KUOPION YLIOPISTON JULKAISUJA D. LÄÄKETIEDE 436
KUOPIO UNIVERSITY PUBLICATIONS D. MEDICAL SCIENCES 436

MARI ERVASTI

Evaluation of Iron Status Using Methods Based on the Features of Red Blood Cells and Reticulocytes

Doctoral dissertation

To be presented by permission of the Faculty of Medicine of the University of Kuopio for public examination in Auditorium ML1, Medistudia building, University of Kuopio, on Friday 13th June 2008, at 12 noon

Institute of Clinical Medicine
Department of Clinical Chemistry
Department of Obstetrics and Gynecology
University of Kuopio

KUOPION YLIOPISTO

KUOPIO 2008

Distributor: Kuopio University Library
P.O. Box 1627
FI-70211 KUOPIO
FINLAND
Tel. +358 17 163 430
Fax +358 17 163 410
www.uku.fi/kirjasto/julkaisutoiminta/julkmyyn.html

Series Editors: Professor Esko Alhava, M.D., Ph.D.
Institute of Clinical Medicine, Department of Surgery

Professor Raimo Sulkava, M.D., Ph.D.
School of Public Health and Clinical Nutrition

Professor Markku Tammi, M.D., Ph.D.
Institute of Biomedicine, Department of Anatomy

Author´s address: Institute of Clinical Medicine
Department of Clinical Chemistry
University of Kuopio
Eastern Finland Laboratory Centre
P.O. Box 1700
FI-70211 KUOPIO
FINLAND
Tel. +358 44 717 8716
Fax +358 17 173 200
E-mail: mari.ervasti@uku.fi

Supervisors: Professor Kari Punnonen, M.D., Ph.D.
Institute of Clinical Medicine
Department of Clinical Chemistry
University of Kuopio

Professor Seppo Heinonen, M.D., Ph.D.
Institute of Clinical Medicine
Department of Obstetrics & Gynecology
University of Kuopio

Reviewers: Professor h.c. Kari Teramo, M.D., Ph.D.
Department of Obstetrics & Gynecology
University Central Hospital, Helsinki

Professor Lothar Thomas, M.D., Ph.D.
Laboratoriumsmedizin
Krankenhaus Nodwest GmbH, Frankfurt/Main, Germany

Opponent: Professor Seppo Parkkila, M.D., Ph.D.
School of Medicine
University of Tampere

ISBN 978-951-27-0956-4
ISBN 978-951-27-1053-9 (PDF)
ISSN 1235-0303

Kopijyvä
Kuopio 2008
Finland

Ervasti, Mari. Evaluation of Iron Status Using Methods Based on the Features of Red Blood Cells and Reticulocytes. Kuopio University Publications D. Medical Sciences 436. 2008. 104 p.
ISBN 978-951-27-0956-4
ISBN 978-951-27-1053-9 (PDF)
ISSN 1235-0303

ABSTRACT

Iron is an essential element in the hemoglobin (Hb), which carry oxygen into the tissues. A shortage of iron causes not only anemia but also disturbances in children's development. Furthermore, iron overload and high iron stores have been associated with a variety of diseases such as hemochromatosis, Type II diabetes mellitus, gestational diabetes mellitus, gestational hypertension and increased risk of acute myocardial infarction. Erythropoietin (EPO) is the principal hormonal stimulator of red blood cell (RBC) production, and EPO synthesis is stimulated in a response to tissue hypoxia.

Laboratory measurements of iron status include both RBC indices reflecting the hematological iron compartment, and biochemical measurements reflecting the store and transferring iron compartments. The development of flow cytometric technique has produced more accurate cell indices reflecting the Hb content of RBCs and reticulocytes.

In the present series of studies, the aims were to investigate the diagnostic markers of iron status, especially the parameters reflecting the features of RBC and reticulocytes, in a cross-sectional population of pregnant women at term and their newborn infants (n = 220). Additionally, a new quantitative flow cytometric method for transferrin receptor (TfR) expression on reticulocytes (reflecting the iron requirement of cells) was developed and tested in a selectively chosen patient group (n = 46). The relationships between markers of hypoxia (EPO and pH), RBC and reticulocyte indices, and serum iron status measurements were also investigated.

On the basis of these studies, cell indices reflecting lower amounts of cellular Hb are the most practical way to evaluate iron deficiency in pregnant women at term, and they have the highest potential diagnostic accuracy. In cord blood, both accelerated erythropoiesis and the magnitude of iron stores contribute to the RBC and reticulocyte indices, thus impairing their value as specific indicators of iron deficiency. TfR expression on reticulocytes can be measured using a quantitative flow cytometric method, and it was shown that in patients with increased demand for iron, TfR expression is higher on reticulocytes than in controls. While the state of anemia is a contributor to the reduced oxygen-carrying capacity, the decreased amount of cellular Hb was also associated with suboptimal tissue oxygenation (higher EPO concentration and lower pH level) in pregnant women and in cord blood at birth.

These studies show that parameters reflecting the properties of the cells in the hematological iron compartment can be used as diagnostic markers for iron deficiency, using techniques that are available in modern hematological cell counters. More clinical studies are needed to confirm these findings but it seems possible that the RBC and reticulocyte indices may allow us to move from screening iron deficiency by Hb to more sensitive and rapid indicators of iron deficiency.

National Library of Medicine Classification: QV 183, WQ 252, WH 150, WH 155, WH 400
Medical Subject Headings: Anemia, Iron-Deficiency; Biological Markers/blood; Blood Cell Count/methods; Case-Control Studies; Erythrocytes; Erythropoiesis; Female; Fetal Blood; Flow Cytometry/methods; Hemoglobins; Humans; Infant, Newborn; Iron/blood; Iron/metabolism; Pregnancy; Receptors, Transferrin; Reticulocytes; Transferrin

Ervasti, Mari. Rautatasapainon arvioiminen punasolujen ja retikulosyyttien ominaisuuksista mitattavien parametrien avulla. Kuopion yliopiston julkaisuja D. Lääketiede 436. 2008. 104 s.
ISBN 978-951-27-0956-4
ISBN 978-951-27-1053-9 (PDF)
ISSN 1235-0303

TIIVISTELMÄ

Punasolujen hemoglobiinissa oleva rauta on elintärkeä alkuaine, sillä happi kulkeutuu siihen sitoutuneena kudoksiin. Raudanpuute voi aiheuttaa mm. anemiaa ja häiriöitä lapsen normaaliin kasvuun. Toisaalta liiallinen rautamäärä ja suuret rautavarastot on yhdistetty lukuisiin sairauksiin, kuten hemokromatoosiin, tyypin II diabetekseen, raskauden aikaiseen diabetekseen, raskauden aikaiseen verenpaineen nousuun ja kohonneeseen akuutin sydäninfarktin riskiin. Erytropoietiini (EPO) on pääasiallinen punasolujen muodostusta säätelevä hormoni, jonka tuotanto lisääntyy kudosten hapenpuutteessa.

Elimistön rautatasapainoa voidaan arvioida monien laboratoriotutkimusten avulla. Soluindeksit kuvastavat punasolumuodostuksessa käytettävän raudan määrää. Seerumista mitattavat biokemialliset tutkimukset kuvastavat mm. rautavarastoja (ferritiini) ja raudankuljetusta (transferriini). Virtaussytometrisen tekniikan kehittyminen on mahdollistanut entistä tarkemman solujen ominaisuuksien mittaamisen. Uudemmat soluindeksit kuvaavat solujen sisältämän hemoglobiinin määrää ja niiden on todettu olevan raudanpuutteisen punasolutuotannon osoittajia.

Tämän tutkimuksen tavoitteena oli tutkia rautatasapainon diagnostisia mittareita ja erityisesti parametreja, jotka kuvastavat punasolujen ja niiden esiasteiden, retikulosyyttien, ominaisuuksia. Tutkimusaineistona olivat synnyttämään tulleilta naisilta ja heidän vastasyntyneiden lastensa napalaskimosta otetut verinäytteet (n = 220). Tässä tutkimuksessa tutkittiin lisäksi hapenpuutetta osoittavien laboratoriotutkimusten [EPO pitoisuuden ja pH tason (n = 67 napalaskimomittausta)] ja rautatasapainoa kuvaavien laboratoriotutkimusten, ja erityisesti punasolu- ja retikulosyytti-indeksien välistä yhteyttä. Tutkimuksessa kehitettiin myös uusi virtaussytometrinen menetelmä retikulosyyttien pinnalla olevien transferriinireseptorien (TfR) (TfR kuvaa solujen raudan tarvetta) määrän arvioimiseksi. Menetelmää tutkittiin valikoidulla potilasaineistolla (n = 46) ja kontrollihenkilöillä (n = 12).

Näiden tutkimusten perusteella alentunutta solujen hemoglobiinisisältöä heijastavilla soluindekseillä on suurin diagnostinen tarkkuus synnyttämään tulleiden naisten raudanpuutteen osoittamisessa. Vastasyntyneen napaverestä mitattuihin punasolu- ja retikulosyytti-indekseihin vaikuttavat sekä kiihtynyt punasolutuotanto että elimistössä olevan raudan määrä, joten punasolu- ja retikulosyytti-indeksejä ei voida käyttää spesifisenä raudanpuutteen osoittajana. Anemia vähentää hapen kuljetuskapasiteettia, mutta näiden tutkimusten perusteella myös vähentynyt solujen hemoglobiinipitoisuus oli yhteydessä alentuneeseen kudosten hapettumiseen (korkeampi EPO pitoisuus, matalampi pH taso) synnyttämään tulleiden naisten näytteissä ja heidän lapsiltaan otetuissa napaverinäytteissä. Retikulosyyttien solupinnan TfR:n määrän mittaaminen on mahdollista kvantitatiivisen virtaussytometrian avulla. Potilailla, joilla oli lisääntynyt raudantarve, havaittiin retikulosyyttien pinnalla olevan enemmän TfR:ja kuin kontrollihenkilöillä.

Solujen ominaisuuksia kuvastavia parametreja voidaan käyttää raudanpuutteen diagnostisina mittareina. Tekniikat ovat nykyään käytössä moderneissa solulaskijoissa. Kliinisiä tutkimuksia tarvitaan lisää varmistamaan näitä löydöksiä, mutta näyttäisi siltä, että punasolu- ja retikulosyytti-indeksit mahdollistavat katseen kääntämisen raudanpuutteen seulonnassa pelkästä hemoglobiinin mittaamisesta herkempiin ja nopeampiin raudanpuutteen osoittajiin.

To my dear family

ACKNOWLEDGEMENTS

This study was carried out at the Institute of Clinical Medicine, Kuopio University, in collaboration with the Department of Clinical Chemistry and the Department of Obstetrics and Gynecology.

I express my deepest gratitude and respect to my principal supervisor, Professor Kari Punnonen, MD, PhD. I wish to thank him not only for his tireless and enthusiastic support during all phases of my research, but also for his warm humanity and never-ending expert guidance in my journey to the scientific world. I have always been able to count on his professional knowledge and it has been a great privilege to work under his guidance. I truly admire his optimism and unfaltering confidence in my work.

I want to express my special gratitude to my second supervisor, Professor Seppo Heinonen, MD, PhD. I feel that I have been fortunate to work with him. I am impressed by his direct way of thinking and rapid expert responses to all my questions and comments. He has had always time to advise me. Additionally, his continuous interest in my research and manuscripts have supported and encouraged me during all these years.

I am thankful to Professor Kari Pulkki, MD, PhD, whose support has been invaluable during the latest months of this work, and I look forward to working with him on many more successful and interesting studies in the future.

I am grateful to Docent Irma Matinlauri, MD, PhD. She introduced me to the world of flow cytometry and the hematology during these years. All her experience has guided me to successive methodological studies.

I owe my sincere thanks to neonatologist Ulla Sankilampi, MD, PhD, for her insights, counsel and constructive criticism about my studies on children. Her great experience opened up new important aspects to our work.

I am also grateful to my co-author, Sanna Kotisaari, MD, who has done great work in planning these studies.

I am grateful to the official reviewers of this thesis, Professor Lothar Thomas, MD, PhD and Professor Kari Teramo, MD, PhD, for their constructive criticism and valuable suggestions for the improvement of this thesis.

I am deeply grateful to my workmates Pirkko Lammi, MD, and Eija Mahlamäki, MD, for their advice with technical aspects of flow cytometry and cell counters. They have also been my tireless teachers during my years in the laboratory. I also want to thank warmly Susanna Luukkonen, MD, Annely Miettinen, MD and Weronika Delezuch, MD for their kind support throughout this work.

I thank laboratory technician Raija Isomäki for her help in analyzing the samples and guiding me through the processes in the laboratory. Additionally, I want to thank her warmly for all her support and encouragement during these years.

I owe my sincere thanks to the laboratory technicians of flow cytometry Riikka Juola, Tarja Kauppinen, Esko Leskinen, and Merja Minkkinen, for their helpful teaching about the analysis of samples.

I also want to thank sincerely my excellent colleagues and my chemist workmates in the laboratory for their invaluable help and for making the work enjoyable.

I wish to thank the personnel of the Department of Clinical Chemistry and Hematology and the Department of Obstetrics and Gynecology of the Kuopio University Hospital for their help in collecting and processing the studied samples. I also wish to express my warm thanks to Olavi Kauhanen, who provided me with important data from the Haikara database. Cordial thanks go also to all the subjects who participated in these studies.

I am deeply grateful to everyone who revised the language of this thesis and of my manuscripts, and special thanks go to Vivian Paganuzzi.

Finally, I express my heartfelt thanks to my dear parents Leena and Pertti, to my sisters, Laura, Lotta and Matleena, and to my brothers Kalle, Pekka, Jussi, Olli, Matti and Juho for their endless love, care and understanding throughout my life. They have always been there for me and shared the moments of despair and the days of success. I gratefully thank especially my father, who never ceased to believe that I could accomplish something great in the scientific field. I also warmly thank my grandmother Sirkka, sisters-in-law Tytti and Mirjami, and my brothers-in-law Ville, Matti and Touko for their encourage and support. All my nieces Viola, Vilma, Minea, Aliisa and Marikki and nephews Johan, Erik, Henrik, Eemeli, Niklas, Nooa and Rasmus have been great delights to me all their lives. I owe many warm thanks to my friends Maija, Hanna-Leena, Sari, Salla, Heidi, Leena, Elli, Anna-Maria, Susanna, Pauliina, Anniina, Eerika and Heppu for their support and encouragement, and for the many happy moments I have shared with them. I also wish to thank all my other relatives for their constant support. Special thanks go to my godmother Tellu, who encouraged me to study medicine.

This work has been financially supported by grants from the Foundation for the Promotion of Laboratory Medicine, the Pohjois-Savo Kidney and Liver Patient Association and the Finnish Association of Haematology.

Kuopio, May 2008

Mari Ervasti

ABBREVIATIONS

ABC	antibody binding capacity
AUC	area under the receiver operating characteristic curve
BFU	burst forming unit
CD71	transferrin receptor
CFU	colony forming unit
CHm	cellular hemoglobin in mature red blood cells
CHr	cellular hemoglobin in reticulocytes
CV%	coefficient of variation
EPO	erythropoietin
FCM	flow cytometer
Fe	iron
FID	functional iron deficiency
FID + ID	functional iron deficiency and iron deficiency
FITC	fluorescein isothiocyanate
Hb	hemoglobin
HbA	hemoglobin A
HbF	fetal hemoglobin
HCT	hematocrit
HIF	hypoxia inducible factor
hsCRP	high sensitivity C-reactive protein
%HYPOm	percentage of hypochromic mature red blood cells
%HYPOr	percentage of hypochromic reticulocytes
IQR	interquartile range
IRE	iron-responsive element
IRF	immature reticulocyte fraction
IRF-H	high immature reticulocyte fraction
IRP	iron regulatory protein
MCH	mean cell hemoglobin
MCHC	mean cell hemoglobin concentration
MCV	mean cell volume of red blood cells
MCVr	mean cell volume of reticulocytes
MESF	molecules equivalent surface fluorochrome
mRNA	messenger ribonucleic acid
NORIP	Nordic Reference Interval Project
PBS	phosphate buffered saline
PE	phycoerythrin
QSC	Quantum™ Simply Cellular®
RBC	red blood cell
RBCHe	red blood cell hemoglobin equivalent
RDW	red cell distribution width
RetHe	reticulocyte hemoglobin equivalent
%Retic	percentage of reticulocytes
rHuEPO	recombinant human erythropoietin
RNA	ribonucleic acid

ROC	receiver operating characteristic
SGA	small for gestational age
sTfR	soluble transferrin receptor
TIBC	total iron binding capacity
TfR	transferrin receptor
TfR-F Index	sTfR / Log Ferritin
%TfR^{+}Ret	percentage of transferrin receptor positive reticulocytes
TfSat	transferrin saturation
TO	thiazole orange
WHO	World Health Organization
ZnPP	zinc protoporphyrin

LIST OF ORIGINAL PUBLICATIONS

I Ervasti M, Kotisaari S, Heinonen S, Punnonen K. Use of advanced red blood cell and reticulocyte indices improves the accuracy in diagnosing iron deficiency in pregnant women at term. Eur J Haematol 2007;79:539-545.

II Ervasti M, Kotisaari S, Sankilampi U, Heinonen S, Punnonen K. The relationship between red blood cell and reticulocyte indices and serum markers of iron status in the cord blood of newborns. Clin Chem Lab Med 2007;45(8):1000-1003.

III Ervasti M, Kotisaari S, Heinonen S, Punnonen K. Elevated serum erythropoietin concentration is associated with coordinated changes in red blood cell and reticulocyte indices of pregnant women at term. Scand J Clin Lab Invest 2008;68(2):160-165.

IV Ervasti M, Sankilampi U, Heinonen S, Punnonen K. Novel red cell indices indicating reduced availability of iron are associated with high erythropoietin concentration and low pH level in the venous cord blood of newborns. Pediatr Res (Article in press).

V Ervasti M, Matinlauri I, Punnonen K. Quantitative flow cytometric analysis of transferrin receptor expression on reticulocytes. Clin Chim Acta 2007;383(1-2):153-7.

The original publications (I-V) have been reproduced with the permission of the publishers. In addition, some unpublished results are presented.

CONTENTS

1. INTRODUCTION

Iron is an essential element in hemoglobin (Hb) in red blood cells (RBC), which carry oxygen into the tissues. A shortage of iron causes not only anemia but also detrimental disturbances in children's development (Tamura *et al*, 2002). On the other hand, an excess of iron has toxic effects due to the induction of oxidative stress, which may be of clinical significance in patients who have hemochromatosis or frequent transfusions (Parkkila, 2000). Furthermore, iron overload and large iron stores have been associated with Type II diabetes mellitus, gestational diabetes mellitus, gestational hypertension and increased risk of acute myocardial infarction (Tuomainen *et al*, 1998; Fernández-Real *et al*, 2002).

Since iron homeostasis plays a crucial role in human life, iron deficiency or iron overload need accurate laboratory analyses already before diseases become manifest. Clinical practice needs simple, straightforward, and cost-effective methods for confirming diagnoses. Conventional laboratory measurements of iron status include both red cell indices reflecting hematological iron compartment and a panel of biochemical measurements, such as serum iron, transferrin, ferritin and transferrin receptor (TfR) reflecting storage and transport iron compartments, and availability of iron (Guyatt *et al*, 1992; Punnonen *et al*, 1997; Brugnara, 2000; Thomas *et al*, 2002). Conventionally used iron status measurements have limitations that may diminish their diagnostic value. In particular, it is challenging to assess the iron status of pregnant women who have ongoing pregnancy specific alterations such as hemodilution and mobilization of iron stores. The development of flow cytometric techniques has allowed the reporting of novel cellular indices along with traditional indices (Brugnara, 2000). These indices reflect more accurately the features of RBCs and reticulocytes, and they have been established to be indicators of iron deficient erythropoiesis.

Erythropoietin (EPO) is the principal hormonal stimulator of erythropoiesis, and EPO synthesis is stimulated as a response to tissue hypoxia (Egrie *et al*, 1985). In severe hypoxia, EPO production increases in a few hours in order to sustain a sufficient amount of Hb in blood circulation as well as to protect the brain and other tissues. EPO

concentrations have also been shown to be associated with iron status parameters (Milman *et al*, 1997).

In the present series of studies, the primary aim was to determine the diagnostic tests of iron status including both the serum markers and the RBC and reticulocyte indices in pregnant women and in cord blood at birth. The second aim was to evaluate the associations between iron status measurements and EPO concentration in these subjects. Furthermore, the association with umbilical vein blood pH and iron status measurements was evaluated in a subgroup consisting of newborn infants with clinical indications for pH measurement. The third aim was to develop the flow cytometric method for quantitative assessment of TfR expression on reticulocytes.

2. REVIEW OF THE LITERATURE

2.1. Body iron distribution and systemic iron homeostasis

Iron (Fe) is vital for all cells in the human body. It is especially needed in hemoglobin (Hb) in red blood cells (RBC), as well as in the myoglobin of muscles and in many cellular enzymes. A systemic homeostasis of iron is under continuously changing balance occurring in many locations of the human body (**FIGURE 1**). The total amount of iron in the human body is about 3.5–4 g in women and 4–5 g in men. The functional iron compartment (80 %) consists of the iron in the hematological compartment (RBCs, erythroid precursors) (app. 2 800 mg, 65 %), in myoglobin (app. 400 mg, 10 %) and in enzymes (app. 200 mg, 5 %) (Wick *et al*, 2000).

Approximately 1–2 mg iron ions per day is absorbed in the upper duodenum (**FIGURE 1**) (Wick *et al*, 2000). In general, the absorption can be increased when the need for iron increases (Whittager *et al*, 1991; Conrad *et al*, 2002). From the apical side of the intestinal cells, iron bounds to circulating transferrin that delivers two iron ions via the bloodstream to tissues. Circulating transferrin-iron complex constitutes the transport iron compartment, which contains a minor part of the body's total iron reserve (2–6 mg) (**FIGURE 1**). Usually, serum iron is bound to transferrin, but if iron-binding capacity is fully saturated, non-transferrin bound iron can be found in serum. The free iron is rapidly eliminated from blood circulation to cells.

Approximately 25 mg of iron is transmitted daily into the hematological compartment for the utilization of iron into the Hb of developing RBCs in erythropoiesis (**FIGURE 1**). Similarly, a total of 25 mg iron per day is released from RBCs after decomposition and obtained by macrophages of the reticuloendothelial system in the spleen (minor amounts also in liver or in bone marrow). Thereafter iron can be reutilized or it is transferred to the storage compartment. The total stored iron compartment (20 %) contains the iron that is stored as forms of ferritin and hemosiderin molecules in deposits of macrophages of reticuloendothelial cells (app. 400 mg), in bone marrow or in the spleen, and in hepatocytes (app. 400 mg) in liver (**FIGURE 1**). Furthermore, about 125 mg of iron is located in erythroid cells of bone marrow. The human body is unable to excrete iron actively, but iron is lost during menstruation or in

other hemorrhages. Minor amounts of iron (1–2 mg per day) are excreted via feces, by desquamation and by perspiration.

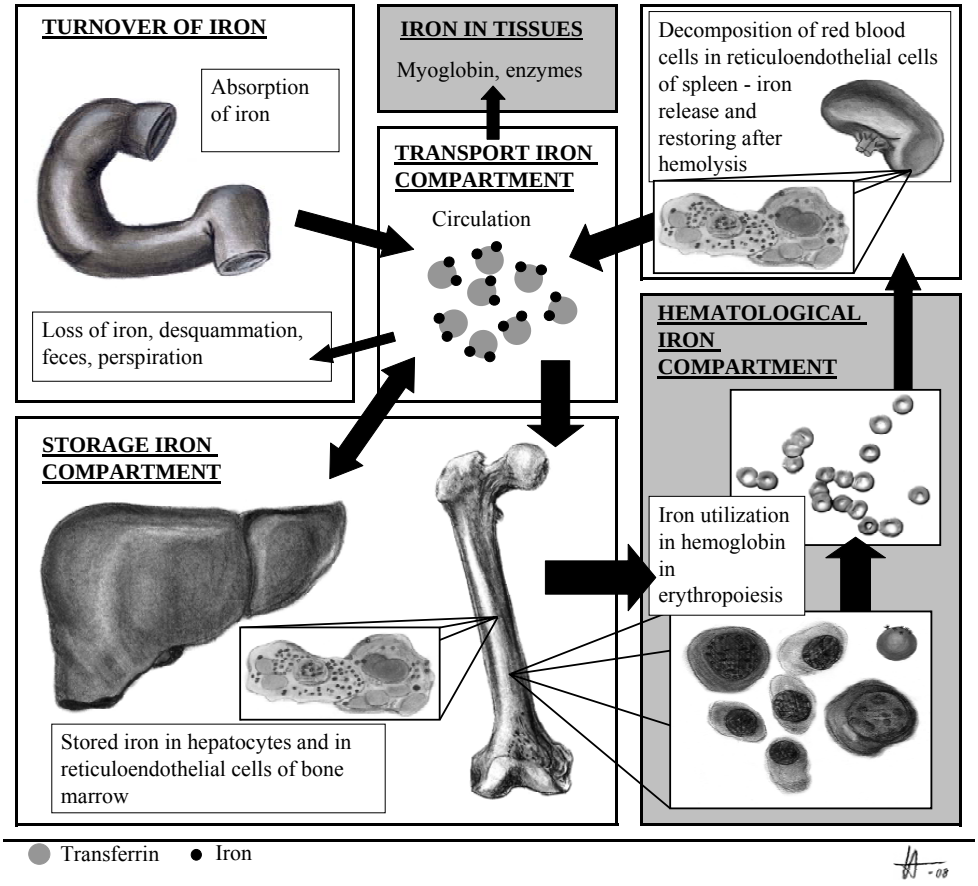


FIGURE 1 Schematic presentation of systemic iron homeostasis in the human body containing storage, transport and hematological iron compartments. Grey areas represent the active iron.

