement),* keskmine väärtus koos 95% ühilduspiiridega (sulgudes) oli 2.9 (2.4–3.6). Rauapuuduse diagnostiliseks kriteeriumiks selles vanuserühmas oli sTfR väärtus > 2.4 mg/L *IDeA*® (tundlikkus 84% ja spetsiifilisus 94%) ning sTfR väärtus > 7.4 mg/L *Tina-quant*® meetodil (tundlikkus 80% ja spetsiifilisus 92%) .

(III) 9–12 kuu vanustel imikutel Eestis oli ferritiini keskmine väärtus 25 µg/L (referentsvahemik 5–55), MCV-l 73 fL (68–80) ja Hb-l 114 g/L (101–128). ROC analüüsil leitud parim diagnostiline väärtus rauapuuduse diagnoosimiseks oli seerumi ferritiini väärtus < 10.9 µg/L (tundlikkus 83% ja spetsiifilisus 80%), MCV < 71 fL (vastavalt 86% ja 83%) ning Hb < 107 g/L (vastavalt 67% ja 87%). Rauapuuduse ja rauapuudusaneemia diagnoosimisel on ferritiini ja MCV tundlikkus ning spetsiifilisus tunduvalt parem kui Hb-l.

Eestis on vajalik sõeltestida 9–12 kuu vanuseid imikuid, määrates Hb, MCV ja ferritiini väärtust.

Järeldused

1. Eestis oli 9–12 kuu vanustel imikutel rauapuudus ja rauapuudusaneemia vastavalt 23%-l ja 9%-l. Seega lähtuvalt Maailma Terviseorganisatsiooni kriteeriumitele (rauapuudusaneemia sagedus > 5%) on Eestis imikutel rauapuudusaneemia sage probleem.
2. Rauapuuduse peamiseks riskiteguriks on sünnimass väiksem kui 3000 g.
3. Eestis on seerumi sTfR referentsvahemik 9–12 kuu vanustel imikutel 1.5–2.7 mg/L *IDeA*® meetodil ja 4.1–7.8 mg/L *Tina-quant*® meetodil. Mõlemal meetodil oli rauapuuduse diagnoosimisel hea tundlikkus ja spetsiifilisus. Eestis on 9–12 kuu vanustel imikutel rauapuuduse

diagnostiliseks väärtuseks seerumi sTfR kontsentratsioon suurem kui 2.4 mg/L *IDeA*® meetodil ja 7.4 mg/L *Tina-quant*® meetodil.

4. Eestis on 9–12 kuu vanustel imikutel seerumi ferritiini referentsvahemik 5–55 µg/L ja MCV-l 68–80 fL. Rauapuuduse diagnostilised väärtused on seerumi ferritiini kontsentratsioon väiksem kui 10.9 µg/L ja MCV väiksem kui 71 fL.
5. Eestis on hemoglobiini referentsvahemik 9–12 kuu vanustel imikutel 101–128 g/L. Rauapuuduse diagnoosimisel on hemoglobiini kontsentratsioon madala efektiivsusega. Seega rauapuuduse sõeltestimisel tuleb vältida ainult Hb määramist.

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APPENDIXES

Appendix I. Questionnaire

The data analysis of the present study was based on the following questions:

- Environment
  - Do you live in a village or a town/city
- Parent's education: primary, basic (up to 15 year olds), secondary (up to 18 year olds), higher education
- Mother's pregnancy – anaemia
  - Have you had anaemia during pregnancy? If yes,
    - What was your haemoglobin level?
    - Have you received any iron supplementation? If yes, which product and how long?
- Nutritional advice
  - Have you received any advice about feeding your baby: from your family doctor, from a nurse, from a relative or from reading?
- Breast-feeding
  - When did you breast-feed your child first time: straight after birth / about 30 minutes after birth / later than 30 minutes after birth?
  - Do you presently breast-feed your child? If not, how old was your child when you stopped breast-feeding?
  - How long was your child receiving only breast-milk (with no added water, juice or tea)?
- Formula feeding
  - Has your child received any infant formula? If yes:
    - How old was your child when you started formula feeding?
    - Which formula do you use?
    - The volume of daily formula.
- Solid foods
  - How old was the child when you started to feed the child with solid foods?
  - Which kind of food was the first?
  - How old was the child when you started feeding:
    - Fruit
    - Vegetables
    - Meat
    - Cereal
    - Milk products
  - How often does your child eat fruit, vegetables, meat, charcuterie, cereal or milk products? Once a day / 2–3 times a day / once a week / 2–3 times a week. The amount of food in ml / g / pieces
- Cow's milk
  - Do you feed your child with cow's milk? If yes:
    - How old was your child when you started to give cow's milk?
    - If you started to feeding your child with cow's milk before 9 months of age then why? Not enough breast-milk and thinking that cow's milk is

19

good for your child / advice from your family doctor / formula milk is expensive / another reason?
    - How much does your child eat cow's milk and with which fat content: less than 2.5% / 2.5% / 3.5%?
- Vitamins / minerals
    - Has your child received any iron supplements? If yes, the name of product, how old was your child and how long were they receiving it?
    - Has your child received any vitamins? If yes, which vitamins and how old was your child when they received it?
- Health of the child
    - How often has your child had a cold, fever, ear infections, runny bowels, urinary tract infections? 0–6 or more times.
    - Has your child had any other illnesses in the last month?
    - Are you satisfied with your child's weight gain? Do you think it is normal or insufficient?

PUBLICATIONS

I

Vendt N, Grünberg H, Leedo S, Tillmann V, Talvik T. Prevalence and causes of iron deficiency anaemia in infants aged 9 to 12 months in Estonia. Medicina (Kaunas). 2007; 43(12):947–952.

II

Vendt N, Talvik T, Leedo S, Tomberg K, Kool P, Tillmann V, Grünberg H. The reference limits and cut-off value for serum soluble transferrin receptors for diagnosing iron deficiency in infants. International Journal of Laboratory Hematology. *(Accepted Feb. 25th 2008)*.

III

Vendt N, Talvik T, Kool P, Leedo S, Tomberg K, Tillmann V, Grünberg H.
Reference and cut-off values for serum ferritin, mean cell volume,
and haemoglobin to diagnose iron deficiency in infants aged 9 to 12 months.
Medicina (Kaunas). 2007; 43(9):698–702.

CURRICULUM VITAE

Neve Vendt

Date and Place of Birth: July 16th 1976, Tartu
Country of Citizenship: Estonian
Status: Married, husband Riho Vendt born in 1972. Children: Martin Vendt born in 2003 and Kristi Vendt born in 2007.
Address: Lunini 6, 51014 Tartu
Telephone: +3727319604
Fax: +3727319608
e-mail: Neve.Vendt@kliinikum.ee.

Education

2005–	University of Tartu, Faculty of Medicine, residency in anaesthesiology and intensive care
2001–	University of Tartu, Faculty of Medicine, PhD studies in paediatrics
2000–2001	County Hospital of Elva, internship in general medicine
1994–2000	University of Tartu, Faculty of Medicine
1991–1994	Hugo Treffneri Gymnasium
1983–1991	Ülenurme Secondary School

Professional employment

2001–2005	South Estonian Emergency Centre, adviser-doctor
1999–2000	County Hospital of Elva, nurse.

Special courses

2006	Intensive care of neonatology, Genova, Italy

Scientific work

2001–	University of Tartu, Department of Paediatrics Iron deficiency and iron deficiency anaemia in children.
2001–2003	Tartu University Hospital, Children's Clinic; Finn- Medi Research Ltd. Eligibility of infant formula
1999–2001	University of Tartu, Department of Paediatrics Feeding problems in early childhood.

A total of 10 publications, 4 CC, 1 publication in Estonian medical journal „Eesti Arst" and 5 presentations in scientific conferences.