2.2. Iron uptake into the cells by transferrin receptor

Transferrin receptor (TfR) is a dimeric cell surface protein (molecular mass 190 kDa) involved in the internalisation of iron into the cells (Kohgo *et al*, 1986; Ward, 1987; Shih *et al*, 1990). The expression of TfR has been found in all proliferating cells and tissues needing iron, e.g. reticulocytes, activated lymphocytes, monocytes, macrophages, skin, liver, pancreas, pituitary, testis and placenta, as well as in malignant

cells (Jandl *et al*, 1963; Gatter *et al*, 1983). The cell surface expression of TfR is by far most abundant in proliferating erythroid precursors requiring the greatest amounts of iron (Kohgo *et al*, 1987), the goal being to sustain constant Hb synthesis. The expression of TfR is maintained during all stages of the maturing erythroid cells until the latest phase, achieving the stage of reticulocytes (Loken *et al*, 1987; Okumura *et al*, 1992; Sposi *et al*, 2000). Importantly, the more immature the reticulocytes are, the stronger is the TfR expression on the cell surface (Loken *et al*, 1987; Serke *et al*, 1992).

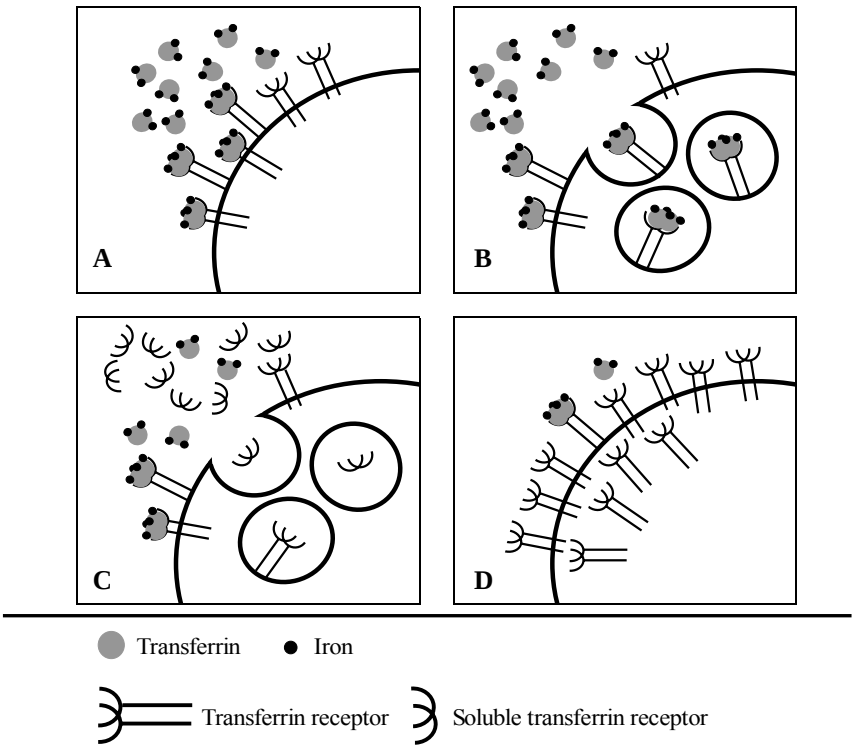


FIGURE 2 Schematic presentation of transferrin receptor (TfR) and cellular iron uptake. **A** Transferrin-iron complex binds into the TfR, **B** which transport the complex into the cells. **C** A small truncated fragment of TfR is exocytosed into the plasma in which the concentration of soluble TfR can be measured. **D** If sufficient iron is not available for cells, abundant amounts of TfR are synthesized on the cell surface.

Transferrin-iron complex binds into TfR (**FIGURE 2 A**), which transports the complex from extracellular space into the cells by endocytosis (**FIGURE 2 B**). Thereafter a small truncated fragment of intact TfR is shed out into the blood circulation

in soluble form (**FIGURE 2 C**) (Shih *et al*, 1990). Circulating soluble TfR (sTfR) is bound into a complex with transferrin with a 2:2 molar ratio in non-iron deficient individuals, but with a 2:1 molar ratio when transferrin saturation (TfSat) is decreased (Kato *et al*, 2002). If cellular iron supply is reduced and demand is not satisfied, abundant amounts of TfR are synthesized onto the cell surface (**FIGURE 2 D**) (Louache *et al*, 1984; Ward, 1987; Kohgo *et al*, 1987; Huebers *et al*, 1990; Kuiper-Kramer *et al*, 1997). Thereby, the level of circulating sTfR is increased both in iron deficiency [pure iron deficiency or functional iron deficiency (described later)] and in conditions in which erythropoietic mass is increased (Kuiper-Kramer *et al*, 1998b; Ervasti *et al*, 2004).

Cellular iron uptake is tightly regulated. Primarily, it has been shown in a cell culture study that when iron is restricted, expression of TfR is increased on the cells, but in the conditions of excess iron the amount of TfR on cells is decreased (Louache *et al*, 1984; Ward, 1987). Accordingly, TfR expression is highly upregulated by erythropoietin (EPO) via activation of iron-responsive elements (IREs) (Weiss *et al*, 1997; Sposi *et al*, 2000). Moreover, hypoxia increases TfR expression when hypoxia-inducible factor 1 (HIF-1) binds to the promoter of TfR (Tacchini *et al*, 1999). In addition, hypoxia stabilizes post-transcriptionally TfR mRNA via iron regulatory proteins (IRPs) (Weiss *et al*, 1997; Tacchini *et al*, 1999; Sposi *et al*, 2000). Of the growth factors, at least insulin-like, epidermal and platelet derived growth factors increase the expression of TfR on the cell surface of fibroblasts (Davis *et al*, 1986).

2.3. Erythropoiesis, reticulocytes and erythrocytes

Erythrocytes i.e. RBCs are essential for all living animals. Their prime function is to carry oxygen into the tissues via blood circulation. As described earlier, novel RBCs are produced continuously in the bone marrow, whereas the oldest ones are decomposed in the macrophages of the spleen or in the liver. In a steady state, approximately 2×10^{11} RBCs are matured daily in the whole developing process lasting about 5–7 days. An adult has approximately five litres of blood containing 5×10^{12} RBCs per litre.

2.3.1. Erythropoiesis in bone marrow

Erythropoiesis means the production of RBCs. In adults as well as in children the RBC production occurs mainly in the bone marrow. In fetuses, erythropoiesis occurs in the first trimester in a primitive form in the yolk sac, and thereafter subsequently in a definitive form in the liver until it shifts during the second trimester to the bone marrow (Palis *et al*, 1998; Dame *et al*, 2000).

The pluripotent stem cell in the bone marrow is the primary cell starting erythropoiesis, dividing either to myeloid or lymphoid lineages. Erythroid cells are developed in the myeloid lineage. The earliest erythroid progenitors are the so-called burst forming unit (BFU) and colony forming unit (CFU) erythroid cells (Loken *et al*, 1987). The earliest morphologically recognisable erythroid cells are proerythroblasts, which are followed by early erythroblasts, intermediate erythroblasts, late erythroblasts and reticulocytes, and eventually mature erythrocytes (**FIGURE 3**).

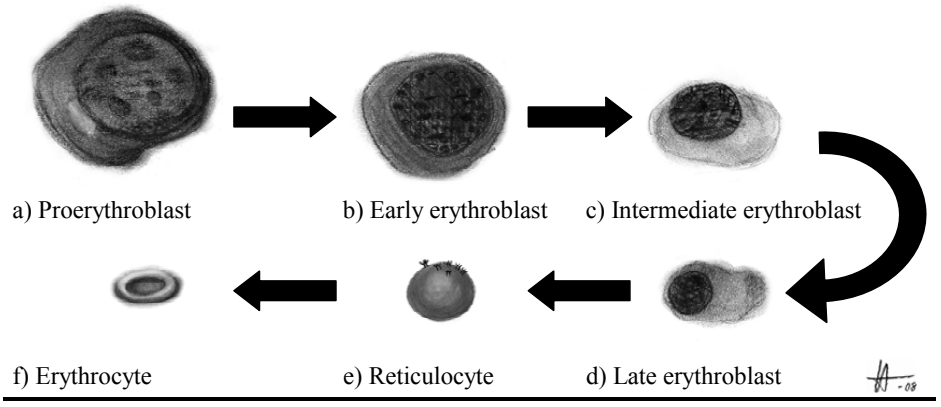


FIGURE 3 Schematic presentation of red blood cell production.

During the maturation of erythrocytes, developing cells are able to divide until the stage of erythroblasts, and their cellular volume decreases simultaneously. The prime focus of erythrocyte development is synthesizing sufficient amounts of Hb at all cell stages up to the reticulocyte. Hb (molecular weight of 68 kDa) consists of four polypeptide globin chains, each of which has a heme with protoporphyrin component and one iron atom in the pocket (Wick *et al*, 2000). Iron plays a key role in transferring

the oxygen from the lungs into tissues via blood circulation, because oxygen binds into the iron atom in Hb molecule. Another function of Hb is to act as an oxygen and acid-base buffer in blood.

If any part of the construction equipment of Hb are not sufficiently available, developing erythroid cells cannot synthesize normal amounts of Hb, thus causing anemia. Each synthesized gram of Hb needs 3.5 mg of elemental iron. The uptake of iron into the erythroid cells is mediated via TfR. The expression of TfR on the maturating erythroid cells is initiated during the early stages of red blood cell production (Loken *et al*, 1987; Okumura *et al*, 1992; Sposi *et al*, 2000). When iron is restricted in the synthesis of heme, zinc can replace the iron. In that case, elevated concentrations of erythrocyte zinc protoporphyrin (ZnPP) can be detected (Blumberg *et al*, 1977).

In human adults, Hb chains are usually α_2 and β_2 chains, forming HbA. During fetal life, when erythropoiesis is involved in the bone marrow and in the liver, most Hb, called fetal Hb (HbF), is composed of γ_2 and α_2 chains (Dame *et al*, 2000). The affinity of oxygen binding is higher in HbF than in HbA, due to the higher diphosphoglycerate binding in HbA, which disturbs the binding of oxygen. This enhances the oxygen transport from mother through the placenta into the fetal blood circulation.

2.3.2. Reticulocytes and mature erythrocytes in blood circulation

Reticulocytes are the youngest immature cells from the erythroid cell lineage that have already entered from bone marrow into the peripherial blood stream. Reticulocytes are round-shaped cells which are larger (mean diameter 8.5 μm, mean volume 112 fL) than erythrocytes (d'Onofrio *et al*, 1995). They still contain ribonucleic acid (RNA) residue in their nucleus and they have cell surface structures on their membranes (Loken *et al*, 1987). Reticulocytes stay in the blood circulation from 1 to 2 days before maturating to erythrocytes. During the maturation of reticulocytes, their cell organelles disappear progressively, the residual nuclei are extruded out from the cell and the size of the cells decreases (Loken *et al*, 1987; d'Onofrio *et al*, 1995). Mature erythrocytes are anucleated biconcave cells, with a mean volume of 90 fL and mean diameter of 8 μm in human

adults. Normal RBCs contains 30 pg of Hb, which is about 90 % of their dry weight. The life cycle of erythrocytes lasts about 120 days.

2.3.3. Erythropoietin induces the production of erythrocytes

A glycoprotein hormone, EPO (30.4 kDa molecular weight in human), is the most important hormone regulator of erythropoiesis (Egrie *et al*, 1985). The main function of EPO is to maintain an adequate Hb level in peripheral blood in order to keep the tissues in normoxic condition. EPO stimulates the proliferation and differentiation of erythroid progenitor cells in bone marrow and inhibits the apoptosis of erythroid progenitors (Jelkmann, 1992; Dame *et al*, 2000). Tissue hypoxia (both acute and chronic) is a key reason for increased EPO production. In the background of tissue hypoxia can be low oxygen-carrying capacity (decreased blood Hb concentration or RBC mass), decreased arterial pO_2, low O_2 affinity and reduced blood flow (Eckardt *et al*, 1989; Jelkmann, 1992). Hence, the level of EPO concentration has an inverse correlation with the degree of anemia as well as the mass of RBCs (Beguin *et al*, 1993; Cazzola *et al*, 1998).

EPO is produced in the kidneys of human adults, and also in fetal liver in the first and second trimesters (Dame *et al*, 2000). Placental EPO production has also been demonstrated in fetal sheep during hypoxia (Davis *et al*, 2003). EPO is not stored in tissues, so its serum concentration reflects the synthesis and elimination. In adults, EPO stimulation occurs in 1–2 hours in response to hypobaric hypoxia (dependent on the severity of hypoxic condition) (Eckardt *et al*, 1989). However, in nearly term fetal sheep, it has been demonstrated that hypoxemia induced by nitric oxide increases EPO production within three hours (Widness *et al*, 1986). The half-life of EPO has been demonstrated to be about five hours after hypobaric hypoxia in human adults (Eckardt *et al*, 1989), whereas in newborns it has been shown to be shorter at least after preeclamptic gestation and in polycythemic infants (3.7 and 2.6 hours, respectively) (Ruth *et al*, 1990).

The oxygen sensing molecular pathway of EPO production is principally mediated via hypoxia inducible factors (HIF) containing α and β subunits (Jelkmann, 2004; Lee *et al*, 2004). HIF-1 α induces EPO production, since in normoxic conditions HIF-1 α is

rapidly degraded enzymatically, whereas in hypoxic conditions degradation does not occur, which leads to the increased EPO levels (Lee *et al*, 2004). Moreover, HIF-1 α mediated hypoxia ensures the iron transport to erythroid cells, since the HIF-1 α also participates in the encoding of transferrin and TfR genes (Rolfs *et al*, 1997; Tacchini *et al*, 1999). This has also been shown in a study on erythroleukemic cells, as EPO was proved to induce the level of TfR mRNA (Weiss *et al*, 1997).

At least in sheep and monkeys at term, EPO does not penetrate the placenta and therefore maternal and newborn EPO reflect their own concentrations (Widness *et al*, 1995). Newborn EPO concentration has been shown to correlate with gestational age (Eckardt *et al*, 1990; Moya *et al*, 1993; Jazayeri *et al*, 1996), but there are also studies showing contradictory results (Forestier *et al*, 1991; Maier *et al*, 1993; Milman *et al*, 1996). Importantly, both serum and amniotic fluid EPO concentrations are related to prolonged fetal hypoxia present in e.g. maternal diabetes, hypertension, preeclampsia or Rh-immunization (Voutilainen *et al*, 1989; Maier *et al*, 1993; Teramo *et al*, 2004a; Teramo *et al*, 2004b). Vaginal birth, as an acute distress for newborns, causes higher EPO levels in newborns than does elective Caesarean section (Widness *et al*, 1984), but there is also a study showing that vaginal delivery does not increase active erythropoietin (Halevi *et al*, 1992). Nevertheless, EPO levels decrease after birth if hypoxic stimulus disappears (Ruth *et al*, 1990). Serum and amniotic fluid EPO concentration has been found to have strong inverse significant correlations with pO_2, pH and base excess (BE) in normal-sized infants, in infants of diabetic pregnancies as well as in samples drawn from cord blood at elective Caesarean section (Rollins *et al*, 1993; Jazayeri *et al*, 1996; Teramo *et al*, 2004a). However, no correlation has been found between the Hb and EPO levels in cord blood specimens of healthy term newborns (Rollins *et al*, 1993; Fahnenstich *et al*, 1996), which might be partly due to the higher affinity of oxygen in the HbF than HbA (Caro *et al*, 1982).

Maternal EPO concentration is elevated in the presence of bleeding, multiple gestations and preeclampsia (Goldstein *et al*, 2000), and it has been shown that maternal EPO concentration rises progressively during pregnancy (with and without iron supplements) (Cotes *et al*, 1983; Riikonen *et al*, 1994; Milman *et al*, 1997; McMullin *et al*, 2003). The EPO concentration in pregnant women has also been shown to be closely

related to iron status parameters, such as serum iron, serum ferritin, total iron binding capacity (TIBC), TfR and reticulocyte count at different time spans during gestation (Beguin *et al*, 1991; Milman *et al*, 1994; Milman *et al*, 1997; McMullin *et al*, 2003).

EPO also has nonhematopoietic roles as it protects progenitor cells in multiple organ systems (brain, gastro-intestinal tract, vasculary system, heart), preventing apoptosis and stimulating proliferation of endothelial cells together with vascular endothelial growth factor (VEGF) (Sakanaka *et al*, 1998; Sirén *et al*, 2001; Cerami *et al*, 2002; Yu *et al*, 2002; Marti, 2004). Additionally, EPO has been shown in animal models to have a role in fetal brain development (Yu *et al*, 2002). On the other hand, fetuses who have had complicated or uncomplicated pregnancies are at risk for impaired neurodevelopmental outcome when EPO levels reflecting fetal hypoxemia are increased (Ruth *et al*, 1988).

2.3.4. Steroid hormones involved in the erythrocyte production

Along with EPO, androgens are stimulative regulators of erythropoiesis in the long run. They have been shown to increase erythropoietic activity, thereby increasing reticulocyte count and blood Hb concentration (Shahidi, 1973). Such progression in growth and hormonal activity occurs after puberty and it explains the higher blood Hb concentration in adult males in comparison with adult females. Androgens have direct and indirect mechanisms in increasing erythropoiesis by accumulating directly the numbers of RBCs and indirectly by increasing the EPO concentration (Shahidi, 1973). Therefore, androgens have been used in the treatment of certain types of anemia. Additionally, athletes have tried to attain higher Hb levels by substance abuse of androgen-anabolic steroids (Hartgens *et al*, 2004). Nevertheless, improved endurance performance has not been able to be demonstrated by the abuse of steroids, although the aerobic capacity of strength athletes has been shown to improve (Hartgens *et al*, 2004).

2.4. Anemia and iron deficiency

2.4.1. Definition and classification of anemias as the basis for differential diagnosis

Anemia is defined as a Hb content under the lower reference limit at the level of 120 g/L for women and 130 g/L for men (WHO, 2001; Beutler *et al*, 2006). The most widely used definition of anemia is the recommendation of the World Health Organization (WHO) (**TABLE 1**) (WHO, 2001). In recent years, the main public laboratories in the Nordic countries have been involved in a project called the Nordic Reference Interval Project (NORIP) to unify the reference values of the most commonly used laboratory measurements in adults (Nordin *et al*, 2004; Rustad *et al*, 2004). According to the NORIP, the reference values of Hb were 117–155 g/L and 134–167 g/L for women and men, respectively, and these are the values used at Kuopio University Hospital. However, no reference limits for infants and children were produced by the NORIP. They are commonly based on a widely used reference textbook, Hematology of Infancy and Childhood (**TABLE 1**) (Nathan *et al*, 1993).

TABLE 1 Reference intervals of hemoglobin and hematocrit by World Health Organization (WHO) and in the book Hematology of Infancy and Childhood (Nathan & Oski, 1993).

WHO				**Hematology of Infancy and Childhood**		
	Lower limit				Hb, g/L	
	Hb	Hematocrit			Lower	Upper
Age or gender group	g/L	mmol/L*	L/L	Age or gender group	limit	limit
Children 6 months to 59 months	110	6.83	0.33	0–1 days	145	225
Children 5–11 years	115	7.13	0.34	2–7 days	135	215
Children 12–14 years	120	7.45	0.36	8–14 days	125	205
Non-pregnant women (above 15 years of age)	120	7.45	0.36	15–29 days	100	180
Pregnant women	110	6.83	0.33	1–2 months	90	130
Men (above 15 years of age)	130	8.07	0.39	3–5 months	95	135

* Conventional conversion factors: 100 g hemoglobin = 6.2 mmol hemoglobin = 0.30 L/L hematocrit.

Hitherto, Wintrobe's anemia classification introduced in 1932 has been the prime guide for categorizing anemias (Wintrobe, 1932). These categories are based on the size of RBCs, which divides anemias into micro-, normo-, and macrocytic diseases. This categorization enables the appropriate differential diagnosis of anemias. The main causes of microcytic anemias are iron deficiency, sideroblastic anemias and thalassemias. Normocytic anemias are commonly consequences of chronic diseases, hemolytic anemias, bleeding or bone marrow infiltration, and rarely are caused by mild iron deficiency. Macrocytic anemias are caused by megaloblastic diseases (e.g. myelodysplastic syndromes, folate or B_{12}-vitamin deficiency), large-scale alcohol consumption, liver diseases, hemolytic (with reticulocytosis) or aplastic anemias.

2.4.2. Iron deficiency and iron deficiency anemia

Iron deficiency is a major nutritional defect worldwide. When iron homeostasis is in a negative balance or if the need for iron increases, there is a risk for iron deficiency anemia. This develops when iron is not adequately available for RBC production and the red cells produced cannot obtain sufficient building materials for normal Hb synthesis. Therefore, RBCs are hypochromic and microcytic in iron deficiency anemia. While anemia is the most conspicuous manifestation of iron deficiency, iron deficiency can have detrimental influences especially on growing children. Indeed, iron is vital for normal brain development and myelination of nervous cells (Yu *et al*, 2002; Lozoff *et al*, 2006). Additionally, there is increasing evidence that iron deficiency and iron deficiency anemia in infants, children and in juvenile rhesus monkeys, and low iron stores in cord blood, are associated with impaired neurologic outcome, cognitive as well as mental and psychomotor development, and behavioral changes (reduced inhibitory response) (Grantham-McGregor *et al*, 2001; Tamura *et al*, 2002; Golub *et al*, 2007).

All living cells need iron so the iron demand increases during growth periods (infancy, childhood, puberty) and during pregnancy, and there can be a discrepancy between supply and demand. In adulthood, the main reasons for iron deficiency are increased loss via hemorrhage: e.g. menstruation during reproductive years in women,

gastro-intestinal bleeding, surgical operations or regular blood donation. Sometimes, absorption of iron from the intestine is restricted (gastrectomy, malabsorption).

2.4.3. Concepts of subclinical and functional iron deficiency

Iron balance can be divided into a number of categories. It can be in a steady state in which the cycle of iron is adequately available where it is needed in the body. In that state iron absorption is restricted by cellular mechanisms, and normally iron stores cannot be overfilled, because of controlled absorption (Conrad *et al*, 2002). However, in certain diseases, e.g. hemochromatosis, iron absorption mechanisms are interrupted and iron accumulates in the body. Moreover, in a negative phase of iron homeostasis, the stored iron is primarily utilized for RBC production. Before manifested anemia, the depletion of the storage iron compartment is asymptomatic and in a latent state, which is also termed subclinical iron deficiency.

Functional iron deficiency is a special form of disturbance in iron homeostasis in which the release of iron from the stores for increased RBC production is not sufficient to satisfy the demand (Ervasti *et al*, 2004). Functional iron deficiency is common in patients receiving EPO substitution treatment (accelerated erythropoiesis), and a shortage of iron has been shown to diminish the response to treatment (Brugnara *et al*, 1993; Brugnara *et al*, 1994a; Schaefer *et al*, 1999).

2.5. Effects of iron overload

Elevated body iron constitutes a disease entity, including diseases with genetic transformations such as hemochromatosis, and iron overload because of RBC transfusions. There are two specific forms of iron overload. In patients who have frequent blood transfusions or intravenous iron therapy, iron accumulates mainly in macrophages, after which excess iron is loaded into parenchymal cells (Papanikolau *et al*, 2005; Siah *et al*, 2005; Batts, 2007). On the other hand, in patients with hemochromatosis, iron overload is a cause of abnormal iron absorption. A majority of hemochromatosis patients have a missense mutation C282Y altering an HFE protein

(Parkkila, 2000). HFE proteins are major histocompatibility–complex class I proteins, which are involved in the regulation of iron absorption. In patients with hemochromatosis, serum iron binding capacity is overwhelmed and excess of iron is rapidly eliminated into the parenchymal cells (mainly in the liver and heart).

Free iron has adverse effects because it has an ability to form free radicals that may increase oxidative damage (Tuomainen *et al*, 2007; Schümann *et al*, 2007). As the underlying mechanism, labile iron mediates the catalysing of hydroxyl and organic radicals, for example (Fenton and Heber-Weiss-reactions) (Papanikolau *et al*, 2005; Schümann *et al*, 2007). This may be a reason for the finding that large iron stores measured by ferritin levels are connected with impaired glucose regulation (type II diabetes mellitus), gestational diabetes mellitus or increased risk of acute myocardial infarction (Tuomainen *et al*, 1998; Jiang *et al*, 2004; Scholl, 2005; Chen *et al*, 2006; Bencaiova *et al*, 2007).

2.6. Anemia and iron status during pregnancy

2.6.1. Prevalence of anemia during pregnancy

There is a high prevalence of anemia during pregnancy in developing and developed countries (van den Broek, 1998a; van den Broek *et al*, 2000; WHO, 2001). It is estimated that approximately 52 % of pregnant women in developing and 25 % in developed countries are anemic (WHO, 2001). The main reason for decreased Hb levels during pregnancy is physiological expansion of plasma volume, which can increase nearly 50 % compared with the volume in non-pregnant women. This hemodilution begins in the first and second trimesters and allows better blood circulation for the placenta, thereby ensuring the well-being of the fetus (Steer *et al*, 1995). Hemodilution occurs individually and reaches its maximum at gestational weeks 24–32. Thereafter, by the time of delivery, blood Hb concentration is increased to near the level of non-pregnant women. This increase during the third trimester is a consequence of accelerated erythropoiesis, which increases the number of RBCs by approximately 25 %. According to the recommendation of the WHO, the lower reference limit for Hb in

pregnant women is 110 g/L, and the limit for severe anemia is 70 g/L (WHO, 2001; WHO, 2006). A recent study on reference intervals for hematological variables in Danish pregnant women suggests as lower limits for Hb concentration 105 g/L, 108 g/L and 118 g/L at 18 weeks, 39 weeks and at 8 postpartum weeks, respectively (Milman *et al*, 2007).

A depletion of iron as well as other nutritional shortages may also be in the background of anemia during pregnancy, but worldwide the most common reasons (especially in developing countries) are parasitic infections, chronic inflammations, socio-economic status and hemoglobinopathies (van den Broek, 1998a; van den Broek *et al*, 2000; Rush, 2000; Milman *et al*, 2007). There are no trustworthy statistics on iron deficient pregnant women in developing countries, since the prevalence of iron deficiency in those countries has only been estimated indirectly via the prevalence of anemia during pregnancy (WHO, 2001). However, since slightly reduced Hb values can be due to physiological hemodilution, iron deficiency is not the ultimate explanation for lowered Hb and there is a need to simultaneously examine primary reasons. Studies in developed countries, such as Denmark, suggest that a shortage of iron is common in women during their fertile years: about 42 %, 13 % and 7 % of 18–30 year women were found to have serum ferritin level ≤ 32 µg/L, < 16 µg/L and < 13 µg/L, respectively (Milman *et al*, 1998). Furthermore, in a population of 30–40 year old women, ferritin was > 30 µg/L, 15–30 µg/L and < 15 µg/L in 60.1 %, 22.7 % and 17.2 % of all women, respectively (Milman *et al*, 1992).

2.6.2. Iron homeostasis during pregnancy

Maternal iron requirement per day is increased during pregnancy on average from three- to seven-fold (from 1 mg to 3–7.5 mg elementary iron) because of the demands of the growing fetus and placenta (**FIGURE 4**) (Milman *et al*, 1999; Baker, 2000; Bothwell, 2000; Wick *et al*, 2000). In addition, the accelerated erythropoiesis in pregnant women especially in the third trimester (Choi *et al*, 2001a) and the blood loss at delivery increase the demand for iron (**FIGURE 4**). On the other hand, iron is conserved in pregnant women, as menstruation does not occur. During pregnancy, maternal bone

marrow iron stores are mobilized in order to satisfy the increased demand (Svanberg *et al*, 1975), and simultaneously the absorption of iron in the small intestine increases (**FIGURE 4**) (Svanberg *et al*, 1975; Whittager *et al*, 1991; Conrad *et al*, 2002). The net requirement of iron during pregnancy has been calculated to be 500–790 mg (Milman *et al*, 1999; Beaton, 2000; Bothwell, 2000).

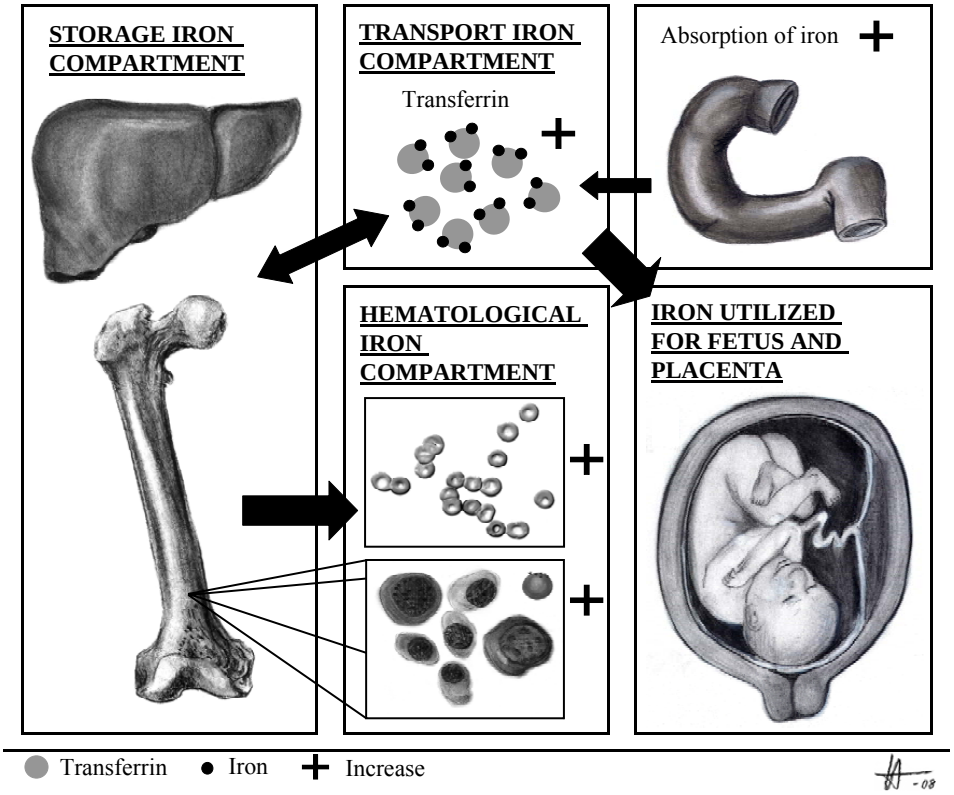


FIGURE 4 Schematic presentation of iron homeostasis during pregnancy. Iron is prioritized for the fetus and placenta by increasing the absorption of iron in the mother's intestine and by mobilizing iron from the store compartment. Furthermore, especially during 3rd trimester of pregnancy, iron is mobilized in order to satisfy the need for increased erythropoiesis of pregnant women.

After delivery, a large amount of the iron that had been utilized from iron stores in the increased maternal RBC mass during pregnancy is restored if blood loss and the fetus have not exhausted utilized maternal iron. RBC and reticulocyte cellular Hb

content has been shown to be normal in the postpartum period (until 42 days) in a study population consisting of non-anemic mothers (Richter *et al*, 1999). While postpartum iron supplementation in iron deficient women has been shown to increase iron stores and reduce iron-deficient erythropoiesis in 12 weeks (Krafft *et al*, 2005), it also occurs apparently physiologically without iron supplements, as 50 % of pregnant women with absent bone marrow iron stores have replenished them during two post-partum months (Svanberg *et al*, 1975).

2.6.3. Risks of iron deficiency during pregnancy

Many studies have been published on the effects of maternal anemia or iron deficiency on the health of pregnant women and pregnancy outcome (Xiong *et al*, 2000; Rush, 2000). However, no consensus view has emerged. It is commonly proposed that anemia plays a major role in maternal mortality, but this view has been criticized in a study reviewing investigations to date (Rush, 2000). The review concluded that only severe anemia has been found to be associated with increased maternal mortality. However the main cause of severe anemia is not iron deficiency, but the aforementioned other causes. On the other hand, in a meta-analysis concerning maternal anemia, early pregnancy anemia has been shown to increase the risk of preterm birth, but during late pregnancy the risk was inverse and statistically not significant (Xiong *et al*, 2000). However, in that analysis many studies were excluded because of variable definitions of anemia and because of incomplete data (Xiong *et al*, 2000).

2.6.4. Advantages and disadvantages of iron supplementation treatment during pregnancy

Daily dietary iron intake is not always sufficient even in developed countries. Recommendations on the real need or routine versus selective iron supplementation during pregnancy vary, because there is no consensus about the benefits and disadvantages of supplementation for both the mother and her offspring, or for a growing child (Taylor *et al*, 1982; Scholl *et al*, 1992; Allen, 1997; Milman *et al*, 1999;

Allen, 2000; Bothwell, 2000; Leinonen *et al*, 2001; Cogswell *et al*, 2003; Milman, 2006; Rioux *et al*, 2007; Schümann *et al*, 2007; Reveiz *et al*, 2007). It is difficult to provide uniform recommendations, because various variables are investigated in studies concerning the advantages and disadvantages of iron supplementation, e.g. optimal Hb or iron stores, outcome of pregnancy, the amount of supplemented iron, and using selective or routine supplementation (Reveiz *et al*, 2007). Additionally, mothers in both developing and developed countries have been studied, and the population often consists only of hospitalized mothers (Rush, 2000). In Finland, iron supplementation during pregnancy is not routinely recommended for all pregnant women (Leinonen *et al*, 2001); in clinical practice the decision about supplementation is usually based simply on a Hb value measured in maternity care units.

To generalize, in countries with a high prevalence of anemia, pregnant women receiving iron supplementation deliver significantly heavier newborns than women receiving placebo, and consistently, low birth weight newborns are less common in women with iron supplementation than in women receiving placebo (Rioux *et al*, 2007). Furthermore, a low dose iron supplement (30 mg/day) lasting from enrollment (before 20 weeks, mean app. 11 weeks) until 28 weeks of pregnancy, after which the amount of iron supplements were determined by ferritin or Hb, improved birth weight even in initially non-anemic women with normal iron status (defined as ferritin $\leq$ 20 μg/L) in comparison with placebo-treated women in those weeks (Cogswell *et al*, 2003).

Nevertheless, there is clear evidence of adverse effects of iron supplementation during pregnancy (see also Chapter 2.5.) (Rioux *et al*, 2007; Schümann *et al*, 2007). Iron supplementation treatment during pregnancy may lead to maternal hemoconcentration and increase lipid peroxidation and oxidative stress (Casanueva *et al*, 2003; Schümann *et al*, 2007). Furthermore, high Hb level is associated with lower birth weight and shorter duration of pregnancy (Steer *et al*, 1995) as well as with increased incidence of gestational hypertension, preeclampsia, low birth weight and low Apgar scores (Rioux *et al*, 2007; Ziaei *et al*, 2007). A recent randomized controlled trial reported that in groups of pregnant women with Hb above 132 g/L receiving a routine (50 mg/d) dose of iron versus placebo supplementation (initiated at the early stage of the second trimester), the prevalences of maternal hypertension and small for gestational

age (SGA) (determined as 10th percentile of birth weight) were higher than in a group with iron supplementation (Ziaei *et al*, 2007). Furthermore, high maternal ferritin indicating high iron stores has been associated with low birth weight of the infant, and especially with asymmetric growth retardation of the fetus (Goldenberg *et al*, 1996; Hou *et al*, 2000; Lao *et al*, 2000), but opposite findings indicating no association between maternal iron status and infant's birth weight have also been published (Jaime-Perez *et al*, 2005). In a 7-year follow-up study after routine or selective iron supplementation in women during pregnancy no significant differences were found in either mortality or morbidity of mothers and their infants, except a serious finding of more frequent hospitalization of infants because of convulsions, in a routinely supplemented group (Hemminki *et al*, 1995).

2.7. Iron status of newborn infants

Iron deficiency was a major cause of anemia in children in the 1960s in Finland (Järvinen *et al*, 1960). However, since then the nutritional status of Finnish women and children has improved. The total iron requirement during gestation consists of the requirements of pregnant women and of the fetus. The availability of iron depends on the amount of iron stores in pregnant women and the ability to absorb the iron in the intestine (Svanberg *et al*, 1975; Conrad *et al*, 2002; O'Brien *et al*, 2003). The total amount of fetal iron uptake is approximately 200–300 mg depending on the size of the newborn at term (2500–3500 g) (Milman *et al*, 1999). There is also reported that the larger the infant and placenta are, the more deprived the maternal iron status is (Ervasti *et al*, 2008c).

During intrauterine life, most of the iron is used in fetal RBC production and after the demand is satisfied, iron is stored as ferritin. The amount of iron stored is increased concomitantly with gestational age (Siddappa *et al*, 2007) and the highest Hb concentration in childhood is found at birth. Because fetal RBCs containing HbF are destroyed more rapidly than other forms of Hb, Hb concentration falls during the first weeks of life. Thereafter, released iron from decomposed RBCs is mainly stored (Rios *et al*, 1975). That is why, delayed cord clamping is used to prevent iron deficiency in

preterm infants and in newborns in developing countries (Rabe *et al*, 2004; Chaparro *et al*, 2006).

The fetus receives iron from the mother across the placenta by an active process in which iron-transferrin complex bound to TfR is endocytosized from the maternal side into the syncytiotrophoblast cell, from where the iron is transferred through the cell into the fetal blood circulation (Wada *et al*, 1979; Srai *et al*, 2002; Rao *et al*, 2002; McArdle *et al*, 2003). This transport system collapses only in severe iron deficiency anemia in pregnant women, and it is usually sufficient to ensure fetal needs, as demonstrated by the ability to ensure iron flux by augmenting cellular expression of iron transport proteins in the placenta (Gambling *et al*, 2003). Therefore, subclinical maternal iron deficiency [measured by zinc protoporphyrin (ZnPP)] does not influence fetal iron supply (Harthoorn-Lasthuizen *et al*, 2001).

The regulation of maternal-fetal iron efflux is still only partly understood. During fetal life the acquisition of iron is greatest during the rapid growth phase during the third trimester, and in preterm newborns (Rao *et al*, 2002; Bradley *et al*, 2004). Iron accumulation in the placenta in the third trimester has also been reported (Bradley *et al*, 2004). Hepcidin, a small (25-amino acid) antimicrobial peptide hormone, which plays a significant role in iron homeostasis, may also be involved in maternal-fetal trans-placental iron passage. Although the exact role of hepcidin in trans-placental iron passage has not yet been elucidated, at least embryonic hepcidin transgene expression has been shown to be associated with down-regulation of placental mRNA TfR (Martin *et al*, 2004). Generally, hepcidin inhibits the iron release (negative feedback) from macrophages and hepatocytes and blocks the iron influx into enterocytes (Ganz, 2006). Hepcidin level is up-regulated in inflammation and iron overload (Nicolas *et al*, 2002). It has also been reported that maternal prohepcidin (a prohormone of hepcidin) at term correlates highly significantly with cord blood prohepcidin at birth, although prohepcidin levels have not been reported to be significantly associated with iron status measurements (Ervasti *et al*, 2008b). Additionally, cord blood pro-hepcidin levels at birth have been shown to correlate with the weight of the placenta (Ervasti *et al*, 2008b).

Severe maternal iron deficiency, maternal hypertension with intrauterine growth restriction and maternal diabetes mellitus have been shown to be associated with lower

iron status of newborns (Chockalingam *et al*, 1987; Rao *et al*, 2002; Lott *et al*, 2005; Verner *et al*, 2007). There is evidence that fetal iron requirements can regulate the absorption of iron in the intestine of pregnant women (O'Brien *et al*, 2003). In this case, the fetus does not receive absorbed iron from the maternal intestine when maternal iron stores are adequate (O'Brien *et al*, 2003). It has also been shown in iron deficient pregnant rats that placental iron transport proteins can be up-regulated to minimize fetal anemia (Gambling *et al*, 2003). The transportation of iron through the placenta is usually sufficient to meet the needs of the fetus except in case of severe maternal iron deficiency (Rios *et al*, 1975; Choi *et al*, 2000b). Thus, the fetus has a parasitic role, exhausting the iron reserves of the mother.

Iron is needed in erythroid cells as well as in the developing brain. Therefore, it is reasonable to suppose that iron deficiency may have adverse effects on the child's development. There is increasing evidence that low iron status at birth and iron deficiency in childhood are associated with impaired cognitive and behavioral development in childhood (Lozoff *et al*, 1991; Grantham-McGregor *et al*, 2001; Tamura *et al*, 2002; Lozoff *et al*, 2006). For this reason, it is important to assure the iron requirement of fetuses. A four-year follow-up study on the effects of a low dose (20 mg/d) iron supplementation during pregnancy found that it did not influence the intelligence quotient in childhood (Zhou *et al*, 2006). However, the incidence of abnormal behavioral total score results were higher in the iron- than in the placebo-supplemented groups (Zhou *et al*, 2006). Furthermore, in a recent study on rhesus monkeys, iron deprivation before birth was reported to be associated with behavioral changes (reduced inhibitory response) (Golub *et al*, 2007).

2.8. Laboratory diagnosis of iron deficiency

Different phases and compartments in iron homeostasis provide a challenge for clinical laboratory measurements (**FIGURE 5**). Bone marrow iron staining has been considered as the gold standard for iron status measurement. However, it does not reflect accurately continuous nuanced overlaps of iron balance. Furthermore, invasive examinations are not appropriate for screening the iron status in patients. Iron status measurements

involve different body compartments, which can be analysed using serum or plasma measurements. Moreover, the Hb content of RBCs reflect hematological aspects of iron homeostasis. While cellular indices reflect iron availability for Hb synthesis of erythropoiesis, biochemical markers reflect circulating iron bound to transferrin (TfSat), iron availability for erythropoiesis (TfR) and iron stores (ferritin). Although there are a variety of iron status measurements, some of them are neither specific nor sensitive, and some have some interfering factors (Borel *et al*, 1991; Guyatt *et al*, 1992; Withold *et al*, 1994).

2.8.1. Biochemical iron status measurements

Serum iron and transferrin contents play in a central role in the exchange of iron homeostasis as they reflect the compartment of circulating iron in serum (**FIGURE 5**). Transferrin and iron have short-term fluctuations as they are dependent on dietary intake, diurnal variation, time of day, iron requirement, and iron release from bone marrow, the reticuloendothelial system or the liver (Borel *et al*, 1991). Most of the serum iron is bound to transferrin, and about 30–40 % of transferrin is normally saturated. Transferrin, with a 79.6 kDa molecular weight, has a variety of isoforms (20 are currently known). Half the transferrin is distributed in serum and the other half extravascularly (Aisen *et al*, 1980). The stimulation of transferrin synthesis in the liver is dependent on iron availability and the demands of the tissues. Transferrin is also regarded as a marker of nutritional status in the human body (Chockalingam *et al*, 1987). Serum iron and transferrin can be combined in calculating the saturated amount of iron in transferrin, as TfSat (Beilby *et al*, 1992; Kasvosve *et al*, 2002). TfSat reflects the iron circulating in serum that can be delivered to the tissues. Before the availability of transferrin analyses, TIBC was measured; it reflects the amount of iron ions that are needed to saturate transferrin (Tsung *et al*, 1975).

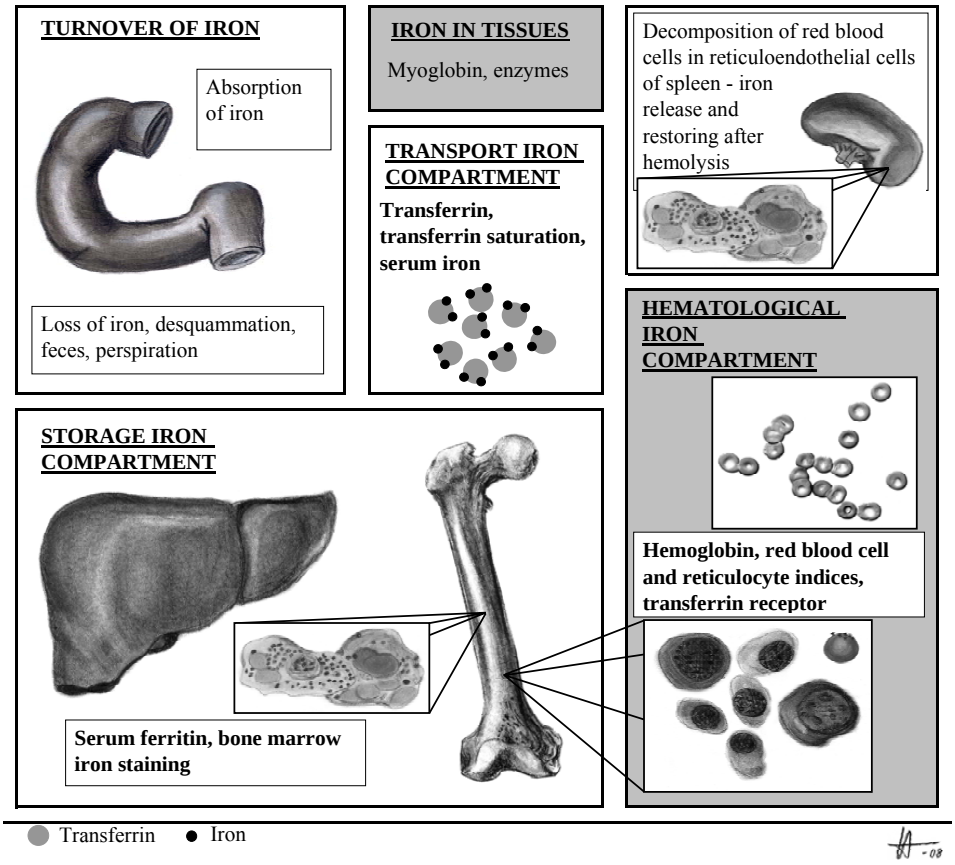


FIGURE 5 Laboratory measurements that reflect different compartments of systemic iron homeostasis.

Ferritin is a macromolecule with a molecular weight of 440 kDa, which has a large amount of iron ions in its core, covered by a protein shell (Aisen *et al*, 1980). Ferritin can be synthesized in all cells, but the liver and spleen have specialized metabolic pathways for storing a large amount of iron. Ferritin has a variety of isoforms that are found in the liver, spleen, bone marrow, placenta, heart and tumors (Bradley *et al*, 2004). Ferritin reflects strictly the compartment of stored iron in the human body (**FIGURE 5**). About 1 μg/L of ferritin circulating in serum is equivalent to 8–10 mg of stored iron (Walters *et al*, 1973). A low ferritin level is a precise and powerful diagnostic tool to indicate iron deficiency (Guyatt *et al*, 1992). However, ferritin as an acute phase protein is also increased in inflammatory and liver diseases, where ferritin

levels of 20 to 70 µg/L can be inaccurate in excluding iron deficiency (Guyatt *et al*, 1992; Punnonen *et al*, 1997; Suominen *et al*, 1998).

A soluble truncated form of intact TfR (molecular weight of 85 kDa) can be measured in serum (Ward, 1987; Shih *et al*, 1990; Cook *et al*, 1993) and it reflects the amount of TfR that is found on the cell surface (Shih *et al*, 1990). TfR is found on all cells, and the main regulator of its synthesis is the availability of iron (Gatter *et al*, 1983; Louache *et al*, 1984). sTfR levels increase immediately when the ferritin level is below 12 µg/L in adults (Suominen *et al*, 1998) and below 9 µg/L in children (Choi *et al*, 2003). The most abundant amount of TfR is found in the erythroid precurcors in the bone marrow (Kohgo *et al*, 1987), and therefore the sTfR concentration is also dependent on the mass of erythropoiesis (Ward, 1987; Huebers *et al*, 1990). The most useful feature of sTfR, as a diagnostic tool in iron deficiency, is that it does not act as an acute phase protein (Ward, 1987; Punnonen *et al*, 1994; Punnonen *et al*, 1997), and thus it distinguishes iron deficiency anemia from the anemia of chronic diseases. sTfR can also be elevated because of subclinical (Suominen *et al*, 1998) or functional iron deficiency (Ervasti *et al*, 2004). One problem in using sTfR is a lack of standardization and the absence of a reference method (Thomas *et al*, 2002; Kolbe-Busch *et al*, 2002).

For the assessment of iron status, ferritin and sTfR are combined in many formulas in order to take into account both the availability of iron for erythropoiesis and the available iron stores (Punnonen *et al*, 1997; Thomas *et al*, 2002). The most commonly used formula is to calculate the TfR-F Index by dividing the sTfR concentration by the logarithmic transformation of the ferritin concentration. The TfR-F Index has also been shown to be an accurate index in distinguishing the anemia of chronic disease from iron deficiency anemia (Punnonen *et al*, 1997), and in detecting iron deficiency in its subclinical phase (Suominen *et al*, 1998; Thomas *et al*, 2002) and in newborn infants (Sweet *et al*, 2001).

2.8.2. Blood count: from hemoglobin to red blood cell indices

While the analytical development of automated cell counters was remarkable during the 1980s and 1990s, analysing cell morphology by microscope is still the basis and

reference method for examining diseases of the blood cells. Hb concentration, initially as a measurement of the oxygen-carrying capacity, was analysed using a colorimetric method already in 1878, although in those days there were misleading errors because of standards (Farr, 1978). In 1901, the reliability of the colorimetric assay was improved by the modification of a more stable standard (Farr, 1978).

In the history of red cell analyses, the major steps in the classification of anemia were taken in 1932, when Wintrobe published the classification based on the size of the RBCs that is still in use (Wintrobe, 1932). In addition to chamber counting by microscope as a measurement of anemia, Wintrobe presented the macrohematocrit (at first called packed red cell volume) that was analysed by spinning the blood samples using centrifuges (Bain, 2002). In the early 1960s, the spectrophotometric cyanmethemoglobin method was introduced for Hb measurements (Savage, 1993). The standardization of Hb measurement begun in 1963 (Zwart *et al*, 1996).

The development of impedance cell counters in the late 1940s was an important phase in the history of cell analysers (Bain, 2002). The first models of analysers were already based on the flow of cells drifting in the solution and passing the aperture. Later, cell counters using optic methods were developed (Mohandas *et al*, 1986).

Nowadays, the basic blood count consists of the number of leukocytes, RBCs and platelets, in addition to Hb, hematocrit (HCT), reticulocyte count, and RBC indices (**TABLE 2**). In the past, all the cell indices were conventionally derived from Hb, the RBC count and hematocrit. Mean cell Hb concentration (MCHC) and mean cell Hb (MCH) are still the counted parameters that reflect the Hb contents of the RBCs. Mean cell volume (MCV) and its distribution curve (red cell distribution width, RDW) was a single direct red cell parameter for a long time (England *et al*, 1974).

In cell analyzers, reticulocytes can be identified by a fluorescent dye labeling the RNA residue of the cells. Importantly, the size and Hb content of reticulocytes can be analyzed in the same way as RBCs. The amount of fluorescently labeled RNA inside the cells has traditionally been used as a method to calculate the number of reticulocytes measured by either flow cytometers (FCM) or hematological analyzers. Moreover, reticulocytes can be divided into subsets on the basis of the amount of fluorescent dye bound to their RNA residue (Serke *et al*, 1992; Choi *et al*, 2001b).

TABLE 2 Abbreviations, explanations, methods and formulas for the conventional and novel red blood cell and reticulocyte indices currently available in automated hematological systems.