Publications

1. Vendt N, Talvik T, Leedo S, Tomberg K, Kool P, Tillmann V, Grünberg H. The reference limits and cut-off value for serum soluble transferrin receptors for diagnosing iron deficiency in infants. International Journal of Laboratory Hematology. *(Accepted for publication onFeb. 25th 2008)*.
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4. Vendt N, Grünberg H, Tuure T, Malminiemi O, Wuolijoki E, Tillmann V, Sepp E, Korpela R. Growth during the first 6 months of life in infants using formula enriched with Lactobacillus rhamnosus GG: double-blind, randomized trial. Journal of Human Nutrition and Diet. 2006; 19(1):51–58.
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Membership of professional societies

2002–	Estonian Pediatric Association, member
2001–	Estonian Junior Medical Association, member
1999–2000	Estonian Medical Students Association, information secretary
1997–2000	Estonian Medical Students Association, member

Grant funding and support: PhD-student grant No DARLA 2295, TARLA 0475, TARLA 2695, Estonian Science Foundation grant No 6683.

Administrative responsibilities and other experiences

2000–2001	University of Tartu, Faculty of Medicine councillor, member
1999–2000	University of Tartu, Faculty of Medicine, information secretary

CURRICULUM VITAE

Neve Vendt

Sünniaeg ja koht: 16. juuli 1976, Tartu
Kodakondsus: Eesti
Perekonnaseis: abielus, abikaasa Riho Vendt sündinud 1972.
Lapsed: Martin Vendt sündinud 2003, Kristi Vendt sündinud 2007.
Aadress: Lunini 6, 51014 Tartu
Telefon: +3727319604
Faks: +3727319608
e-post: Neve.Vendt@kliinikum.ee

Haridus

2005–	Tartu Ülikool, arstiteaduskond, anestesioloogia- ja intensiivravi residentuur
2001–	Tartu Ülikool, arstiteaduskond, doktoriõpe
2000–2001	Elva Haigla MTÜ, üldarstlik internatuur
1994–2000	Tartu Ülikool, arstiteaduskond, arstiteaduse põhiõpe
1991–1994	Hugo Treffneri Gümnaasium
1983–1991	Ülenurme Keskkool

Teenistuskäik

2001–2001	Lõuna-Eesti Häirekeskus, arst-nõustaja
1999–2000	Elva Haigla MTÜ, meditsiiniõde

Erialane enesetäiendus

2006	Vastsündinute intensiivravi, Genova, Itaalia

Teadustegevus

2001–	Tartu Ülikooli lastekliinik Rauapuudus ja rauapuudusaneemia lastel.
2001–2003	SA Tartu Ülikooli Kliinikum, lastekliinik; Finn- Medi Research Ltd. Ravimuuring: Imiku piimasegu taluvus.
1999–2001	Tartu Ülikooli lastekliinik Imikute ja väikelaste toitumine Eestis.

Kokku ilmunud 10 publikatsiooni, neist 4 rahvusvahelistes ajakirjades, 1 ajakirjas „Eesti Arst” ja 5 konverentsi teesidena.

Publikatsioonid

1. Vendt N, Talvik T, Leedo S, Tomberg K, Kool P, Tillmann V, Grünberg H. The reference limits and cut-off value for serum soluble transferrin receptors for diagnosing iron deficiency in infants. International Journal of Laboratory Hematology. *(Publitseerimiseks vastu võetud 25. veebr. 2008)*.
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Muu teaduslik organisatsiooniline ja erialane tegevus

2002– Eesti Lastearstide Selts, liige
2001– Eesti Nooremarstide Ühendus, liige
1999–2000 Eesti Arstiteadusüliõpilaste Selts, infosekretär
1997–2000 Eesti Arstiteadusüliõpilaste Selts, liige

Uurimistoetused ja lepingud: doktorantide sihtfinantseerimine DARLA 2295, sihtfinantseerimine TARLA 0475, TARLA 2695, ETF grant 6683.

Administratiivtöö ja muud kohustused

2000–2001 Tartu Ülikool, arstiteaduskonna nõukogu, liige
1999–2000 Tartu Ülikool, arstiteaduskond, pressisekretär

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