Abbreviation	Unit	Explanation	Method or formula
Hb	g/L	Hemoglobin	Cyanmethemoglobin analysis (standardized)
HCT	L/L	Hematocrit	(RBC x MCV) / 10
RBC	1 x 10^{12} cells/L	Red blood cell count	Number of RBCs
Retic or %Retic	1 x 10^{9} cells/L or %	Absolute amount or percentage of reticulocytes	Reticulocyte dyes
MCV or MCVr	fL	Mean cell volume of mature red blood cells or reticulocytes	Mean of the cell volume for the mature RBC or reticulocyte population, respectively
RDW	%	Red cell distribution width	100 x (SD of MCV / MCV)
MCH	pg	Mean cell hemoglobin	Calculated mean cell Hb using the formula (Hb / RBC) x 10
MCHC	g/L	Mean cell hemoglobin concentration	Calculated mean cell Hb concentration using the formula [Hb / (RBC x MCV)] x 1000
CHCMm or CHCMr	g/L	Cellular hemoglobin concentration mean in mature red blood cells or reticulocytes	Mean of the cellular Hb concentration for the mature RBC or reticulocyte population, respectively
CHm or CHr	pg	Cellular hemoglobin of mature red blood cells or reticulocytes	Mean of the cell Hb mass for the RBC or reticulocyte population, respectively
%HYPOm or %HYPOr	%	Percentage of hypochromic mature red blood cells or reticulocytes	Percentage of mature RBC or reticulocyte population with Hb concentration less than 280 g/L
%HYPERm or %HYPERr	%	Percentage of hyperchromic mature red blood cells or reticulocytes	Percentage of mature RBC or reticulocyte population with Hb concentration higher than 410 g/L
%MICROm or %MICROr	%	Percentage of microcytic mature red blood cells or reticulocytes	Percentage of mature RBC or reticulocyte population with mean cell volume less than 60 fL
%MACROm or %MACROr	%	Percentage of macrocytic mature red blood cells or reticulocytes	Percentage of mature RBC or reticulocyte population with mean cell volume higher than 120 fL
IRF-H, IRF-M+H	%	Immature reticulocyte fraction high, and immature reticulocyte fraction medium + high	Percentages of the immature reticulocyte fractions derived from the scattergram of reticulocytes
RBC-He or RET-He	Arbitrary unit	Red blood cell or reticulocyte hemoglobin equivalent	Hb content of RBCs or reticulocytes quantitated by the arbitrary unit

2.8.3. Basic principles of automated cell counters

Analysis of cells drifting in medium is a technique used in modern hematological analysers. Impedance analyzers utilize the poor conductiveness of the cells as they

cause an increasing impedance pulse when they bypass the orifice in the conductive solution. Each bypassing cell causes one pulse according to their cell volume, and an oscilloscope detects this pulse. Whereas impedance analysers measure the electricity of the flowing cells, optical analysers detect the scatters shed out by each cell flowing through the laser light. In photo-optical detectors, the light scattering instruments detect the laser reflection that is passed by particles (**FIGURE 6**) (Mohandas *et al*, 1986).

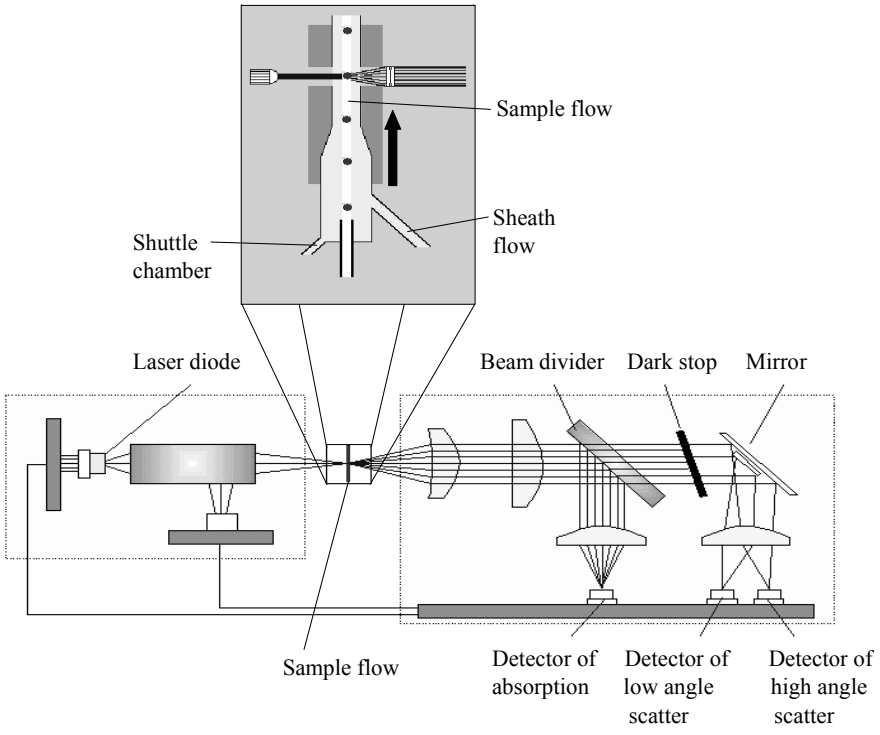


FIGURE 6 Schematic presentation of the principle of an optical cell counter. Light scattering instruments detect via photo-optical detectors the laser reflection that is passed by particles. (Modified from Instructions of ADVIA 120, by kind permission of Siemens, Health Care Diagnostics, Tarrytown, NY, USA).

Low- and high-angle detectors are used in optical analysers, by means of which the size and intracellular Hb content of the individual particles scatter the laser light, which is recorded. Each dot in the scattergram indicates a cell whose position is based on its size and internal composition (forward light scatter reflects the size of the cells

and side light scatter reflects the composition) (**FIGURE 7 A**). **FIGURE 7 B** shows the linear cell scattergram of normal RBCs with a volume of 60–120 fl and Hb concentration of 280–410 g/L.

FCMs are used nowadays as basic equipment in the differential diagnosis of malignant hematological diseases. In the most sophisticated FCMs, between 1 and 3 lasers are used. Basically, forward and side scatters allow the evaluation of the size and granularity of the flowing cells. In addition, cell surface structures can be detected using monoclonal antibodies labeled by different fluorochromes. Normal cells express certain antigens, and thus the erroneous antigen expression on the cells classifies the disease. The permeabilization of cells is used to dye intracellular organelles. The emitted fluorescence signals of each cell passing the aperture in sheath fluid are detected using photo-optical detectors.

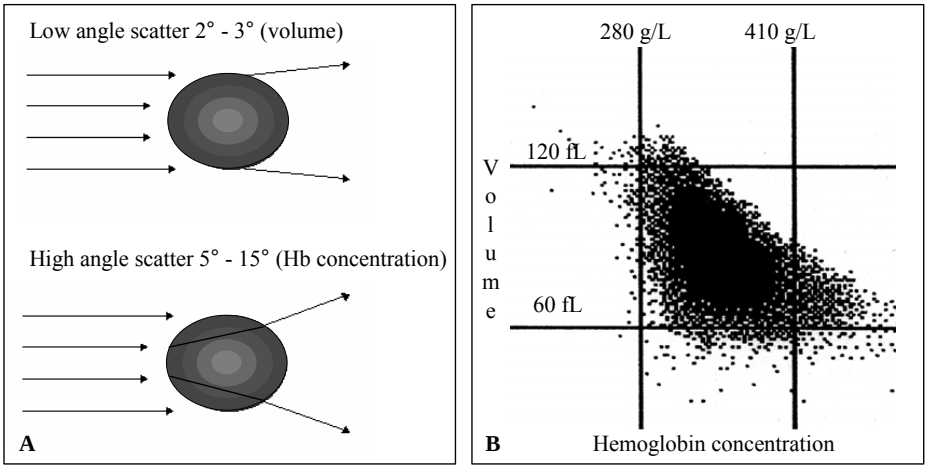


FIGURE 7 A Example of an optical cell counter that is able to record the size and complexity of the individual particles using low- and high-angle scatters. **B** Scatter plot of red blood cells. X-axis indicates hemoglobin concentration cut-offs for normochromic cells. Y-axis indicates volume range of normocytic cells. (Modified from Instructions of ADVIA 120, by kind permission of Siemens, Health Care Diagnostics, Tarrytown, NY, USA).

Basically, in FCM analyses the number of antigen positive cells is counted as a percentage of the total number of cells. However, the development of quantitative FCM methods has enabled the evaluation of the amount of antigen expression. Fluorescence

intensity calibrators are used in quantitative methods (Serke *et al*, 1998; Lenkei *et al*, 1998). Currently, there are three different calibration techniques available in the quantitative assessment of cell surface antigen expression (Serke *et al*, 1998). In quantitative FCM analyses the saturated amounts of fluorescent dye are used for calibrating beads and cells, after which the amount of antigen expression on cells can be calculated as units of antibody binding capacity (ABC) or molecules of equivalent surface fluorochrome (MESF). Antibody binding capacity (ABC) reflects the number of antibodies bound on the cells, which is equal to cellular antigen binding sites (Serke *et al*, 1998; Lenkei *et al*, 1998). Different fluorochromes of a single monoclonal antibody may yield different numbers of cellular binding sites (Serke *et al*, 1998; Lenkei *et al*, 1998). When using quantitative FCM, separate titration measurements are needed for fluorescent ligands on cells and on calibration beads, because they are different binding sites (Serke *et al*, 1998).

2.8.4. Use of red blood cell indices in the diagnosis of iron deficiency

MCV has been widely used for decades in the differential diagnosis of anemias, which has been a basis for categorizing the anemias (Wintrobe, 1932). Iron deficiency causes typically hypochromic and microcytic RBCs, since normal amounts of Hb cannot be synthesized. Hypochromacy has been demonstrated to develop before microcytosis in recently phlebotomized individuals with mild iron deficiency (Patton *et al*, 1991). An incipient iron deficiency can be also normocytic, because the development of microcytosis is a slow process and MCV reflects the whole RBC population during the previous 120 days. A measurement of ZnPP (using a hematofluorometer) is another index reflecting insufficient availability of iron in erythropoietic cells, because in iron deficiency elevated amounts of zinc replaces the iron ion needed for the heme molecule (Blumberg *et al*, 1977; Harthoorn-Lasthuizen *et al*, 1998).

The introduction of light scattering methods has made possible a more strict and sensitive evaluation of RBCs. Novel red cell indices have been established as indicators of iron availability for Hb synthesis of erythropoiesis as they reflect the amounts of Hb inside the red cells and the percentage of hypochromic red cells (Mohandas *et al*, 1986;

Brugnara *et al*, 1993; d'Onofrio *et al*, 1995; Brugnara, 1998; Brugnara, 2000; Mast *et al*, 2002; Kotisaari *et al*, 2002; Franck *et al*, 2004; Thomas *et al*, 2005; David *et al*, 2006). The percentage of hypochromic RBCs (%HYPOm) has been used to monitor functional iron deficiency in patients treated with recombinant human EPO (rHuEPO) (Braun *et al*, 1997; Bovy *et al*, 1999; Schaefer *et al*, 1999). Additionally, cellular Hb in reticulocytes (CHr) has been shown to be a useful tool to check the availability of iron when attaining the target HCT or Hb level in patients with chronic kidney disease, myeloma or lymphoma during rHuEPO treatment (Fishbane *et al*, 2001; Kaneko *et al*, 2003; Katodritou *et al*, 2007). In particular, it has been proposed that CHr is useful in the evaluation and screening of iron status in healthy infants (9- to 12-month-old), children (mean age 2.9 ± 2.0), adolescents and pre-menopausal women, and in a canine model (Brugnara *et al*, 1999; Ullrich *et al*, 2005; Stoffman *et al*, 2005; Fry *et al*, 2006; Luo *et al*, 2007). Additionally, red cell and reticulocyte indices have been shown to provide a simple way to screen the iron status in blood donors (Radtke *et al*, 2005). The documentation of blood doping has also been performed using cell indices such as CHr and RDW in order to demonstrate abnormal hematological profiles, e.g. in elite cross-country skiers (Stray-Gundersen *et al*, 2003). Although CHr and %HYPOm can be determined by only one brand of hematological analyzer (Siemens), other cell counter manufacturers (Sysmex, Abbott, Beckman Coulter) have also provided alternative indices of RBC and reticulocyte features which can be used in a comparable manner in the assessment of iron deficient states. Reticulocyte and RBC Hb equivalents (RetHe, RBCHe) (**TABLE 2**) correlate highly with CHr and cellular Hb in RBCs (CHm), respectively (Briggs *et al*, 2001; Franck *et al*, 2004; Thomas *et al*, 2005; Canals *et al*, 2005; David *et al*, 2006; Brugnara *et al*, 2006).

By measuring RBC and reticulocyte populations, the different time spans of iron homeostasis can be assessed, since the time spans of CHr and %HYPOm are different. Reticulocytes stay in the blood circulation for only 1–2 days before maturing, so their features are rapidly affected by changes in the availability of iron for Hb synthesis of erythroid bone marrow. In contrast, RBC indices reflect iron status for a longer period of time, since their life span is about 120 days. In young iron-deficient females, the response of iron supplementation treatment has been shown to be evident in 7 days on

CHr or percentage of hypochromic reticulocytes (%HYPOr) (Kotisaari *et al*, 2003). Similarly, in hemodialyzed patients receiving frequent EPO treatment, the response of reticulocyte indices to simultaneous intravenous iron therapy occurs in a few days (Brugnara *et al*, 1994b; Fishbane *et al*, 2001). Moreover, %HYPOm has been shown to decrease significantly within two weeks during iron supplementation of 200 mg per day (Kotisaari *et al*, 2003) as well as in 12 weeks during the postpartum period with supplements of 80 mg per day (Krafft *et al*, 2005). Nowadays, at least %HYPOm is recommended for clinical use in patients receiving rHuEPO treatment during frequent dialysis (Schaefer *et al*, 1999).

However, cellular indices have some limitations in specificity, which is a consequence of other hematological conditions such as thalassemias (d'Onofrio *et al*, 1992). This is because thalassemia patients have impaired globin synthesis due to abnormal hereditary features which cause their erythropoiesis to produce microcytic RBCs with low cellular Hb. However, the differential diagnosis between thalassemias and microcytic anemias can also be done by novel indices of cell counters by calculating the ratio between the microcytic and hypochromic red cells (d'Onofrio *et al*, 1992).

The question whether the acute phase response contributes to red cell indices (especially to reticulocyte indices) has also been raised. A slight decrease in reticulocyte Hb content may occur, although the differentiating of iron deficiency anemia and anemia of chronic disease might also be done using reticulocyte indices under inflammatory conditions (Canals *et al*, 2005). The biological background for the changes in the reticulocyte indices is that during acute phase responses iron is blocked inside the macrophages, which may decrease iron availability for Hb synthesis. However, no studies on this topic have been published.

2.9. Common problems in clinical practice of iron status measurements in pregnant women and newborn infants

The iron status measurements currently used are not reliable indicators of iron status during pregnancy. Hb is not an accurate indicator of iron status, especially during

pregnancy, since hemodilution causes a substantial decrease in Hb level during the second and third trimester. Even so, Hb is used in Finland as an indicator for iron supplementation during pregnancy.

In the background of decreased Hb level there may also be iron deficiency. However, iron status cannot be evaluated accurately during pregnancy using current markers. Similarly to Hb, ferritin concentration decreases during pregnancy, because of hemodilution and the subsequent decrease in iron stores, which are utilized for the needs of the fetus, placenta and increased maternal erythropoiesis (Svanberg *et al*, 1975; Scholl *et al*, 1992; Milman *et al*, 1999). Because sTfR is dependent on the availability of iron and the amount of erythropoietic tissue, the concentrations are increased during the later stages of pregnancy (Choi *et al*, 2000a). Transferrin concentration and TIBC are also raised during pregnancy (Morgan, 1961). Of the conventional RBC indices, MCV is increased during pregnancy, possibly due to the contribution of high numbers of reticulocytes, which are larger than mature RBCs (Chanarin *et al*, 1977; Milman *et al*, 2007).

In newborn infants, all laboratory measurements show great variation. This is also found in iron status measurements, and there is also a large variation in red cell indices in cord blood at birth. Therefore, it is hard to set the cut-off limits for abnormal results for the measurements used. Ferritin is the most commonly used indicator of iron status in newborns, and cut-off limits of 60–100 µg/L have been suggested (Rao *et al*, 2002; Siddappa *et al*, 2007). Some investigators have also suggested the use of sTfR or the TfR-F Index (Rusia *et al*, 1995; Kuiper-Kramer *et al*, 1998a; Sweet *et al*, 2001); however, this has been critized because of the influence of increased erythropoietic mass and the day-to-day variation in sTfR in the early days.

2.10. Summary of the literature: what is known and what remains to be discovered?

Iron is essential for life, whereas an excess of iron is toxic. Iron deficiency is easy to treat, in contrast to iron overload. Moreover, the adverse effects of iron deficiency can cause permanent severe damage to the child's cognitive development, for example,

which could be prevented by early identification. The evaluation of iron status using serum iron status measurements is not satisfactory in pregnant women and newborns. Systemic iron homeostasis during pregnancy is in a delicate balance in order to sustain the availability of iron in fetus and for accelerated RBC production in pregnant women.

Our current knowledge of the effects on obstetric outcome of anemia and iron deficiency in pregnant women is based only on findings of studies using traditional methods such as Hb and serum iron status measurements, although these measurements show pregnancy specific alterations. Additionally, they are used to evaluate possible advantages of iron supplementation during pregnancy. Since pregnancy-specific alterations cannot be controlled, the conclusions of the available studies vary.

Iron status can be evaluated using several laboratory analyses involved in the transport, storage and hematological compartments. The different gradations and physiological changes in iron homeostasis hamper the use of available measurements. The development of cell counters has made it possible to address new concepts on the evaluation of iron status using the indices reflecting the Hb content of RBCs and reticulocytes. These indices have been shown to be extremely valuable in screening early changes in the availability of iron in various populations.

EPO synthesis is dependent on tissue oxygenation, which depends on the oxygen-carrying capacity, oxygen affinity, arterial pO_2 and tissue blood flow. The oxygen-carrying capacity depends on the Hb content and RBC mass. However, the relationship between EPO and the quality of RBCs has not yet been fully investigated.

As for serum iron status measurements, the concentration of sTfR depends on both the availability of iron and accelerated erythropoiesis. Currently, quantitative FCM measurement allows the assessment of the amount of cell surface receptors. While reticulocytes are newly formed red cells reflecting the status of bone marrows, the quantitation of reticulocyte TfR expression would reflect the iron availability for erythropoiesis. Thus, the impact of erythropoiesis on sTfR may be avoided.

3. AIMS OF THE STUDY

These studies were carried out in order to determine the iron status parameters which reflect the features of RBCs and reticulocytes and the association of red cell indices with EPO concentration. The study settings were a cross-sectional population of pregnant women and their newborn infants at term in the Pohjois-Savo health care district, in central Finland, and selected patient samples with different gradations of iron status. The specific aims of the studies were as follows:

- To screen the iron status and to test the usefulness and diagnostic accuracy of iron status measurements in pregnant women at term using both serum and cellular iron status markers. (I)

- To determine the usefulness of cellular iron status measurements in diagnosing iron deficiency in newborn infants and to define the reference intervals of iron status measurements in cord blood specimens at birth. (II)

- To discover the associations between serum EPO concentration, pH level and, RBC and reticulocyte indices (reflecting cellular Hb contents) in pregnant women at term and in cord blood at birth. (III, IV)

- To develop a quantitative FCM analysis for TfR expression on reticulocytes in order to use TfR expression as a measure of iron requirement without having the rate of erythropoiesis as an interfering factor. (V)

4. SUBJECTS AND METHODS

4.1. Study designs

4.1.1. Studies on the diagnostic markers of iron status in pregnant women and in their newborn infants (I, II)

The aims of these cross-sectional studies were to screen the iron status in an unselective study population consisting of pregnant women at term and in newborn infants at birth using serum measurements and RBC and reticulocyte indices. The usefulness of the cell indices as indicators of iron deficiency was compared with that of serum iron status measurements in pregnant women and in their newborn infants. The collected data were also used to define the diagnostic efficiency by means of a receiver operating characteristic (ROC) curve and area under the ROC curve (AUC) analyses (I). TfSat was used as an *a priori* reference test for iron deficiency in pregnant women (I). The clinical performance of cell indices was also compared with that of the currently used combination of iron status measurements during pregnancy (Hb, MCV, ferritin) (I). In newborn infants, the associations between cellular and serum iron status measurements were analysed (II). Additionally, the reference values were determined in cord blood at birth of non-anemic newborn infants (II).

4.1.2. Studies on the associations between red blood cell indices and serum erythropoietin concentration (III, IV)

These studies were undertaken to investigate the influence of cellular iron deficiency on the EPO concentration in order to advance the knowledge of associations between the cellular and serum iron status measurements and EPO concentration. The association between iron status measurements and EPO concentrations was primarily studied in pregnant women at term (III).

Additionally, in a secondary study on newborn infants, the aim was to investigate the associations between serum EPO concentration, and RBC and reticulocyte indices as well as between serum iron status measurements (IV). Cellular and serum iron status

measurements were also investigated in three groups of potentially asphyxiated children: “no signs of clinical asphyxia” (umbilical vein cord blood pH not controlled), normal pH (> 7.15) and low pH (≤ 7.15) in order to examine the association between the red cell indices and pH level (IV). The population consisted of the same pregnant women and newborn infants investigated in studies I and II.

4.1.3. Developing the flow cytometric method for transferrin receptor expression on reticulocytes (V)

In this study, an FCM analysis of TfR expression on reticulocytes was developed in order to assess the iron need of erythropoietic tissue at the cellular level. The patient samples used in this study were selectively chosen by red cell index results indicating iron deficiency. A quantitative FCM analysis was performed to calculate the ABCs of TfR (CD71) expression on reticulocytes. The amounts of TfR positive reticulocytes as percentages of all reticulocytes [thiazole orange (TO) positive events] were also recorded. The results of the quantitative FCM method were compared with those of cellular and serum iron status measurements.

4.2. Study subjects

4.2.1. Pregnant women (I, III) and newborn infants (II, IV)

The ethics committee of the Pohjois-Savo Health Care District approved the study. A total of 220 pregnant women and their newborn infants, all of ethnic Finnish background, were enrolled during a 10-month period in 2004–2005 at the Kuopio University Hospital. Informed consent forms had been signed by the pregnant women before delivery. The number of the subjects in the individual studies varied, since adequate amounts were not obtained for each analysis in the samples for some of the subjects, or the gestation was not at term (< 37 weeks). Furthermore, two women with twins were excluded.

The main outcome laboratory measurements were the complete blood count including novel RBC and reticulocyte indices, such as %HYPOm, %HYPOr, CHm and

CHr (**TABLE 2**) before delivery in pregnant women and in cord blood at birth. The serum iron status of the pregnant women and of cord blood were evaluated for iron, transferrin, ferritin and sTfR. TfSat was calculated using the formula [S-Iron (μmol/L) x 0.038]/S-Transferrin (g/L)*100. In addition, the TfR-F Index (sTfR/LogFerritin) was calculated. Serum EPO and high sensitivity C-reactive protein (hsCRP) concentrations were also measured in pregnant women and in cord blood at birth.

Data on maternal characteristics and pregnancy outcome were collected from the birth register of Kuopio University Hospital. These data included the number of previous pregnancies, parity, smoking during pregnancy, pregnancy complications, mode of delivery, gestational age, newborn gender, birth weight and length, placental weight, and Apgar scores at one and five minutes of age. Hb levels in different trimesters were also collected from the register. Most of the Hb results were obtained from the women's maternity cards. Iron supplementation had not been recommended for all the pregnant women during the course of their pregnancy, but they were asked whether they had used iron supplementation or iron-containing multivitamin tablets at any time during the pregnancy.

A total of 67 newborn infants had umbilical vein blood pH measurements at birth. In these newborn infants, the umbilical vein blood pH results was controlled after cord clamping.

4.2.2. Subjects in the flow cytometric study (V)

The ethics committee of the Pohjois-Savo Health Care District approved the study. The studied blood count samples (n = 46) had been drawn in 2005 and in 2007 for clinical purposes from patients treated at Kuopio University Hospital. The samples were selected on the basis of blood count and advanced cell indices. Twelve healthy volunteers served as controls. The whole population consisted of 22 men and 36 women. There were a variety reasons for the basic blood count analyses: surgical procedures (n = 13), hematological malignancies (n = 3), solid tumors (n = 7), cardiovascular diseases (n = 5), kidney failure (n = 3), polycytemia vera (n = 1), and other micellaneous diseases (n = 14). The patient groups were divided retrospectively

according to their iron status based on their %HYPOm, %HYPOr, ferritin and TfR-F Index. Of the 46 patients, 24 were assigned to the functional iron deficiency (FID) group, while the combined FID and iron deficiency (FID + ID) group consisted of 10 patients, and the other 12 patients were assigned to the group with "replete iron status".

4.3. Laboratory methods

4.3.1. Blood count and red blood cell indices (I-V)

Blood counts were analysed on an ADVIA 120 Hematology System (Siemens, Health Care Diagnostics, Tarrytown, NY, USA). Analysis of Hb concentration as a main part of the basic blood count was performed on the ADVIA 120 analyzer using the standardized cyanmethemoglobin measurement (Zwart *et al*, 1996) with a slightly modified application by colorimeter at 546 nm wavelength. All Hb variants were determined with this analysis. Of the red cell indices, Hb, RBC count, HCT, the percentage of reticulocytes (%Retic), MCV, MCVr, MCH, MCHC, CHm, CHr, %HYPOm, %HYPOr and high immature reticulocyte fraction (IRF-H) were recorded.

The advanced red cell indices of the ADVIA 120 System were directly measured based on a cell-by-cell analysis using a single laser for counting and sizing the cells (Mohandas *et al*, 1986). The volume and Hb concentration of isovolumetrically sphered red cells were analyzed using the forward and side light scatters that were measurered on photo-optical detectors at a low (2–3˚) and a high (5–15˚) angle, respectively. Therefore, MCV as well as CH reflecting the mean of the red cell Hb concentration for the red cell population were directly measured parameters on cell-by-cell analysis. MCH and MCHC were derived from the Hb, RBC count and MCV.

The analysis of reticulocytes on the ADVIA 120 System was based on the staining of reticulocyte RNA using a non-fluorescent nucleic acid dye Oxazine 750 (Siemens, Health Care Diagnostics, Tarrytown, NY, USA) after which they were detected by light scattering. As with RBC indices, reticulocyte features can be analyzed with the size and Hb content. In addition, the maturation of reticulocytes can be staged as having low, medium or high RNA-staining intensity (Brugnara, 1998).

TABLE 2 shows the explanations, methods and formulas of the indices that were measured by the ADVIA 120 Hematology System. The scatterplot of the RBC population shows the size (y-axis) and Hb content (x-axis) of the cells (**FIGURE 7**). The normal limits for the red cell populations were set to contain Hb of 280–410 g/L and to be in the size range of 60–120 fL. The red cells that disperse to the area where Hb content is below 280 g/L were defined as hypochromic, and the red cells that were small for size (< 60 fL) were defined as microcytic. MCV and CH are the means of the size and Hb content of cell-by-cell measured cell population histograms.

From the methodological point of view, novel red cell indices are time sensitive, i.e. they are stable for only 12 h after sampling (Brugnara, 2000). In these series, the samples were sent to the laboratory immediately after collection via pneumatic mail, and the cell counter analyses were performed with routine samples as usual within six hours of sampling.

4.3.2. Serum (I-IV) and plasma (V) iron status measurements

Concentrations of ferritin, EPO and high sensitivity C-reactive protein (hsCRP) were measured using an automated Immulite2000-analyzer (IEMA) (Diagnostic Products Corporation, Los Angeles, CA, USA). Serum iron was measured with a Konelab 60i unit (Thermo Fisher Scientific, Vantaa, Finland). Serum transferrin was analyzed using an Immage analyzer (Beckman Coulter Inc., Fullerton, CA, USA). Serum or plasma sTfR was measured using an automated immunoturbidometric IDeA TfR-IT assay (Orion Diagnostica, Espoo, Finland) with a Konelab 60i unit (Thermo Fisher Scientific, Vantaa, Finland) (Suominen *et al*, 1997; Punnonen *et al*, 2000). The umbilical vein blood pH levels were analyzed using Rapidlab 1265 (Siemens, Health Care Diagnostics, Tarrytown, NY, USA).

4.4. Quantitative flow cytometric method for transferrin receptor expression on reticulocytes (V)

The development of quantitative FCM methods has enabled the evaluation of the amount of cellular antigen expression by quantification of fluorescence intensity calibrators. The Quantum™ Simply Cellular® (QSC mouse antibody binding standards, Bangs Laboratories Inc., IN, USA) assay is designed for detecting direct immunofluorescence obtained from labeled antibodies bound on the bead surface including blank and four bead standards for known amounts of antibody binding sites. This is done producing a calibration curve to compare the analysed cell populations with the curve. ABC reflects the number of antibodies bound on the cells, which is equal to the number of cellular antigen binding sites (Serke *et al*, 1998; Lenkei *et al*, 1998).

Venous blood samples were collected in EDTA tubes (Vacutainer™, Becton Dickinson Vacutainer Systems, Plymouth, UK) for the analysis of TfR expression on reticulocytes. A total of 50 µl blood or 25 µl of each QSC standard with 50 µl phosphate buffered saline (PBS) (Isoton® II diluent, Coulter Corporation, Miami, FL, USA) was incubated with 30 µl of CD71-PE monoclonal antibody (mAb) (mouse anti-human-CD71-PE, clone M-A712, BD-Pharmingen, San Diego, CA, USA) for 20 minutes in the dark at room temperature, after which the beads and samples were washed twice with 3 ml PBS (centrifugation 300 g, 5 min, room temperature). The beads were resuspended in 1.0 ml PBS and kept in the dark before analysis. For thiazole orange (TO) labeling, 5 µl of CD71-PE-labeled cells was pipetted into new separate tubes, after which 1.0 ml PBS and 1.0 ml TO-FITC solution (Retic-COUNT reagent, BD Biosciences, San Jose, CA, USA) were added and the cells were incubated for a further 30 minutes in the dark at room temperature.

At least 1 000 events from blank and 4 000 events from standard beads, and at least 200 000 cells from blood samples were acquired using the same flow cytometer [Coulter Epics XL MCL FCM (Coulter Corporation, Miami, FL, USA)] and the same settings. EXPO32 ADC software [XL 4 color and Analysis (build 219.1), Coulter Corporation, Miami, FL, USA] was used for data acquisition and analysis. Blood samples were gated in order to obtain peripheral erythroid cells (gate A), and

reticulocytes (gate B, TO positive events) (**FIGURE 8**). Another gate (gate C) was created to evaluate the amount of TfR positive reticulocytes as percentages of all reticulocytes (%TfR^{+}Ret). Platelets were excluded (gate P). The median channel values of the gated CD71-PE positive reticulocytes were compared with the calibration curve based on QSC beads to calculate the ABC values of cell samples using QuickCal software (version 2.3, Bangs Laboratories Inc., IN, USA). After the FCM analyses, the EDTA-samples were finally centrifuged and plasma was separated and stored frozen at –20 °C.

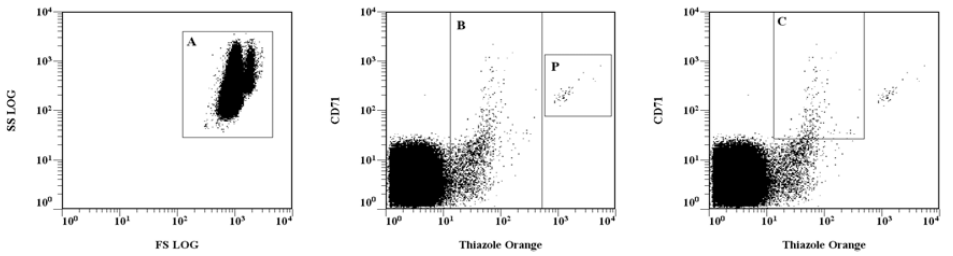


FIGURE 8 Gating strategy in flow cytometric analysis for reticulocytes. Gate A was produced to detect the peripheral red blood cells. Gate B comprised the thiazole orange (TO) positive cells, indicating reticulocytes. The TO positive cells with transferrin receptor (CD71) on the cell surface were included in gate C. Gate P excluded platelets from the analyses.

The stability of the samples for TfR expression was tested on three samples immediately after sampling, and at one, two, four, 24 and 28 hours after storing both at room temperature and at + 4 °C. In addition, the stability of the stained samples was analysed immediately and after storing for one, two, three and four hours at room temperature, and for one, two and three hours storing at + 4 °C. The intra-assay CV% was computed from the results of three different samples that were analysed eight times. The day-to-day variation was analysed between the three days in one individual without signs of iron deficiency.

4.5. Statistical analyses (I–V)

The median and interquartile range (IQR) were calculated. The normality of the laboratory variables was analyzed by means of the Kolmorogov-Smirnov test with the Lilliefors significance correction. The Mann-Whitney U test was used to determine the significances of the differences between the means of continuing variables. The significance of differences for categorical variables was calculated with the Chi-Square test with Yate's correction.

For the pregnant women, primary cut-offs for iron status measurements were selected on the basis of the reference limits used for clinical interpretation in our laboratory for non-pregnant women (I) (Kotisaari *et al*, 2003; Nordin *et al*, 2004; Rustad *et al*, 2004). The reference intervals for the TfR-F Index were calculated from a study population consisting of healthy volunteers (unpublished data). TfSat was used as an *a priori* reference test of iron deficiency, since TfSat is in the centre of iron homeostasis, as it reflects the overlap of absorbed iron, iron released from red cell destruction, iron bypass from the liver and the transfer of iron to the bone marrow or other tissues (I). The efficiency of red cell indices (I) in detecting iron deficiency was evaluated using ROC curve analysis including AUCs and likelihood ratios (LR) (Boyd, 1997). Furthermore, sensitivities and specificities at the optimal cut-off point (maximum point of efficiency curve with a minimal false negative, and false positive results) were derivated (I).

Reference intervals for cord blood at birth (II) were calculated by the non-parametric direct method with use of the 2.5 % and 97.5 % reference limits in non-anemic newborn infants.

Pearson or Spearman correlations were used to evaluate the associations between the variables (I–V). Subgroups were based on the quartiles or on the different disease categories (III–V). One-way ANOVA with the significances of Bonferroni (III) or Tukey (V), or Kruskall-Wallis tests (IV) were used to calculate the differences between the groups. For independent variables, multivariate stepwise linear regression analyses were performed (III).

Statistical significance was defined as $p < 0.05$. Microsoft Excel 2000 for Windows (Microsoft Office, Microsoft Corp., Redmond, WA, USA) and SPSS 11.5 for

Windows (SPSS Inc., Chicago, ILL, USA) were used as statistical software. The figures were produced with the GraphPad Prism 4.0 for Windows (GraphPad Software Inc., San Diego, CA, USA) and with Microsoft Excel 2000. For calculating reference intervals, GraphRoc for Windows, Version 2.0 (GraphROC, developed by V. Kairisto and A. Poola) was used (Kairisto *et al*, 1995).

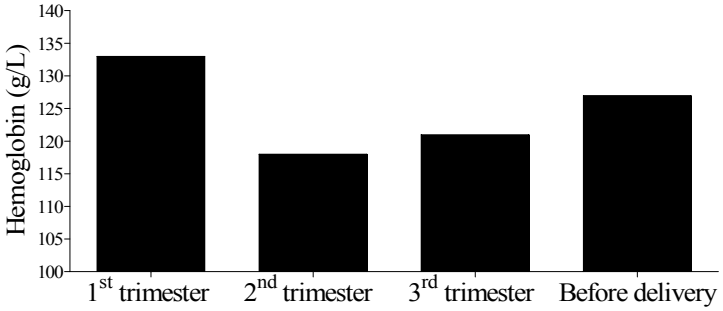
5. RESULTS

5.1. Screening of iron deficiency in pregnant women and newborn infants (I, II)

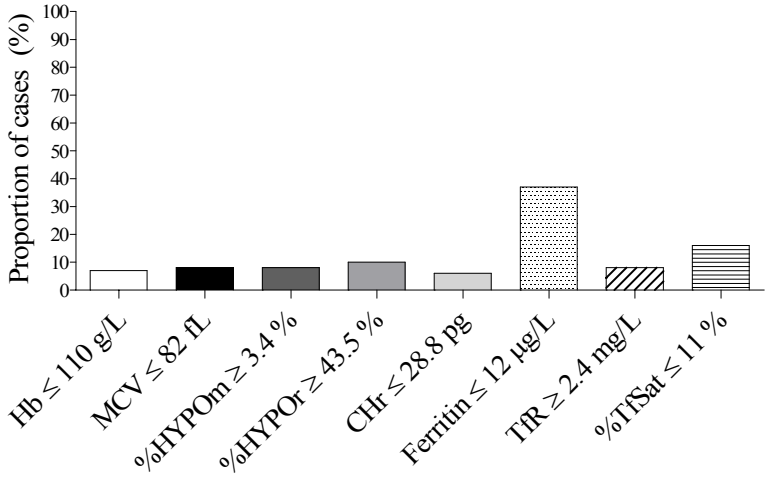
In study I there were 202 pregnant women who had results of Hb, %HYPOm, %HYPOr, CHr, sTfR, TfSat and ferritin, with the exception of four pregnant women who did not have TfSat results. Of these women, 31 (16 %) were classified as iron deficient based on TfSat according to the definition (TfSat ≤ 11 %). Anemia was rare (7 %, n = 14) among pregnant women at term, but much more frequent when the entire pregnancy was taken into account: 3 during the first, 45 during the second and 31 during the third trimesters. **FIGURE 9 A** shows a trend of mean Hb level of pregnant women during pregnancy. Low ferritin results (≤ 12 µg/L) were found in 38 % of the pregnant women, and sTfR was elevated in 8 %. Cellular iron deficiency was found at term in 17, 21 and 13 pregnant women judged by %HYPOm, %HYPOr and CHr, respectively (**FIGURE 9 B**).

Not all the women who had anemia or who had a single serum marker indicating iron deficiency had a low CHr or an increased %HYPOm (**TABLE 3**). If the iron deficiency diagnosis was based on CHr, all the woman had lowered ferritin. However, a low ferritin result was found in a number of the women without signs of decreased Hb content in RBCs or reticulocytes (**TABLE 3**).

A total of 199 newborn infants were studied. Although many newborn infants were anemic (n = 38), only nine had iron deficiency as defined by a TfSat level less than 30 % (Nathan *et al*, 1993). Ferritin was low (< 60 µg/L) in 13 newborn infants. Among the anemic newborn infants only one had low ferritin (34 µg/L), and another one had low TfSat (24 %). Therefore, in the study population there were only two newborn infants with iron deficiency anemia.



A



B

FIGURE 9 A Trend of hemoglobin results during pregnancy from the women's maternity cards and from measured blood count before delivery. **B** The percentages of pregnant women classified as iron deficient on the basis of a single marker of iron status.

TABLE 3 The comparison of CHr and %HYPOm with other iron status markers in diagnosing iron deficiency in 202 pregnant women at term.

	CHr	
	CHr ≤ 28.8 pg (n = 13)	CHr > 28.8 pg (n = 189)
Hb ≤ 110 g/L	5 (38.5 %)	9 (4.8 %)
MCV ≤ 82 fL	9 (69.2 %)	8 (4.2 %)
%HYPOm ≥ 3.4 %	8 (61.5 %)	9 (4.8 %)
%HYPOr ≥ 43.5 %	12 (92.3 %)	9 (4.8 %)
Ferritin ≤ 12 µg/L	13 (100 %)	61 (32.3 %)
sTfR ≥ 2.4 mg/L	5 (38.5 %)	11 (5.8 %)
	%HYPOm	
	%HYPOm ≥ 3.4 % (n = 17)	%HYPOm < 3.4 % (n = 185)
Hb ≤ 110 g/L	6 (35.3 %)	8 (4.3 %)
MCV ≤ 82 fL	9 (52.9 %)	8 (4.3 %)
CHr ≤ 28.8 pg	8 (47.1 %)	5 (2.7 %)
%HYPOr ≥ 43.5 %	11 (64.7 %)	10 (5.4 %)
Ferritin ≤ 12 µg/L	13 (76.5 %)	61 (33.0 %)
sTfR ≥ 2.4 mg/L	4 (23.5 %)	12 (6.5 %)

5.2. The features of red cell indices in pregnant women and in newborn infants (I, II)

The means and medians of RBC and reticulocyte indices of the pregnant women at term and of the non-pregnant controls were within the reference limits for non-pregnant women (**TABLE 4**). The percentage of reticulocytes was the only index that was near to the upper reference limit in the pregnant women. Additionally, in this population of pregnant women at term, cellular indices (%HYPOm, %HYPOr and CHr) did not show remarkable signs of iron deficiency and their descriptives were similar to those of non-pregnant women (**TABLE 4**).

TABLE 4 Red cell indices in pregnant women at term and in non-pregnant control women.

	Pregnant women at term (n = 202)				**Non-pregnant women controls (n = 77)**				**Reference limits for non-pregnant control women**
	Mean	SD	Median	IQR	Mean	SD	Median	IQR	
Hb (g/L)	127	± 10	127	13	133	± 8	132	11	117 - 155
MCV (fL)	88	± 5	88	6	88	± 4	88	6	82 - 98
MCH (pg)	30	± 2	30	2	30	± 2	30	2	27 - 33
MCHC (g/L)	339	± 10	339	12	339	± 10	336	15	315 - 360
%Retic (%)	1.8	± 0.4	1.8	0.5	1.2	± 0.4	1.2	0.5	0.6 - 2.0
%HYPOm (%)	1.1	± 2	0.4	0.9	1.5	± 2.4	0.7	1.2	0.1 - 3.4
%HYPOr (%)	20.2	± 16.8	14.4	20.2	19.7	± 16.3	14.4	17.3	3.7 - 43.5
CHr (pg)	32.3	± 2.2	32.5	2.6	31.5	± 1.7	31.6	2.6	28.8 - 34.5

During the last weeks before labor most pregnant women had adequate amounts of iron for Hb synthesis since they did not have iron deficient reticulocytes or iron deficient erythrocytes at term. Furthermore, in most of the women studied (93 %), the CHr/CHm ratio was above 1.00 (mean ± SD, 1.07 ± 0.05), which suggests stable ongoing erythropoiesis (Brugnara, 1998). **FIGURE 10** shows examples of scatter plots of RBC populations in pregnant women with normal %HYPOm and with highly increased (16.7 %) %HYPOm.

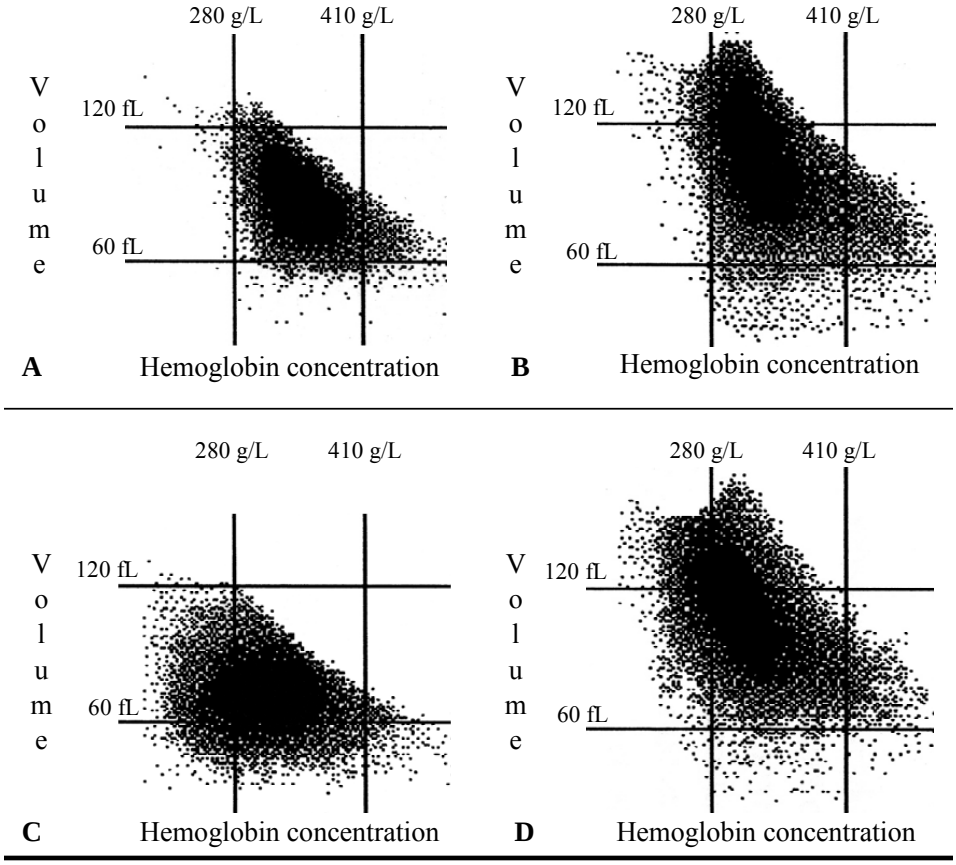


FIGURE 10 The upper columns show the scatter plots of red blood cells in a pregnant woman (**A**) and in a newborn infant (**B**) without iron deficiency. The lower columns show the scatter plots of red blood cells in a pregnant woman (**C**) and in a newborn infant (**D**) with iron deficiency.

In newborn infants, RBC and reticulocyte indices have their own distinctive features: the normal variation of the individual RBC population is wider than in pregnant women or non-pregnant adults (**FIGURE 10**) (Brugnara, 2000). The RBC populations in newborns' contain a considerable amount of red cells that are macro- or microcytic, so, RDW is large. Mean CH of RBC and reticulocyte populations are also higher in cord blood at birth than later in life (Brugnara, 2000). If the main RBC population is located near the lower x-axis or on the left side in a scattergram (hypochromic site), indicating iron deficiency in adults (**FIGURE 10**), a similar location is also evident in iron-deficient newborns. However, newborn infants without iron deficiency typically also have a great amount of hypochromic RBCs, which is associated with an increase in macrocytic red cells. Consequently, in many cord blood samples, macrocytic RBCs contain less Hb (showing a shift to the left in a scatter gram in cells of high volume) (**FIGURE 10**). MCH, mean cell volume of reticulocytes (MCVr), RDW and the absolute reticulocyte count of newborn infant are larger than later in life (Brugnara, 2000). On the other hand, there are no marked shifts in the values of cellular Hb contents or in the cellular volumes, because the means of the histograms remain relatively stable. Generally, however, the mean cell volume is higher in cord blood than in adults (**FIGURE 10**).

The reference ranges of the red cell indices and serum iron status measurements in cord blood are shown in **TABLE 5**. Along with the large variation evident in the cell population of individual cord blood samples, a wide variation in red cell indices was observed. There were no statistically significant gender differences in the serum iron markers or in the cellular indices (Mann-Whitney U test).

5.3. Diagnostic accuracy of red blood cell indices in diagnosing iron deficiency in pregnant women at term (I)

The diagnostic accuracy of RBC and the reticulocyte indices were calculated using TfSat as an *a priori* reference test with a cut-off value of ≤ 11 %. In ROC and AUC analyses, advanced red cell indices provided remarkably high AUCs: %HYPOr 0.80, CHr 0.79 and %HYPOm 0.75. Additionally, ferritin alone and in combined with the

TfR (TfR-F Index) provided high AUCs (0.77 and 0.75, respectively), but their likelihood ratios were low (study I, Table 3). TfSat (≤ 11 %) was also used as a reference test when calculating the optimal cut-off levels (maximum point of efficiency curve) for the other iron status measurements such as Hb, MCV and ferritin. The optimal cut-off levels were for Hb ≤ 122 g/L, MCV ≤ 87 fL and ferritin ≤ 11 µg/L. When using the combination of Hb, MCV and ferritin (with these calculated cut-off points) as a reference test, the AUCs for individual RBC or reticulocyte indices were high: CHr 0.95, %HYPOm 0.96, %HYPOr 0.96 (study I, Table 4). Since only a few iron-deficient anemic pregnant women were identified, the presentation of diagnostic accuracies was not considered meaningful, and the same was true for the iron-deficient newborn infants.

TABLE 5 Results of iron status measurements in 199 newborns infants at term. Reference intervals in cord blood at birth (2.5 % and 97.5 % reference limits) for iron status measurements are calculated for 163 non-anemic (Hb > 146 g/L) newborn infants.

Variable	Mean	SD	Median	Reference intervals
Blood count and cellular indices				
Hb (g/L)	159	15	160	146 - 189
HCT (L/L)	0.49	0.05	0.49	0.44 - 0.58
MCV (fL)	109	4	109	102 - 118
MCVr (fL)	124	6	124	115 - 136
MCH (pg)	35	1	35	33 - 38
MCHC (g/L)	325	10	325	306 - 342
%Retic (%)	4.0	0.8	3.9	2.6 - 5.4
IRF-H (%)	24.1	7.8	23.2	10.2 - 40.0
CHm (pg)	34.9	1.3	34.8	32.5 - 37.2
CHr (pg)	35.6	1.3	35.5	33.1 - 38.6
%HYPOm (%)	3.0	3.0	2.1	0.4 - 9.9
%HYPOr (%)	42.0	15.6	40.7	18.3 - 76.8
Serum measurements				
sTfR (mg/L)	2.0	0.7	1.9	1.2 - 4.0
Ferritin (µg/L)	198	137	166	45 - 636
TfR-F Index	0.95	0.43	0.87	0.49 - 2.1
Iron (µmol/L)	27.4	7.7	27.0	12.2 - 42.1
Transferrin (g/L)	2.0	0.4	1.9	1.2 - 2.9
TfSat (%)	55	19	54	21 - 111

Abbreviations are explained in **TABLE 2**.

5.4. Correlations between serum and cellular iron status measurements in pregnant women and newborn infants (I, II)

The correlation matrix of the serum and cellular iron status measurements in the pregnant women is presented in **TABLE 6** (I). A similar matrix for newborn infants is shown in study II (Table 3). In the women, the correlations between the CH and HYPO indices were highly significant, whereas in newborn infants they were not. In the women, Hb correlated more tightly with cellular indices (%HYPOm, %HYPOr) than with serum iron status markers (ferritin, iron, TfSat). Moreover, the correlation between Hb and transferrin as well as sTfR was not significant in the women. The results were opposite in newborn infants, where Hb correlated significantly with sTfR but not with %HYPOm, %HYPOr, CHm and CHr (II).

5.5. Association between serum erythropoietin concentration and cellular indices (III, IV)

Cellular iron status indices correlated significantly with serum EPO in the pregnant women and in cord blood at birth (III, IV). The most significant correlations were found between the EPO concentration and %HYPOr ($r = 0.57$, $p < 0.001$), %HYPOm ($r = 0.52$, $p < 0.001$) and CHr ($r = - 0.45$, $p < 0.001$) in the women, and between EPO and %HYPOr ($r = 0.56$, $p < 0.001$), MCVr ($r = 0.50$, $p < 0.001$) and %HYPOm ($r = 0.45$, $p < 0.001$) in cord blood. Furthermore, serum iron status measurements correlated significantly with serum EPO concentrations in the women (sTfR $r = 0.26$, $p < 0.001$; ferritin $r = - 0.32$, $p < 0.001$; transferrin $r = 0.14$, $p = 0.046$) and in cord blood (sTfR $r = 0.42$, $p < 0.001$; iron $r = - 0.28$, $p < 0.001$; transferrin $r = 0.31$, $p < 0.001$; TfSat $r = - 0.44$, $p < 0.001$) (III, IV). Similarly, in the quartile analyses, significantly higher %HYPOm and %HYPOr values were found in the highest EPO concentration quartiles of the women, and of cord blood, than in the lowest EPO concentration quartiles (**FIGURE 11**).

TABLE 6 The correlation (Spearman) matrix of serum and cellular iron status measurements in pregnant women (n = 202).

	MCV	**CHm**	**CHr**	**%HYPOm**	**%HYPOr**	**sTfR**	**Ferritin**	**TfR-F Index**	**Transferrin**	**Iron**	**%TfSat (n = 198)**
Hb	0.12	0.27**	0.25**	- 0.36**	- 0.38**	- 0.03	0.28**	- 0.15*	0.07	0.18*	0.16*
MCV		0.87**	0.63**	- 0.48**	- 0.24**	- 0.16*	0.26**	- 0.22**	- 0.24**	0.11	0.20**
CHm			0.76**	- 0.70**	- 0.48**	- 0.22**	0.35**	- 0.30**	- 0.21**	0.23**	0.31**
CHr				- 0.68**	- 0.79**	- 0.34**	0.57**	- 0.49**	- 0.22**	0.28**	0.37**
%HYPOm					0.77**	0.41**	- 0.54**	0.53**	0.25**	- 0.19**	- 0.29**
%HYPOr						0.38**	- 0.64**	0.55**	0.18*	- 0.31**	- 0.38**
sTfR							- 0.40**	0.90**	0.30**	- 0.16*	- 0.26**
Ferritin								- 0.73**	- 0.26**	0.24**	0.34**
TfR-F Index									0.32**	- 0.23**	- 0.34**
Transferrin										0.16*	- 0.14*

Two-tailed correlation is significant at the ** 0.01 and * 0.05 level, respectively.

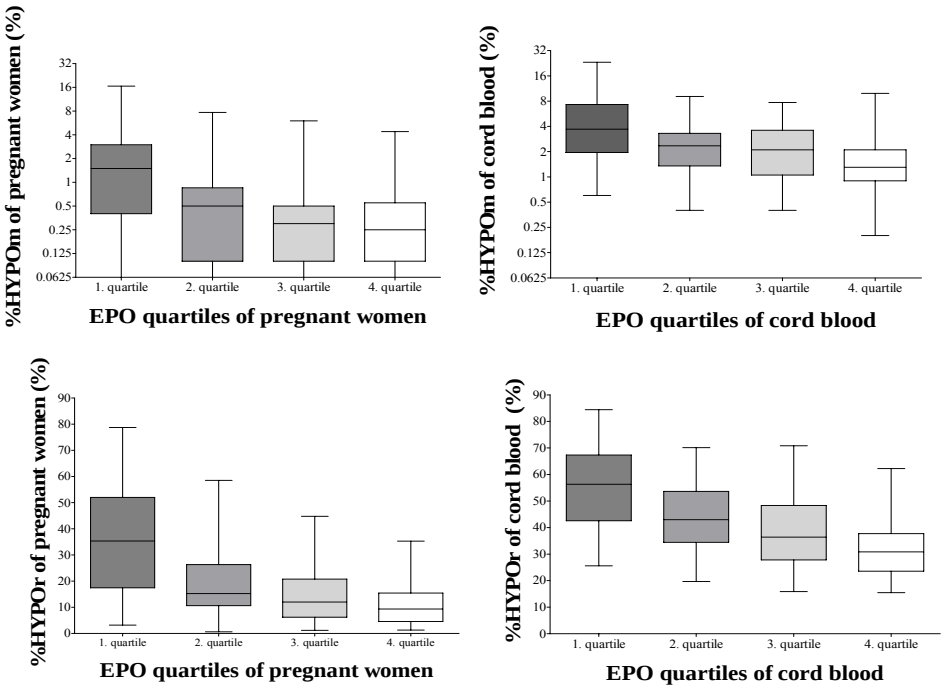


FIGURE 11 Box-plots of %HYPOm and %HYPOr in the quartile groups of erythropoietin (EPO) concentration of pregnant women and cord blood at birth.

5.6. Association between pH and cellular indices in cord blood at birth (IV)

In **FIGURE 12**, box-plots of the Hb, MCHC, %HYPOm and %HYPOr results are shown in the groups of newborn infants with no signs of clinical asphyxia (n = 126, pH not analysed), normal pH (> 7.15, n = 51) and low pH (≤ 7.15, n = 16). Newborn infants with low umbilical vein blood pH had significantly higher %HYPOm, %HYPOr, and IRF-H, and lower MCHC than newborn infants with normal pH. In addition, their EPO levels were higher, as were sTfR and the TfR-F Index (study IV, Table 4). The correlations between pH level, serum and cellular iron status measurements are shown in Table 3 in study IV.

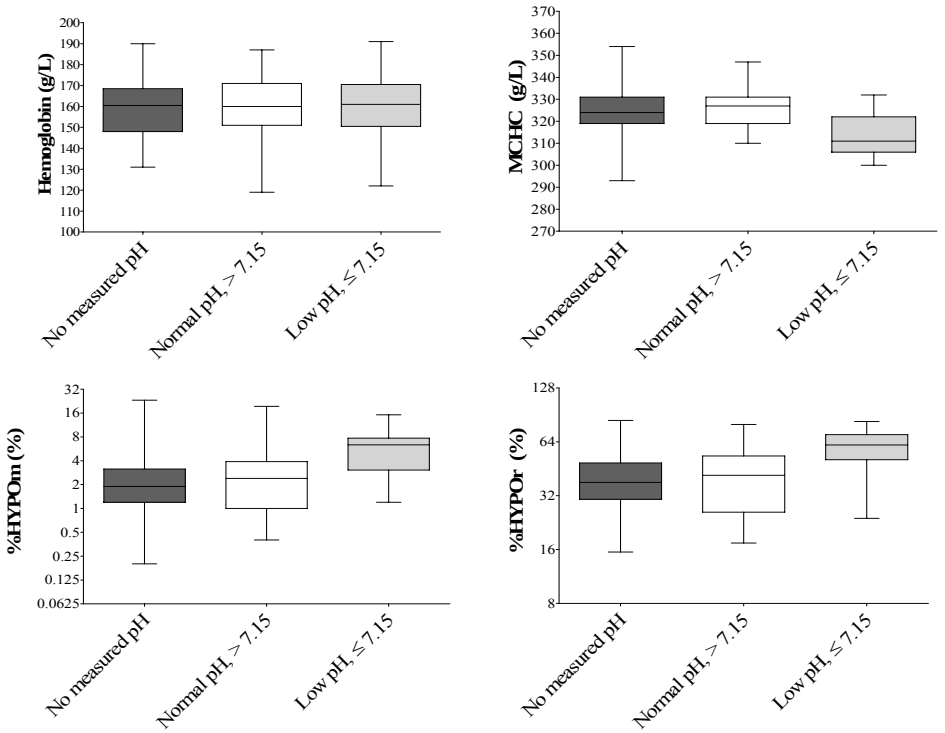


FIGURE 12 Box-plots of Hb, MCHC, %HYPOm and %HYPOr in the groups of newborn infants without clinical signs of asphyxia (no pH measurements, n = 126), with normal pH (> 7.15, n = 51) and with low pH (≤ 7.15, n = 16).

5.7. Quantitative flow cytometric method for transferrin receptor expression on reticulocytes (V)

The quantitative expression of the TfR on reticulocytes was measurable by FCM (V). The stability of TfR expression on reticulocytes in EDTA samples was excellent, as the ABC of TfR expression of three samples remained stable for up to 28 hours (CV% 2.6–3.0 %) when the samples were stored at room temperature or at + 4 °C (unpublished data). However, after the samples were stained, the median of TfR expression increased progressively (unpublished data), which is why the samples should be analysed immediately after incubation in TO-dye. Intra-assay variability (CV%) ranged from 4.2 % to 12.4 % for ABC of TfR expression, and from 2.4 % to 10.6 % for %TfR^{+}Ret (V). The day-to-day variation was calculated between the three days in one individual, and

the CV% ranged from 3.3 % to 8.7 % for ABC of TfR expression and from 3.3 % to 22.8 % for %TfR$^+$Ret (V).

The Spearman correlation coefficient between ABC and %TfR$^+$Ret was highly significant, being 0.96 ($p < 0.001$) (**TABLE 7**). ABC and %TfR$^+$Ret correlated significantly especially with the reticulocyte indices, but also with Hb, HCT and RBC indices (**TABLE 7**). There were no significant correlations between serum sTfR or ferritin, and ABC or %TfR$^+$Ret (**TABLE 7**). In addition, there was no correlation between absolute amounts of reticulocytes and ABC or %TfR$^+$Ret, although %Retic correlated significantly with ABC and %TfR$^+$Ret. This may be partly due to iron-deficient patients having higher %Retic.

Low ABC levels and a low %TfR$^+$Ret were seen especially in the control group without iron deficiency. The patients who were categorized by their iron status into the FID or FID+ID groups had significantly higher ABC values than controls or patients with replete iron status (**TABLE 8**). Moreover, patients in the iron deficient groups had higher %TfR$^+$Rets (**TABLE 8**).

TABLE 7 Spearman correlations between antibody binding capacity (ABC), percentage of transferrin receptor positive reticulocytes (%TfR$^+$Ret) and serum and cellular iron status measurements in 58 subjects.

	ABC	**%TfR$^+$Ret**	**Reticulocyte count**	**%Reticulocytes**
Hb	- 0.528**	- 0.554**	- 0.410**	- 0.500**
Hct	- 0.528**	- 0.540**	- 0.414**	- 0.514**
IRF-H	0.652**	0.682**	0.500**	0.632**
MCV	- 0.167	- 0.149	0.414**	0.354**
CHm	- 0.353**	- 0.344**	0.306*	0.181
CHr	- 0.602**	- 0.596**	0.055	- 0.022
%HYPOm	0.598**	0.578**	0.094	0.271*
%HYPOr	0.718**	0.746**	0.204	0.369**
sTfR	0.246	0.258	- 0.099	0.026
Ferritin	0.189	0.227	0.305*	0.307*
TfR-F Index	0.052	0.047	- 0.243	- 0.167
ABC		0.956**	0.188	0.362**
%TfR$^+$Ret			0.214	0.366**
Reticulocyte count				0.932**

Two-tailed correlation is significant at the ** 0.01 and * 0.05 level.

TABLE 8 Antibody binding capacity (ABC) and percentage of transferrin receptor positive reticulocytes (%TfR^{+}Ret) in 58 subjects in control, replete, functional iron deficiency or functional iron deficiency + iron deficiency groups.

	Controls (n = 12)	**Replete** (n = 12)	**FID** (n = 24)	**FID + ID** (n = 10)
ABC (gate B)	663 ± 110	897 ± 329	1763 ± 922**	1441 ± 727*
%TfR^{+}Ret (%)	17.9 ± 6.2	22.9 ± 8.9	40.1 ± 9.8**	36.1 ± 9.8**

Difference between the controls and the patient groups (one-Way-ANOVA with Tukey) is significant at the **0.01 and *0.05 level.

6. DISCUSSION

The basic goal of clinical laboratories is to serve clinical practice and help clinicians to make diagnoses, to monitor patients, or to make treatment decisions by developing the most appropriate, simple, straightforward, and cost-effective methods. The goal can be reached by developing more accurate tests, using the applications of modern medical laboratory sciences.

Since iron homeostasis plays a crucial role in human life, both iron deficiency and iron overload need accurate measurement already before diseases become manifest. There are many laboratory tests for the evaluation of iron balance, some of which are quite sensitive and specific markers in clinical settings. However, they are inaccurate and unable to reflect accurately the dynamic process and different phases of iron homeostasis with particular patient groups (e.g. pregnant women, children) and in some conditions (e.g. increased erythropoiesis). Since most of the iron is in Hb molecules of RBCs, and the appropriate laboratory technique is available, there is increasing interest in evaluating iron status at the cellular level. In the present studies, the parameters reflecting cellular iron status were investigated in pregnant women and their newborn infants. The correlations between the iron status measurements and EPO concentration were also studied in these populations.

Additionally, as a result of the development of quantitative FCM, new methods have been introduced to measure the amount of antigen expression on the cell surface. This was investigated by measuring TfR expression on the surface of reticulocytes in order to analyse the cellular need for iron in selectively chosen hospital patients with different stages of iron status.

6.1. Validity of the results: strengths and weaknesses of the studies

In studies I–IV on pregnant women and their newborn infants, the subjects were recruited unselectively at Kuopio University Hospital, which covers the area of the Pohjois-Savo Health Care District (population about 150 000). In this area, the deliveries are concentrated in one major hospital, with approximately 2 400 deliveries

per year. The pregnant women were recruited consecutively during working hours from the women who were admitted to the hospital for delivery and who were willing to participate in the study, which investigated both pregnant women and cord blood at birth. Only a minority of the pregnant women were recruited from the ward before delivery. An unselective approach like this provides an appropriate representative population for screening and cross-sectional comparison of the subjects, and also makes it possible to establish reference values for laboratory tests, which was one purpose in the present studies. In these studies (I–IV), only a few subjects (2–10 %) were excluded from particular sets of data analysis because some of the laboratory test results were missing. Two pregnant women were excluded because they gave birth to twins, and four were excluded because their babies' gestational age was less than full-term (< 37 weeks). Therefore, excluding subjects did not affect the validity of the results. When evaluating iron status using the RBC and reticulocyte indices, we should take into account the possibility that thalassemias may be an interfering factor (d'Onofrio *et al*, 1992). However, since these studies (I–V) involved only a population with an ethnic Finnish background living in the Pohjois-Savo area, we can assume that it did not contain thalassemia patients, as the thalassemias are so rare in the Finnish population.

The iron status of pregnant women (I–IV) was determined only at term. However, since measurement at the time of delivery reflects the final balance of iron distribution between the mother and fetus, we expected to find considerable differences in the iron status of the pregnant women, which, in turn, could have provided good population estimates for possible differences between various parameters. However, this collection protocol does not provide results for evaluating iron status during pregnancy, and therefore further studies are warranted to evaluate the usefulness of RBC and reticulocyte indices during the course of pregnancy.

A weakness of the studies I–IV is that it was not possible to adjust the amounts of supplemented iron taken by the subjects. The data on iron supplementation were obtained only at the time of delivery, after consent forms had been signed. Therefore, the iron medication taken by subjects may have influenced the iron status in both the pregnant women and their newborn infants. It may be assumed that providing iron medication reduces the likelihood of finding iron-deficient subjects and improves the

iron status of the subjects. In any case, these studies were not performed in order to evaluate the benefits or disadvantages of iron supplementation on pregnancy outcome, pregnant women or newborn infants. Again, on the basis of these results, only the final balance of iron homeostasis between the mother and the fetus can be estimated.

Study IV investigated the relationship between EPO and cord blood pH results and iron status. A weakness in this study was that pH results were not available for all newborn infants because pH measurements were done only if a newborn had had clinical indications for pH measurements. This is why statistical comparisons between the newborn infants with or without clinical signs of asphyxia could not be performed. Consequently, only the descriptives of the iron status analyses in newborn infants with incomplete pH data were presented.

In study V, in which the quantitative FCM analysis for evaluating the iron need of developing erythroid cells was developed, the use of a selective study population was the most appropriate in order to achieve samples in different stages of iron status. The samples were chosen on the basis of cell counter indices, which have been shown to reflect different phases of iron-deficient erythropoiesis.

6.2. Screening the iron status: from hemoglobin to red blood cell indices

The basic blood count, including the number of leucocytes, erythrocytes and thrombocytes, and Hb concentration, is one of the main laboratory tests in clinical medicine. Although Hb concentration is of great value in judging and monitoring anemia, the current techniques give more accurate measurements to evaluate cellular Hb (Mohandas *et al*, 1986). The main clinical interest in the RBC and reticulocyte indices has been their use in distinguishing between different anemias on the basis of the size of the red cells (Wintrobe, 1932). The main purpose of the new RBC and reticulocyte indices has been the monitoring of iron status in patients receiving rHuEPO treatment (Fishbane *et al*, 2001; Katodritou *et al*, 2007).

TABLE 9 Studies on diagnostic accuracy using cellular indices as markers of iron deficiency or iron-deficiency anemia.

Study	Patients	Diagnostic criteria	Variable	AUC
Brugnara *et al*, 1999	210 children (mean ± age, 2.9 ± 2.0)	TfSat < 20 %	CHr	0.78
			Ferritin	0.57
Mast *et al*, 2002	78 patients undergoing bone marrow examination	Lack of iron in bone marrow	MCV	0.505 or 0.570 (in patients with MCV > 100 fL)
			CHr	0.642 or 0.735 (in patients with MCV > 100 fL
			Ferritin	0.660 or 0.690 (in patients with MCV > 100 fL)
Kotisaari *et al*, 2002	34 anemic female students	sTfR ≥ 2.4 mg/L	CHr	0.86
			CHm	0.88
			%HYPOr	0.90
			%HYPOm	0.98
	95 anemic hospitalized patients	sTfR ≥ 2.4 mg/L	%HYPOm	0.77
			%HYPOr	0.67
Ullrich *et al*, 2005	202 infant (9–12 months old)	TfSat < 10 %	Hb	0.73
			CHr	0.85
Radtke *et al*, 2005	1142 blood donors	*Log TfR/Ferritin ≥ 2.5	Hb	0.7350
			CHr	0.8301
**Note that ferritin is included in the ID diagnosis (Log TfR/Ferritin)*			CHm	0.8493
			%HYPOm	0.8331
			%HYPOr	0.8263
			*Ferritin	0.9777
Fry and Kirk 2006	7 dogs	TfSat < 28 %	MCV	0.748
			MCHC	0.654
			CHr	0.881
			%HYPOr	0.841
		TIBC > 365 µg/dL	MCV	0.852
			MCHC	0.653
			CHr	0.899
			%HYPOr	0.945
Ervasti *et al*, 2007	198 pregnant women at term	TfSat ≤ 11 %	MCV	0.63
			CHr	0.79
			%HYPOm	0.75
			%HYPOr	0.80
Luo *et al*, 2007	172 premenopausal women	Ferritin ≤ 14 µg/L and Hb ≥ 110 g/L	Hb	0.827
			MCV	0.817
			CHr	0.892
		Ferritin ≤ 14 µg/L and Hb ≤ 110 g/L	Hb	0.900
			MCV	0.886
			CHr	0.928

On the basis of several studies, RBC and reticulocyte indices offer the possibility to diagnose, monitor and screen the iron balance. RBC and reticulocyte indices reflect iron status at the cellular level, so they are dependent on the relationship between iron

supply and demand in bone marrow. When insufficient iron is available, more RBCs containing less Hb are produced. The novel red cell indices have been proved to be more accurate than Hb and ferritin in screening iron status (**TABLE 9**) (Brugnara *et al*, 1999; Mast *et al*, 2002; Kotisaari *et al*, 2002; Ullrich *et al*, 2005; Radtke *et al*, 2005; Fry *et al*, 2006; Luo *et al*, 2007; Ervasti *et al*, 2007a). Although in these studies the diagnostic criteria of iron deficiency vary or are combined with anemia, the AUCs of novel cellular indices exceed those of ferritin or Hb as well as conventional RBC indices such as MCV or MCHC. If iron-deficiency anemia was evaluated in these studies, the RBC and reticulocyte indices provided greater AUCs in comparison with the evaluation of iron deficiency alone. This may be due to the more severe stage of iron deficiency in the former (**TABLE 9**).

The lack of an appropriate gold standard has been a problem in evaluating the diagnostic accuracies of iron status markers. In some cases, bone marrow iron staining has been used as the gold standard. The accuracy of RBC indices from bone marrow samples was assessed in only one of the studies in **TABLE 9** (Mast *et al*, 2002). However, since bone marrow sampling is invasive, it is inappropriate in many clinical situations and especially for screening purposes. Using bone marrow examination, it is also impossible to assess accurately the amount of iron stores, and only rough stages can be graded.

6.3. Advantages and disadvantages of red blood cell indices in the evaluation of iron status

While Hb alone has been used as a parameter for screening the iron status for decades, it is now possible to estimate the changes in the Hb content of RBCs and reticulocytes. Tools (mechanic, electronic and optic) to measure cell indices are available and the development of cell counters concentrates on the handling of data and on the development of the software. An important benefit of advanced RBC and reticulocyte indices is the rapid real-time monitoring of the changes in erythropoiesis, as the indices can be measured in RBC and reticulocyte populations. This facilitates the detection of iron deficiency already in the subclinical phase before manifested anemia. Moreover,

the cell indices are cost-effective in clinical practice, because the results can be printed out along with the data included in the basic blood count without any need for other reagents (Brugnara *et al*, 1999; Ullrich *et al*, 2005; Radtke *et al*, 2005). However, it should be kept in mind that by using the RBC and reticulocyte indices, the assessment of iron availability for erythropoiesis in cellular compartment can be performed, but that is not a measure of the total amount of iron in the body.

The major disadvantage of the use of novel red cell indices has been for a long time the fact that only one modern cell counter manufacturer has provided and patented the method of the indices (Mohandas *et al*, 1986). However, several cell counter manufacturers have now developed the corresponding indices (Franck *et al*, 2004; Thomas *et al*, 2005; David *et al*, 2006; Brugnara *et al*, 2006), which have been shown to accurately reflect iron-deficient erythropoiesis.

According to one study (Mast *et al*, 2002), the presence of variable numbers of macrocytic RBCs may hamper the use of the RBC and reticulocyte indices in accurately diagnosing iron status. Actually, this phenomenon of increased RBC volume has also been documented in patients undergoing cardiac surgery who are receiving rHuEPO treatment, and in newborn infants (study II), who typically have accelerated erythropoiesis (Sowade *et al*, 1998; Ervasti *et al*, 2007b). Thus, this may be due to increased amounts of reticulocytes rather than the impact of MCV itself. This might be one component contributing to the great variation in decision limits for %HYPO (3.7–10 %) that have been suggested for the assessment of iron status in patients with chronic kidney disease who are receiving regular rHuEPO treatment (Fishbane *et al*, 2001; Kaneko *et al*, 2003). This phenomenon might also be a reason for the impaired response of %HYPOm to intravenous iron treatment in patients who need increasing rHuEPO doses, even though the correct Hb level has been attained (Sunder-Plassmann *et al*, 1997).

If sufficient iron is not available for Hb synthesis, the progression of iron deficiency causes changes in all iron compartments (Suominen *et al*, 1998). Basically, in pure progressive iron deficiency, an inadequate supply of iron to the erythroid precursors in bone marrow results in decreased Hb content in new RBCs. There are so far no studies determining the limit for ferritin concentration which defines the level

when iron-deficient reticulocytes are developed. A schematic presentation (**FIGURE 13**) of the progression of iron deficiency shows that progressively decreasing iron stores are not able to provide sufficient iron into the whole developing erythroid mass. Simultaneously, reduced Hb synthesis can be observed in the RBCs that are found in the blood circulation. However, blood Hb concentration may remain for a while within the reference limits even if the iron stores are fully depleted (**FIGURE 13**).

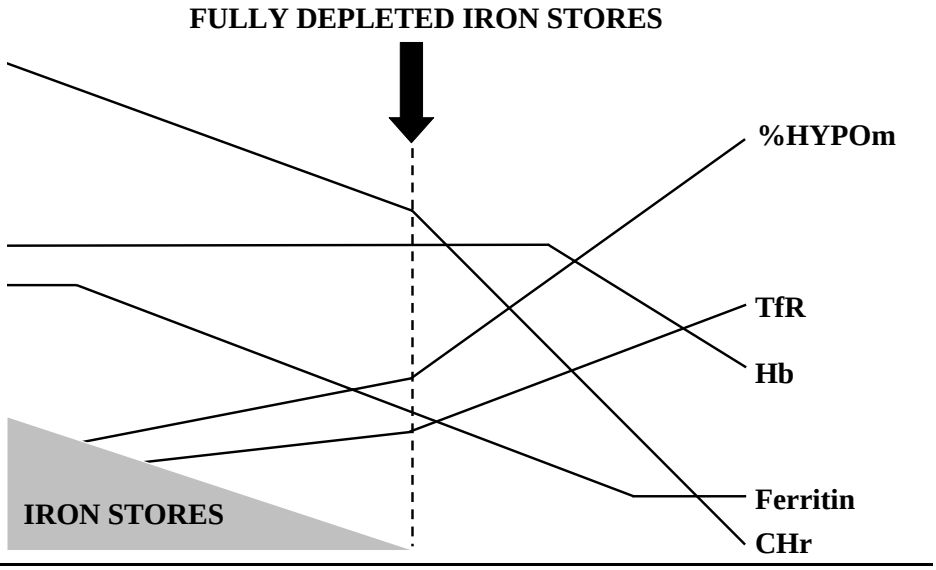


FIGURE 13 Schematic presentation of the development of iron deficiency when iron stores are being exhausted. Before the iron stores are fully depleted, cells containing reduced amounts of hemoglobin are produced and this is reflected by decreased cellular hemoglobin content in reticulocytes (CHr) and more hypochromic red blood cells (%HYPOm). Hemoglobin concentration may remain for a while within the reference limits even if the iron stores are fully depleted.

6.4. The use of red blood cell and reticulocyte indices, and the serum iron status measurements in pregnant women at term

The clinical performance of the current markers of iron status is far from perfect during pregnancy. Nevertheless, too often the current clinical routine in Finland is to assess the iron status of pregnant women by merely measuring Hb using a simple bedside device as a rapid screening marker in maternity care units. On the basis of study I, the new

RBC and reticulocyte indices were the most accurate diagnostic markers of iron status in pregnant women at term, which is consistent with findings of earlier studies with other populations (Mast *et al*, 1998; Brugnara *et al*, 1999; Kotisaari *et al*, 2002; Ullrich *et al*, 2005; Radtke *et al*, 2005; Fry *et al*, 2006; Luo *et al*, 2007). Before cell indices are used in the course of pregnancy, further studies are needed to evaluate their usefulness in monitoring the iron availability for erythropoiesis in the course of pregnancy.

While the need for iron increases during pregnancy, the accuracy of iron status measurements falls. In addition to diurnal and day-to-day variations, there are also pregnancy-specific alterations in serum proteins and Hb concentration during pregnancy (Borel *et al*, 1991; Scholl *et al*, 1992; Allen, 1997; Choi *et al*, 2000a; Milman *et al*, 2007). Maternal iron stores are mobilized from bone marrow stores because of the need for iron in, for example, the maternal and fetal RBCs (Svanberg *et al*, 1975), which reduces the total ferritin concentration in pregnant women during gestation (Milman *et al*, 1999). However, as with the decreased Hb due to hemodilution during pregnancy, this is also an apparent reason for decreased serum iron status measurement (iron, ferritin) (Bentley, 1985; Milman *et al*, 1999). Therefore, the specificity of low ferritin in reflecting depleting iron stores also falls during pregnancy. These alterations have been shown to be evident in pregnant women with or without iron supplementation (Svanberg *et al*, 1975; Milman *et al*, 1995). In pregnant women, the transferrin concentration in serum typically increases without evidence of iron deficiency, so transferrin saturation decreases. Hence, measuring the changes in cellular Hb content provides a significant improvement in the evaluation of iron status in pregnant women, because the iron is mobilized from the stores to Hb synthesis in the RBC. Additionally, since iron is accumulated in the fetus at the expence of the mother, we can assume that maternal iron status is too low if maternal RBCs have insufficient amounts of iron and hypochromic cells are formed. Therefore, evaluating the features of the largest compartment (hematological) of iron status may be an accurate tool in evaluating the iron status of pregnant women.

Moreover, the RBC volume increases usually in the course of pregnancy, and MCV is increased by about 4 fL (Chanarin *et al*, 1977; Milman *et al*, 2007). This is assumed to be a consequence of the increased number of reticulocytes in late pregnancy

(Chanarin *et al*, 1977; Milman *et al*, 2007). MCV is also a markedly late marker of iron deficiency, so it is not a reliable measure of iron deficiency during pregnancy especially if the normal lower cut-off limit (82 fL) is used.

Only a few studies have investigated the new RBC and reticulocyte indices in pregnant women or during the postpartum period. %HYPOm has been shown to reflect iron status during rHuEPO and iron supplementation in the course of pregnancy (Breymann, 2002). Moreover, postpartum oral iron supplementation (of dose 80 mg) has been shown to reduce the %HYPOm with iron (Krafft *et al*, 2005). It has also been reported that on the basis of cellular Hb content, there was no evidence of postpartum (up to day 42) iron deficiency (Richter *et al*, 1999). Importantly, %HYPOm has been reported not to be affected by inflammatory response at term of pregnancy (Krafft *et al*, 2003).

It has been reported that ferritin was the most useful laboratory measurement of iron status during pregnancy in severely anemic pregnant Malawi women (van den Broek *et al*, 1998b). However, although ferritin concentration decreases significantly during pregnancy, the duration of pregnancy was not adjusted in that study. Moreover, bone marrow iron could have been redistributed to meet the need for increased amounts of RBCs in these women, which might be the basis for decreased iron stores, but not the whole iron content of the body. A valuable suggestion regarding the use of ferritin in obstetrics is that the need for iron supplementation during pregnancy should be analysed in early pregnancy, when iron stores are not yet mobilized (Milman *et al*, 1995; Milman *et al*, 2006).

Some investigators suggest that sTfR reflects iron status also during pregnancy and the improvement of iron status by iron supplementation, and hence it is a useful marker of iron status (Carriaga *et al*, 1991; Åkesson *et al*, 1998; Rusia *et al*, 1999). However, it has also been shown that sTfR is elevated with increasing gestation concomitantly with increased erythropoiesis but without evidence of iron deficiency (Beguin *et al*, 1991; Åkesson *et al*, 1998; Choi *et al*, 2000a; Choi *et al*, 2001a). In study I, the diagnostic accuracy of sTfR was very low in comparison with that of RBC and reticulocyte indices, ferritin or Hb. Additionally, it has been speculated that maternal sTfR might be partly produced in the placenta (Carriaga *et al*, 1991).

6.5. Limitations of iron status markers in newborn infants

There is no appropriate iron status measurement for newborn infants. Ferritin is the most widely used marker of iron status in newborns, and a value below 60 µg/L may reflect low iron status (Rao *et al*, 2007). Physiologically, fetuses accumulate iron from the maternal side throughout pregnancy and the most abundant amounts of iron are stored during the third trimester (Siddappa *et al*, 2007). Iron is transferred to the bone marrow in fetuses, in order to fulfil the demand for accelerated erythropoiesis, after which iron is stored as ferritin. The longer the pregnancy lasts, the larger the iron store in the fetus will be (Rao *et al*, 2007). Consequently, there is wide variation in newborn ferritin values.

The use of sTfR concentration as the only iron status marker in the newborn period has been questioned, although some studies claim that sTfR or TfR-F Index may be useful markers of iron status in newborns (Rusia *et al*, 1995; Kuiper-Kramer *et al*, 1998a; Sweet *et al*, 2001). sTfR cannot be considered only as an indicator of iron requirements for erythropoiesis in newborns (Rusia *et al*, 1995; Choi *et al*, 2003), since changes in the rate of erythropoiesis may significantly contribute to the serum concentrations of sTfR (Carpani *et al*, 1996; Kuiper-Kramer *et al*, 1998a; Sweet *et al*, 2001). Furthermore, the usefulness of sTfR as an iron status marker in newborns may also be impaired by significant day-to-day variations in sTfR concentration during the first days of life (Kuiper-Kramer *et al*, 1998a).

In study II, %HYPOm and %HYPOr correlated with TfSat. However, in newborn infants hypochromacy indices were also positively correlated with the size of the RBCs and reticulocytes (MCV and MCVr). The rate of fetal erythropoiesis is accelerated towards term, and there is a marked release of reticulocytes and young erythrocytes, containing less Hb, into the blood stream (Palis *et al*, 1998), which in turn is reflected as high IRF-H (Ervasti *et al*, 2007b). However, mean CHr and CHm were quite stable and no significant correlations between CHr, CHm and serum iron status measurements were found in newborn infants.

In study V, the quantitative FCM measurement of the cellular iron need was developed. Since we only presented the assay in the study, further studies are needed to establish its clinical usefulness. The purpose of this method was to assess iron need

even if the rate of erythropoiesis is accelerated (Ervasti *et al*, 2004). Measuring TfR expression on reticulocytes might help in the evaluation of the iron need in newborn infants, because they have physiologically highly accelerated erythropoiesis, and accumulate primarily their iron in the developing erythrocytes. This method might also provide an opportunity to reduce the sample volume, which is important during the early weeks of life in premature newborns.

6.6. Transferrin receptor expression as a marker of iron demand in reticulocytes

Although serum sTfR concentration is a remarkably good indicator of the availability of iron, it is also increased when the number of erythropoietic cells in bone marrow is increased (they contain the most abundant numbers of TfR in the body) (Punnonen *et al*, 1997; Kuiper-Kramer *et al*, 1998b; Ervasti *et al*, 2004). The quantitative flow cytometric analysis for TfR expression on reticulocytes was developed (study V) in order to analyse the iron requirement at the cellular level in newly-formed erythroid cells. Using this method, the contribution of erythropoietic mass to the observed increase in plasma sTfR level may be eliminated (Ervasti *et al*, 2004). While the measurement of TfR expression was feasible, the day-to-day variation as well as the inter- and intra-variable variations were remarkably low. The stability of the samples for up to 28 hours advocates the use of quantitative FCM analysis (study V). However, the stability of TO dye limits the assay, but it should be taken into consideration when analysing the samples.

Similarly to %HYPOr and CHr, TfR expression on reticulocytes also reflects a real-time requirement for iron. The ABC level or the $\%TfR^{+}$Rets correlated significantly with the cellular iron status indices, which can also be assumed to be related to the need for iron in erythroid cells. No correlations between sTfR and TfR expression were found, which can be due to the fact that the plasma sTfR concentration is also dependent on the whole mass of erythropoietic tissue.

A challenge for this FCM TfR expression measurement might be the rarity of reticulocytes in proportion to the total amount of the RBCs in the blood samples, as well as the rate of the biological process in erythroid marrow. However, no correlations

between the ABC values or the %TfR$^+$Rets and the reticulocyte count were found. Therefore, the mass of erythropoiesis seems not to be a major determinant of the %TfR$^+$Rets or the ABC values. Moreover, a uniform rise in TfR expression in all maturation stages of reticulocytes was found in iron-deficient patients. Thus, the maturation stage of reticulocytes is not the only cause of high TfR expression: the availability of iron is also a cause of high TfR expression (Serke *et al*, 1993; Kuiper-Kramer *et al*, 1998b). The results of study V are consistent with those of previous studies that have shown increased TfR expression in iron-deficient conditions (Kuiper-Kramer *et al*, 1997; Kuiper-Kramer *et al*, 1998b). This method needs to be evaluated in clinical studies, especially in patients with accelerated erythropoiesis and in patients who are at risk for functional iron deficiency, such as in patients undergoing rHuEPO treatment.

6.7. Iron-deficient cells may impair oxygenation

Tissue oxygenation is dependent on the oxygen-carrying capacity, arterial pO_2, O_2 affinity and the blood flow in tissues. The oxygen-carrying capacity in turn depends on the cardiac output and blood oxygen content (Hb saturation and concentration). Hypoxia can be caused by low cardiac output, hypoxemia (low arterial pO_2) and low blood Hb content (Brouillette *et al*, 1997). Perinatal asphyxia is a condition in which placental gas exchange is inadequate, and fetal metabolism changes from aerobic to anaerobic, which leads to metabolic acidosis and lower pH levels. The pH level is higher in umbilical cord vein than in umbilical arteries (Brouillette *et al*, 1997). Perinatal asphyxia causes many problems (mortality and morbidity in newborns and neurological disturbances in later life), but umbilical artery pH level predicts poorly perinatal brain damage (Ruth *et al*, 1988). When the fetus suffers from tissue hypoxia, the serum EPO concentration increases, which is why fetuses with chronic hypoxia (e.g. diabetes mellitus, preeclampsia or growth retardation) have increased serum and amniotic fluid EPO concentrations (Teramo *et al*, 2004a). Hence, increased serum and amniotic fluid EPO concentrations can be used as markers of chronic and subchronic fetal hypoxia (Teramo *et al*, 2004a; Teramo *et al*, 2004b; Teramo, 2006).

When tissue oxygenation is inadequate, EPO synthesis is stimulated, which in turn accelerates erythropoiesis, leading to an increase in the number of erythrocytes, and the production of them consumes iron reserves (**FIGURE 14**). It has been shown that infants of diabetic mothers have suffered from chronic hypoxia causing a redistribution of body iron, and this is reflected by the changes in the serum markers of iron status (decreased ferritin, elevated ZnPP and increased TIBC) (Georgieff *et al*, 1990). Importantly, it is characteristic of stillborn fetuses who have died of chronic hypoxia (e.g. diabetes mellitus) that their iron stores have been fully depleted (Petry *et al*, 1992). Increased amounts of RBC ZnPP reflecting impaired iron availability for erythropoiesis have also been found to be associated with high EPO levels in newborn infants (Lott *et al*, 2005). Additionally, iron status has been shown to be connected with high EPO levels in pregnant women (Milman *et al*, 1997; McMullin *et al*, 2003).

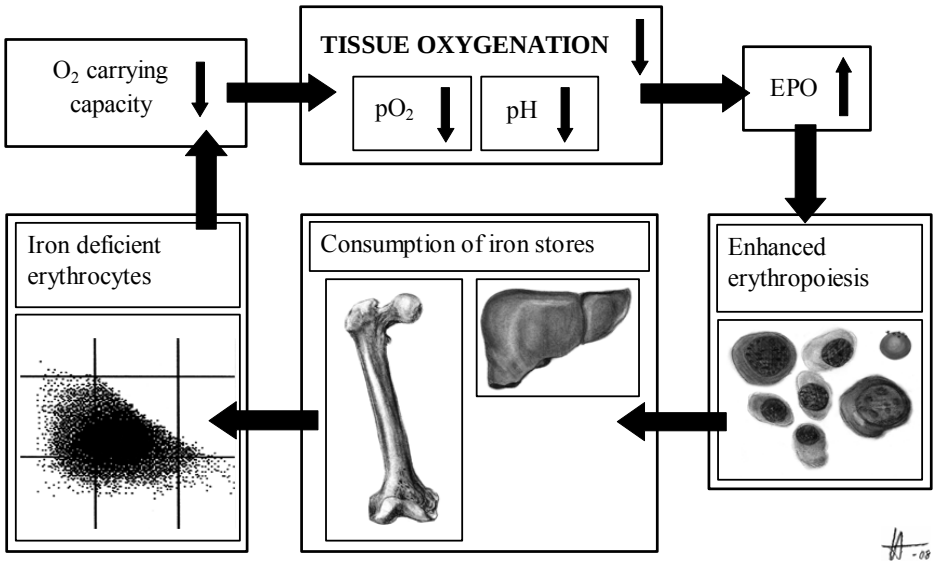


FIGURE 14 Schematic presentation of the association of tissue hypoxia with iron metabolism. Tissue hypoxia causes an increased EPO concentration. In conditions where metabolism is anaerobic, pH levels decrease. Increased EPO concentration stimulates erythropoiesis in order to get the maximal amounts of erythrocytes to improve oxygenation. Increased erythropoiesis consumes body iron reserves and if the iron reserves are depleted, cells containing less hemoglobin develop. If the cells have less hemoglobin, they may have impaired oxygen-carrying capacity and the tissue hypoxia increases.

If insufficient iron is available, RBCs will become hypochromic because of the low Hb content (**FIGURE 14**). Consequently, hypochromic RBCs may not carry sufficient oxygen to the tissues, which may result in more severe tissue hypoxia (**FIGURE 14**). This was suggested in studies III and IV, in which the oxygen-carrying capacity appeared to be impaired when high amounts of hypochromic cells containing less Hb were present (**FIGURE 14**). Thereafter, tissue oxygenation may decrease and induce stimulation of EPO production.

There were no significant correlations between hypoxia markers (EPO and pH) and iron stores (ferritin), and RBC count or blood Hb concentration (IV). However, of the cellular indices, %HYPOm, %HYPOr, MCHC and MCV correlated with the serum EPO concentrations and pH levels of the cord blood at birth (IV). Additionally, the correlation between serum EPO concentration, and sTfR and TfSat was significant in cord blood at birth (IV). This could be explained by the changes in Hb content of RBC and reticulocytes influencing the oxygenation of tissues even if the fetal blood Hb concentration is regarded as normal. Hence, along with RBC mass (Beguin *et al*, 1993; Cazzola *et al*, 1998), the quality of the RBCs may have influenced the oxygen carrying capacity (studies III and IV). However, in this study setting, it was not possible to evaluate precisely which was the first step, placental insufficiency or simply the reduced availability of iron at the same time.

EPO concentration is also known to increase due to the distress at delivery (Widness *et al*, 1984). This was also found in study IV, since newborn infants who were born by emergency section or by vaginal delivery had significantly higher EPO concentration than newborn infants with elective section.

6.8. Conclusions and future research

Ferritin, a marker of the stored iron compartment, reflects the reserve of body iron (**FIGURE 15**). Transferrin and TfSat reflect iron transferred in the serum (**FIGURE 15**), and they have diurnal variation and many interfering factors. The RBCs reflecting the hematological iron compartment contain a significant amount (65 %) of the whole iron content of the body (**FIGURE 15**). Using currently available RBC and reticulocyte

indices, it is possible to measure the whole Hb concentration and the quality of the cells (size and Hb content) (**FIGURE 15**). Additionally, the cellular iron availability of reticulocytes (%TfR^{+}Ret), which reflect the state of erythropoiesis, can now also be measured (**FIGURE 15**). When more clinical studies have been made, indices reflecting the features of cells might offer a way to move from screening iron deficiency by Hb to more sensitive and rapid indicators of iron deficiency.

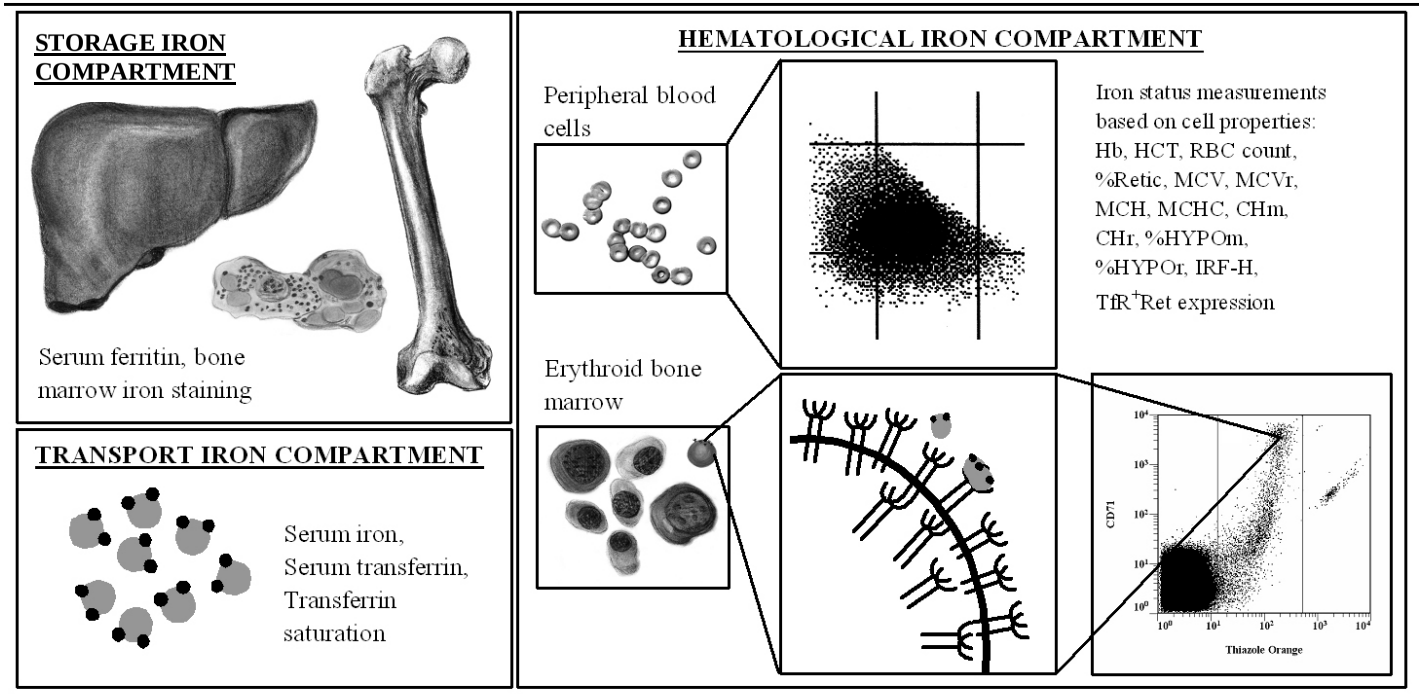


FIGURE 15 Schematic presentation of currently available iron status measurements reflecting the different compartments of systemic iron homeostasis. While iron status can be measured by serum markers, cellular features are appropriate tools for evaluating the Hb contents and the need for iron.

7. CONCLUSIONS

RBC and reticulocyte indices are the most practical way to evaluate iron deficiency in pregnant women, at least at term. Advanced RBC and reticulocyte indices, such as CHr and %HYPOm, are available on automated cell analysers and they allow a simple and precise estimation of iron status.

In newborn infants, both accelerated erythropoiesis and the magnitude of iron stores contributes to the RBC and reticulocyte indices, thus impairing the value of RBC and reticulocyte indices as specific indicators of iron deficiency. In cord blood at birth, reference values of iron status measurements show wide variations in both serum and cellular iron markers.

While the state of anemia is a major contributor of oxygen-carrying capacity, the decreased amount of cellular Hb may also be associated with suboptimal tissue oxygenation based on alterations of the indicators of hypoxic conditions (EPO and pH) in pregnant women at term and in their newborn infants.

TfR expression on reticulocytes can be quantified using the FCM method. High expression of TfR is found in patients with iron deficiency or functional iron deficiency, but not in controls or patients with adequate iron stores. Thus, reticulocytes with high TfR expression reflect a high demand for iron in RBC production.

Indices reflecting the features of the RBCs and reticulocytes are available as diagnostic markers of iron status in modern laboratory cell analysers. More clinical studies might provide a way to move from screening iron deficiency by Hb to more sensitive and rapid indicators of iron deficiency.

8. REFERENCES

Aisen P and Listowsky I. Iron transport and storage proteins. Ann Rev Biochem 1980;49:357-393.

Åkesson A, Bjellerup P, Berglund M, Bremme K and Vahter M. Serum transferrin receptor: a specific marker of iron deficiency in pregnancy. Am J Clin Nutr 1998;68:1241-1246.

Allen L H. Anemia and iron deficiency: effects on pregnancy outcome. Am J Clin Nutr 2000;71:1280S-1284S.

Allen L H. Pregnancy and iron deficiency: unresolved issues. Nutr Rev 1997;55:91-101.

Bain B J. Performing a blood count. Blood cells, A practical guide. 3rd ed, 2002. Blackwell Science Ltd.

Baker W F. Iron deficiency in pregnancy, obstetrics, and gynecology. Hematol Oncol Clin North Am 2000;14:1061-1077.

Batts K P. Iron overload syndromes and the liver. Mod Pathol 2007;20:S31-S39.

Beaton G H. Iron needs during pregnancy: do we need to rethink our targets. Am J Clin Nutr 2000;72:265S-271S.

Beguin Y, Clemons G K, Pootrakul P and Fillet G. Quantitative assessment of erythropoiesis and functional classification of anemia based on measurements of serum transferrin receptor and erythropoietin. Blood 1993;81:1067-1076.

Beguin Y, Lipscei G, Thoumsin H and Fillet G. Blunted erythropoietin production and decreased erythropoiesis in early pregnancy. Blood 1991;78:89-93.

Beilby J, Olynyk J, Ching S, Prins A, Swanson N, Reed W, et al. Transferrin index: an alternative method for calculating the iron saturation of transferrin. Clin Chem 1992;38:2078-2081.

Bencaiova G, Krafft A, Burkhardt T and Zimmerman R. Hemoglobinopathies, body iron stores and gestational diabetes mellitus. Haematologica 2007;90:1138-1139.

Bentley D P. Iron metabolism and anaemia in pregnancy. Clin Haematol 1985;14:613-628.

Beutler E and Waalen J. The definition of anemia: what is the lower limit of normal of the blood hemoglobin concentration. Blood 2006;107:1747-1750.

Blumberg W E, Elsinger J, Lamola A A and Zuckerman D M. The hematofluorometer. Clin Chem 1977;23:270-274.

Borel M J, Smith S M, Derr J and Beard J L. Day-to-day variation in iron-status indices in healthy men and women. Am J Clin Nutr 1991;54:729-735.

Bothwell T H. Iron requirements in pregnancy and strategies to meet them. Am J Clin Nutr 2000;72(suppl):257S-264S.

Bovy C, Tsobo C, Crapanzano L, Rorive G, Beguin Y, Albert A, et al. Factors determining the percentage of hypochromic red blood cells in hemodialysis patients. Kidney Int 1999;56:1113-1119.

Boyd J C. Mathematical tools for demonstrating the clinical usefulness of biochemical markers. Scand J Clin Lab Invest Suppl 1997;227:46-63.

Bradley J, Leibold E A, Harris Z L, Wobken J D, Clarke S, Zumbrennen K B, et al. Influence of gestational age and fetal iron status on IRP activity and iron transporter protein expression in third-trimester human placenta. Am J Physiol Regul Integr Comp Physiol 2004;287:894-901.

Braun J, Lindner K, Schreiber M, Heidler R A and Hörl W H. Percentage of hypochromic red blood cells as predictor of erythropoietic and iron response after i.v. iron supplementation in maintenance haemodialysis patients. Nephrol Dial Transplant 1997;12:1173-1181.

Breymann C. Iron deficiency and anaemia in pregnancy: modern aspects of diagnosis and therapy. Blood Cells Mol Dis 2002;29:506-516.

Briggs C, Rogers R, Thompson B and Machin S J. New red cell parameters on the Sysmex XE-2100 as potential markers of functional iron deficiency. Sysmex Journal International 2001;11:63-68.

Brouillette R T and Waxman D H. Evaluation of the newborn's blood gas status. Clin Chem 1997;43:215-221.

Brugnara C. Use of reticulocyte cellular indices in the diagnosis and treatment of hematological disorders. Int J Clin Lab Res 1998;28:1-11.

Brugnara C. Reticulocyte cellular indices: a new approach in the diagnosis of anemias and monitoring of erythropoietic function. Crit Rev Clin Lab Sci 2000;37:93-130.

Brugnara C, Chambers L A, Malynn E, Goldberg M A and Kruskall M S. Red blood cell regeneration induced by subcutaneous recombinant erythropoietin: iron-deficient erythropoiesis in iron-replete subjects. Blood 1993;81:956-964.

Brugnara C, Colella G M, Cremins J, Langley R C, Schneider T J, Rutherford C J, et al. Effects of subcutaneous recombinant human erythropoietin in normal subjects: development of decreased reticulocyte hemoglobin content and iron-deficient erythropoiesis. J Lab Clin Med 1994a;123:660-667.

Brugnara C, Laufer M R, Friedman A J, Bridges K and Platt O. Reticulocyte hemoglobin content (CHr): early indicator of iron deficiency and response to therapy. Blood 1994b;83:3100-3101.

Brugnara C, Schiller B and Moran J. Reticulocyte hemoglobin equivalent (Ret He) and assessment of iron-deficient states. Clin Lab Haem 2006;28:303-308.

Brugnara C, Zurakowski D, DiCanzio J, Boyd T and Platt O. Reticulocyte hemoglobin content to diagnose iron deficiency in children. JAMA 1999;281:2225-2230.

Canals C, Remacha A F, Sardá M P, Piazuelo J M, Royo M T and Romero M A. Clinical utility of the Sysmex XE 2100 parameter - reticulocyte hemoglobin equivalent - in the diagnosis of anemia. Haematologica 2005;90:1133-1134.

Caro J, Erslev A J, Silver R, Miller O and Birgegard G. Erythropoietin production in response to anemia or hypoxia in the newborn rat. Blood 1982;60:984-988.

Carpani G, Buscaglia M, Ghisoni L, Pizzotti D, Vozzo N, Bellotti M, et al. Soluble transferrin receptor in the study of fetal erythropoietic activity. Am J Hematol 1996;52:192-196.

Carriaga M, Skikne B, Finley B, Cutler B and Cook J. Serum transferrin receptor for the detection of iron deficiency in pregnancy. Am J Clin Nutr 1991;54:1077-1081.

Casanueva E and Viteri F. Iron and oxidative stress in pregnancy. J Nutr 2003;133:1700S-1708S.

Cazzola M, Guarnone R, Cerani P, Centenara E, Rovati A and Beguin Y. Red blood cell precursor mass as an independent determinant of serum erythropoietin level. Blood 1998;91:2139-2145.

Cerami A, Brines M, Ghezzi P, Cerami C and Loretta M I. Neuroprotective properties of epoetin alfa. Nephrol Dial Transplant 2002;17:8-12.

Chanarin I, McFadyen I R and Kyle R. The physiological macrocytosis of pregnancy. Br J Obstet Gynaecol 1977;84:504-508.

Chaparro C M, Neufeld L M, Teno Alavez G, Eguia-Líz Cedillo R and Dewey K G. Effect of timing of umbilical cord clamping on iron status in Mexican infants: a randomized controlled trial. Lancet 2006;367:1997-2004.

Chen X, Scholl T O and Stein T P. Association of elevated serum ferritin levels and the risk of gestational diabetes mellitus in pregnant women. Diabetes Care 2006;29:1077-1082.

Chockalingam U M, Murphy E, Ophoven J C, Weisdorf S A and Georgieff M K. Cord transferrin and ferritin values in newborn infants at risk for prenatal uteroplacental insufficiency and chronic hypoxia. J Pediatr 1987;111:283-286.

Choi J W, Im Moon W and Pai S H. Serum transferrin receptor concentrations during normal pregnancy. Clin Chem 2000a;46:725-727.

Choi J W, Kim C S and Pai S H. Erythropoietic activity and soluble transferrin receptor level in neonates and maternal blood. Acta Paediatr 2000b;89:675-679.

Choi J W and Pai S H. Change in erythropoiesis with gestational age during pregnancy. Ann Hematol 2001a;80:26-31.

Choi J W and Pai S H. Reticulocyte subpopulations and reticulocyte maturity index (RMI) rise as body iron status falls. Am J Hematol 2001b;67:130-135.

Choi J W and Pai S H. Associations between serum transferrin receptor concentrations and erythropoietic activities according to body iron status. Ann Clin Lab Sci 2003;33:279-284.

Cogswell M E, Parvanta I, Ickes L, Yip R and Brittenham G M. Iron supplementation during pregnancy, anemia, and birth weight: a randomized controlled trial. Am J Clin Nutr 2003;78:773-781.

Conrad M E and Umbreit J N. Pathways of iron absorption. Blood Cells Mol Dis 2002;29:336-355.

Cook J D, Skikne B S and Baynes R D. Serum transferrin receptor. Annu Rev Med 1993;44:63-74.

Cotes P M and Canning C E. Changes in serum immunoreactive erythropoietin during the menstrual cycle and normal pregnancy. Br J Obstet Gynaecol 1983;90:304-311.

d'Onofrio G, Chirillo R, Zini G, Caenaro G, Tommasi M and Micciulli G. Simultaneous measurement of reticulocyte and red blood cell indices in healthy subjects and patients with microcytic and macrocytic anemia. Blood 1995;85:818-823.

d'Onofrio G, Zini G, Ricerca B M, Mancini S and Mango G. Automated measurement of red blood cell microcytosis and hypochromia in iron deficiency and β-thalassemia trait. Arch Pathol Lab Med 1992;116:84-89.

Dame C and Juul S E. The switch from fetal to adult erythropoiesis. Clin Perinatol 2000;27:507-526.

David O, Grillo A, Ceoloni B, Cavallo F, Podda G, Biancotti P P, et al. Analysis of red cell parameters on the Sysmex XE 2100 and ADVIA 120 in iron deficiency and in uraemic chronic disease. Scand J Clin Lab Invest 2006;66:113-120.

Davis L E, Widness J A and Brace R A. Renal and placental secretion of erythropoietin during anemia or hypoxia in the ovine fetus. Am J Obstet Gynecol 2003;189:1764-1770.

Davis R J and Czech M P. Regulation of transferrin receptor expression at the cell surface by insulin-like growth factors, epidermal growth factor and platelet-derived growth factor. EMBO J 1986;5:653-658.

Eckardt K-U, Boutellier U, Kurtz A, Schopen M, Koller E and Bauer C. Rate of erythropoietin formation in humans in response to acute hypobaric hypoxia. J Appl Physiol 1989;66:1785-1788.

Eckardt K-U, Hartmann W, Vetter U, Pohlandt F, Burkhardt T and Kurtz A. Serum immunoreactive erythropoietin of children in health and disease. Eur J Pediatr 1990;149:459-464.

Egrie J C, Browne J, Lai P and Lin F-K. Characterization of recombinant monkey and human erythropoietin. Prog Clin Biol Res 1985;191:339-350.

England J M and Down M C. Red-cell-volume distribution curves and the measurement of anisocytosis. Lancet 1974;1:701-703.

Ervasti M, Kotisaari S, Heinonen S and Punnonen K. Use of advanced red blood cell and reticulocyte indices improves the accuracy in diagnosing iron deficiency in pregnant women at term. Eur J Haematol 2007a;79:539-545.

Ervasti M, Kotisaari S, Heinonen S and Punnonen K. Elevated serum erythropoietin concentration is associated with coordinated changes in red blood cell and reticulocyte indices of pregnant women at term. Scand J Clin Lab Invest 2008a;68:160-165.

Ervasti M, Kotisaari S, Romppanen J and Punnonen K. In patients who have stainable iron in the bone marrow an elevated plasma transferrin receptor value may reflect functional iron deficiency. Clin Lab Haematol 2004;26:205-209.

Ervasti M, Kotisaari S, Sankilampi U, Heinonen S and Punnonen K. The relationship between red blood cell and reticulocyte indices and serum markers of iron status in the cord blood of newborns. Clin Chem Lab Med 2007b;45:1000-1003.

Ervasti M, Luukkonen S, Sankilampi U, Heinonen S and Punnonen K. The correlation between maternal and cord blood pro-hepcidin concentrations, and between their iron status measurements at term. Manuscript 2008b.

Ervasti M, Matinlauri I and Punnonen K. Quantitative flow cytometric analysis of transferrin receptor expression on reticulocytes. Clin Chim Acta 2007c;383:153-157.

Ervasti M, Sankilampi U, Heinonen S and Punnonen K. Early signs of maternal iron deficiency do not influence the iron status of the newborn, but are associated with higher birth weight of the infant. Submitted 2008c.

Ervasti M, Sankilampi U, Heinonen S and Punnonen K. Novel red cell indices indicating reduced availability of iron are associated with high erythropoietin concentration and low pH level in the venous cord blood of newborns. Pediatr Res 2008d;Article in press.

Fahnenstich H, Dame C, Alléra A and Kowalewski S. Biochemical monitoring of fetal distress with serum-immunoreactive erythropoietin. J Perinat Med 1996;24:85-91.

Farr A D. Some problems in the history of hemoglobinometry (1878-1931). Med Hist 1978;22:151-160.

Fernández-Real J M, López-Bermejo A and Ricart W. Cross-talk between iron metabolism and diabetes. Diabetes 2002;51:2348-2354.

Fishbane S, Shapiro W, Dutka P, Valenzuela O F and Faubert J. A randomized trial of iron deficiency testing strategies in hemodialysis patients. Kidney Int 2001;60:2406-2411.

Forestier F, Daffos F, Catherine N, Renard M and Andreux J-P. Developmental hematopoiesis in normal human fetal blood. Blood 1991;77:2360-2363.

Franck S, Linssen J, Messinger M and Thomas L. Potential utility of Ret-Y in the diagnosis of iron-restricted erythropoiesis. Clin Chem 2004;50:1240-1242.

Fry M M and Kirk C A. Reticulocyte indices in a canine model of nutritional iron deficiency. Vet Clin Pathol 2006;35:172-181.

Gambling L, Danzeisen R, Gair S, Lea R G, Charania Z, Solanky N, et al. Effect of iron deficiency on placental transfer of iron and expression of iron transport proteins *in vivo* and *in vitro*. Biochem J 2003;356:883-889.

Ganz T. Molecular pathogenesis of anemia of chronic disease. Pediatr Blood Cancer 2006;46:554-557.

Gatter K C, Brown G, Trowbridge I S, Woolston R-E and Mast A E. Transferrin receptors in human tissues: their distribution and possible clinical relevance. J Clin Pathol 1983;36:539-545.

Georgieff M K, Landon M B, Mills M M, Hedlund B E, Faassen A E, Schmidt R L, et al. Abnormal iron distribution in infants of diabetic mothers: Spectrum and maternal antecedents. J Pediatr 1990;117:455-461.

Goldenberg R L, Tamura T, DuBard M, Johnston K E, Copper R L and Neggers Y. Plasma ferritin and pregnancy outcome. Am J Obstet Gynecol 1996;175:1356-1359.

Goldstein J D, Garry D J and Maulik D. Obstetric conditions and erythropoietin levels. Am J Obstet Gynecol 2000;182:1055-1057.

Golub M S, Hogrefe C E and Germann S L. Iron deprivation during fetal development changes the behavior of juvenile rhesus monkeys. J Nutr 2007;137:979-984.

Grantham-McGregor S and Ani C. A review of studies on the effect of iron deficiency on cognitive development in children. J Nutr 2001;131:649S-668S.

Guyatt G, Oxman A, Ali M, Willan A, McIlroy W and Patterson C. Laboratory diagnosis of iron-deficiency anemia: an overview. J Gen Intern Med 1992;7:145-153.

Halevi A, Dollberg S, Manor D, Nussinovich R, Kaempfer R and Gale R. Is cord blood erythropoietin a marker of intrapartum hypoxia? J Perinatol 1992; XII:215-219.

Hartgens F and Kuipers H. Effects of androgenic-anabolic steroids in athletes. Sports Med 2004;34:513-554.

Harthoorn-Lasthuizen E J, Lindemans J and Langenhuijsen M M A C. Does iron-deficient erythropoiesis in pregnancy influence fetal iron supply? Acta Obstet Gynecol Scand 2001;80:392-396.

Harthoorn-Lasthuizen E J, Lindemans J and Langenhuijsen M M A C. Zinc protoporphyrin as screening test in female blood donors. Clin Chem 1998;44:800-804.

Hemminki E and Meriläinen J. Long-term follow-up of mothers and their infants in a randomized trial on iron prophylaxis during pregnancy. Am J Obstet Gynecol 1995;173:205-209.

Hou J, Cliver S P, Tamura T, Johnston K E and Goldenberg R. Maternal serum ferritin and fetal growth. Obstet Gynecol 2000;95:447-452.

Huebers H, Beguin Y, Pootrakul P, Einspahr D and Finch C. Intact transferrin receptors in human plasma and their relation to erythropoiesis. Blood 1990;75:102-107.

Jaime-Perez J C, Herrera-Garza J L and Gomez-Almaguer D. Sub-optimal fetal iron acquisition under a maternal environment. Arch Med Res 2005;36:598-602.

Jandl J H and Katz J H. The plasma-to-cell cycle of transferrin. J Clin Invest 1963;42:314-326.

Järvinen L, Takkunen R, Tiisala R and Hallman N. [Helsinkiläislasten hemoglobiiniarvoista sekä rautalääkityksen vaikutuksesta niihin]. Duodecim 1960;LXXVI:4-16.

Jazayeri A, Tsibris J C M, Hunt L T and Spellacy W N. Umbilical plasma erythropoietin correlations with blood gases and gestational age in appropriately grown infants. Am J Perinatol 1996;13:227-230.

Jelkmann W. Molecular biology of erythropoietin. Intern Med 2004;43:649-659.

Jelkmann W. Erythropoietin: structure, control of production, and function. Physiol Rev 1992;72:449-489.

Jiang R, Manson J A E, Meigs J B, Ma J, Rifai N and Hu F B. Body iron stores in relation to risk of type 2 diabetes in apparently healthy women. JAMA 2004;291:711-717.

Kairisto V and Poola A. Software for illustrative presentation of basic clinical characteristics of laboratory tests - GraphROC for Windows. Scand J Clin Lab Invest 1995;55:43-60.

Kaneko Y, Miyazaki S, Hirasawa Y, Gejyo F and Suzuki M. Transferrin saturation versus reticulocyte hemoglobin content for iron deficiency in Japanese hemodialysis patients. Kidney Int 2003;63:1086-1093.

Kasvosve I and Delanghe J. Total iron binding capacity and transferrin concentration in the assessment of iron status. Clin Chem Lab Med 2002;40:1014-1018.

Kato J, Kobune M, Kohgo Y, Fujikawa K, Takimoto R, Torimoto Y, et al. Ratio of transferrin (Tf) to tf-receptor complex in circulation differs depending on tf iron saturation. Clin Chem 2002;48:181-183.

Katodritou E, Terpos E, Zervas K, Speletas M, Kapetanos D, Kartsios C, et al. Hypochromic erythrocytes (%): a reliable marker for recognizing iron-restricted erythropoiesis and predicting response to erythropoietin in anemic patients with myeloma and lymphoma. Ann Hematol 2007;86:369-376.

Kohgo Y, Niitsu Y, Kondo H, Kato J, Tsushima N, Sasaki K, et al. Serum transferrin receptor as a new index of erythropoiesis. Blood 1987;70:1955-1958.

Kohgo Y, Nishisato T, Kondo H, Tsushima N, Niitsu Y and Urushizaki I. Circulating transferrin receptor in human serum. Br J Haematol 1986;64:277-281.

Kolbe-Busch S, Lotz J, Hafner G, Blanckaert N J C, Claeys G, Togni G, et al. Multicenter evaluation of a fully mechanized soluble transferrin receptor assay on the Hitachi and Cobas Integra analyzers. The determination of reference ranges. Clin Chem Lab Med 2002;40:529-536.

Kotisaari S, Romppanen J, Ågren U, Eskelinen S and Punnonen K. Reticulocyte indices rapidly reflect an increase in iron availability for erythropoiesis. Haematologica 2003;88:1422-1423.

Kotisaari S, Romppanen J, Penttilä I and Punnonen K. The Advia 120 red blood cell and reticulocyte indices are useful in diagnosis of iron-deficiency anemia. Eur J Haematol 2002;68:150-156.

Krafft A, Huch R and Breymann C. Impact of parturition on iron status in nonanaemic iron deficiency. Eur J Clin Invest 2003;33:919-923.

Krafft A, Perewusnyk G, Hänseler E, Quack K, Huch R and Breymann C. Effect of postpartum iron supplementation on red cell and iron parameters in non-anaemic iron-deficient women: a randomised placebo-controlled study. BJOG 2005;112:445-450.

Kuiper-Kramer E P A, Baerts W, Bakker R, van Eyck J, van Raan J and van Eijk H G. Evaluation of the iron status of the newborn by soluble transferrin receptors in serum. Clin Chem Lab Med 1998a;36:17-21.

Kuiper-Kramer E P A, Coenen J L L M, Huisman C M S, Abbes A, van Raan J and van Eijk H G. Relationship between soluble transferrin receptors in serum and membrane-bound transferrin receptors. Acta Haematol 1998b;99:8-11.

Kuiper-Kramer E P A, Huisman C M S, van der Molen-Sinke J, Abbes A and van Eijk H G. The expression of transferrin receptors on erythroblasts in anaemia of chronic disease, myelodysplastic syndromes and iron deficiency. Acta Haematol 1997;97:127-131.

Lao T T, Tam K-F and Chan L Y. Third trimester iron status and pregnancy outcome in non-anaemic women; pregnancy unfavourably affected by maternal iron excess. Hum Reprod 2000;15:1843-1848.

Lee J-W, Bae S-H, Jeong J-W, Kim S-H and Kim K-W. Hypoxia-inducible factor (HIF-1)α: its protein stability and biological functions. Exp Mol Med 2004;36:1-12.

Leinonen P and Teramo K. [Kenelle rautaa raskauden aikana?]. Suom Lääkäril 2001;56:2143-2147.

Lenkei R, Gratama J W, Rothe G, Schmitz G, D'hautcourt J L, Årekrans A, et al. Performance of calibration standards for antigen quantitation with flow cytometry. Cytometry 1998;33:188-196.

Loken M R, Shah V O, Dattilio K L and Civin C I. Flow cytometric analysis of human bone marrow: I. Normal erythroid development. Blood 1987;69:255-263.

Lott D G, Zimmermann M B, Labbé R F, Kling P J and Widness J A. Erythrocyte zinc protoporphyrin is elevated with prematurity and fetal hypoxemia. Pediatrics 2005;116:414-422.

Louache F, Testa U, Pelicci P, Thomopoulos P, Titeux M and Rochant H. Regulation of transferrin receptors in human hematopoietic cell lines. J Biol Chem 1984;259:11576-11582.

Lozoff B and Georgieff M K. Iron deficiency and brain development. Semin Pediatr Neurol 2006;13:158-165.

Lozoff B, Jimenez E and Wolf A W. Long-term developmental outcome of infants with iron deficiency. N Engl J Med 1991;325:687-694.

Luo D, Chen Y, Wu W, Zhang F, Xu J, Cui W, et al. Reticulocyte hemoglobin content in diagnosis of iron deficiency in Chinese pre-menopausal women. Chin Med J 2007;120:1010-1012.

Maier R F, Böhme K, Dudenhausen J W and Obladen M. Cord blood erythropoietin in relation to different markers of fetal hypoxia. Obstet Gynecol 1993;81:575-580.

Marti H H. Erythropoietin and the hypoxic brain. J Exp Biol 2004;207:3233-3242.

Martin M E, Nicolas G, Hetet G, Vaulont S, Grandchamp B and Beaumont C. Transferrin receptor 1 mRNA is downregulated in placenta of hepcidin transgenic embryos. FEBS letters 2004;574:187-191.

Mast A, Blinder M, Gronowski A, Chumley C and Scott M. Clinical utility of the soluble transferrin receptor and comparison with serum ferritin in several populations. Clin Chem 1998;44:45-51.

Mast A, Blinder M, Lu Q, Flax S and Dietzen D. Clinical utility of the reticulocyte hemoglobin content in the diagnosis of iron deficiency. Blood 2002;99:1489-1491.

McArdle H J, Danzeisen R, Fosset C and Gambling L. The role of the placenta in iron transfer from mother to fetus and the relationship between iron status and fetal outcome. BioMetals 2003;16:161-167.

McMullin M F, White R, Lappin T, Reeves J and MacKenzie G. Haemoglobin during pregnancy: relationship to erythropoietin and haematinic status. Eur J Haematol 2003;71:44-50.

Milman N. Iron prophylaxis in pregnancy - general or individual and in which dose? Ann Hematol 2006;85:821-828.

Milman N, Agger A O and Nielsen O J. Iron status markers and serum erythropoietin in 120 mothers and newborn infants. Acta Obstet Gynecol Scand 1994;73:200-204.

Milman N, Bergholt T, Byg K-E, Eriksen L and Graudal N. Iron status and iron balance during pregnancy. A critical reappraisal of iron supplementation. Acta Obstet Gynecol Scand 1999;78:749-757.

Milman N, Bergholt T, Byg K-E, Eriksen L and Hvas A-M. Reference intervals for haematological variables during normal pregnancy and postpartum in 434 healthy Danish women. Eur J Haematol 2007;79:39-46.

Milman N, Byg K E, Bergholt T, Eriksen L and Hvas A M. Body iron and individual iron prophylaxis in pregnancy - should the iron dose be adjusted according to serum ferritin? Ann Hematol 2006;85:567-573.

Milman N, Clausen J and Byg K-E. Iron status in 268 Danish women aged 18-30 years: influence of menstruation, contraceptive method, and iron supplementation. Ann Hematol 1998;77:13-19.

Milman N, Graudal N and Agger A O. Iron status markers during pregnancy No relationship between levels at the beginning of the second trimester, prior to delivery and post partum. J Intern Med 1995;237:261-267.

Milman N, Graudal N, Nielsen O J and Agger A O. Serum erythropoietin during normal pregnancy: relationship to hemoglobin and iron status markers and impact of iron supplementation in a longitudinal, placebo-controlled study on 118 women. Int J Hematol 1997;66:159-168.

Milman N, Graudal N, Nielsen O J and Agger A O. Cord serum erythropoietin in 90 healthy newborn term infants: relationship to blood gases and iron status markers. Int J Hematol 1996;64:197-201.

Milman N and Kirchhoff M. Iron stores in 1359, 30- to 60-year-old Danish women: evaluation by serum ferritin and hemoglobin. Ann Hematol 1992;64:22-27.

Mohandas N, Kim Y R, Tycko D H, Orlik J, Wyatt J and Groner W. Accurate and independent measurement of volume and hemoglobin concentration of individual red cells by laser light scattering. Blood 1986;68:506-513.

Morgan E H. Plasma-iron and haemoglobin levels in pregnancy. The effect of oral iron. The Lancet 1961;1:9-12.

Moya F R, Grannum P A T, Widness J A, Clemons G K, Copel J A and Hobbins J C. Erythropoietin in human fetuses with immune hemolytic anemia and hydrops fetalis. Obstet Gynecol 1993;82:353-358.

Nathan D G and Oski F A. Hematology of Infancy and Childhood. 4th ed, 1993. Saunders.

Nicolas G, Viatte L, Bennoun M, Beaumont C, Kahn A and Vaulont S. Hepcidin, a new iron regulatory peptide. Blood Cells Mol Dis 2002;29:327-335.

Nordin G, Mårtensson A, Swolin B, Sandberg S, Christensen N J, Thorsteinsson V, et al. A multicentre study of reference intervals for haemoglobin, basic blood cell counts and erythrocyte indices in the adult population of Nordic countries. Scand J Clin Lab Invest 2004;64:385-398.

O'Brien K O, Zavaleta N, Abrams S A and Caulfield L E. Maternal iron status influences iron transfer to the fetus during the third trimester of pregnancy. Am J Clin Nutr 2003;77:924-930.

Okumura N, Tsuji K and Nakahata T. Changes in cell surface antigen expression during proliferation and differentiation of human erythroid progenitors. Blood 1992;80:642-650.

Palis J and Segel G B. Developmental biology of erythropoiesis. Blood Rev 1998;12:106-114.

Papanikolau G and Pantopoulos K. Iron metabolism and toxicity. Toxicol Appl Pharmacol 2005;202:199-211.

Parkkila S. [Perinnöllinen hemokromatoosi]. Duodecim 2000;116:829-836.

Patton W N, Cave R J and Harris R I. A study of changes in red cell volume and haemoglobin concentration during phlebotomy induced iron deficiency and iron repletion using the Technicon H1. Clin Lab Haematol 1991;13:153-161.

Petry C D, Eaton M A, Wobken J D, Mills M M, Johnson D E and Georgieff M K. Iron deficiency of liver, heart, and brain in newborn infants of diabetic mothers. J Pediatr 1992;121:109-114.

Punnonen K, Irjala K and Rajamaki A. Iron-deficiency anemia is associated with high concentrations of transferrin receptor in serum. Clin Chem 1994;40:774-776.

Punnonen K, Irjala K and Rajamäki A. Serum transferrin receptor and its ratio to serum ferritin in the diagnosis of iron deficiency. Blood 1997;89:1052-1057.

Punnonen K, Kaipainen-Seppänen O, Riittinen L, Tuomisto T, Hongisto T and Penttilä I. Evaluation of iron status in anemic patients with rheumatoid arthritis using an automated immunoturbidimetric assay for transferrin receptor. Clin Chem Lab Med 2000;38:1297-1300.

Rabe H, Reynolds G and Diaz-Rossello J. Early versus delayed umbilical cord clamping in preterm infants. Cochrane Database Syst Rev 2004; DOI: 10.1002/14651858.CD003248.pub2-.

Radtke H, Meyer T, Kalus U, Röcker L, Salama A, Kiesewetter H, et al. Rapid identification of iron deficiency in blood donors with red cell indexes provided by Advia 120. Transfusion 2005;45:5-10.

Rao R and Georgieff M K. Perinatal aspects of iron metabolism. Acta Paediatr Suppl 2002;91:124-129.

Rao R and Georgieff M K. Iron in fetal and neonatal nutrition. Semin Fetal Neonatal Med 2007;12:54-63.

Reveiz L, Gyte G M L and Cuervo L G. Treatments for iron-deficiency anaemia in pregnancy. Cochrane Database Syst Rev 2007; DOI: 10.1002/14651858.CD003094.pub2-.

Richter C, Weitzel H, Huch A and Huch R. Failure to demonstrate postpartum functional iron deficiency using quantitative red blood cell analysis. Eur J Obstet Gynecol Reprod Biol 1999;84:69-74.

Riikonen S, Saijonmaa O, Järvenpää A-L and Fyhrquist F. Serum concentrations of erythropoietin in healthy and anaemic pregnant women. Scand J Clin Lab Invest 1994;54:653-657.

Rios E, Lipschitz D A, Cook J D and Smith N J. Relationship of maternal and infant iron stores as assessed by determination of plasma ferritin. Pediatrics 1975;55:694-699.

Rioux F M and LeBlanc C P. Iron supplementation during pregnancy: what are the risks and benefits of current practices? Appl Physiol Nutr Metab 2007;32:282-288.

Rolfs A, Kvietikova I, Gassmann M and Wenger R H. Oxygen-regulated transferrin expression is mediated by hypoxia-inducible factor-1. J Biol Chem 1997;272:20055-20062.

Rollins M D, Maxwell A P, Afrasiabi M, Halliday H L and Lappin T R J. Cord blood erythropoietin, pH, PaO2 and haematocrit following caesarean section before labour. Biol Neonate 1993;63:147-152.

Rush D. Nutrition and maternal mortality in the developing world. Am J Clin Nutr 2000;72:212S-240S.

Rusia U, Flowers C, Madan N, Agarwal N, Sood S and Sikka M. Serum transferrin receptors in detection of iron deficiency in pregnancy. Ann Hematol 1999;78:358-363.

Rusia U, Flowers C, Madan N, Agarwal N, Sood S and Sikka M. Serum transferrin receptor levels in the evaluation of iron deficiency in the neonate. Acta Paediatr Jpn 1995;38:455-459.

Rustad P, Felding P, Franzson L, Kairisto V, Lahti A, Mårtensson A, et al. The Nordic reference interval project 2000: recommended reference intervals for 25 common biochemical properties. Scand J Clin Lab Invest 2004;64:271-284.

Ruth V, Autti-Rämö I, Granström M-L, Korkman M and Raivio K O. Prediction of perinatal brain damage by cord plasma vasopression, erythropoietin, and hypoxanthine values. J Pediatr 1988;113:880-885.

Ruth V, Widness J A, Clemons G and Raivio K O. Postnatal changes in serum immunoreactive erythropoietin in relation to hypoxia before and after birth. J Pediatr 1990;116:950-954.

Ruth V and Raivio K. Perinatal brain damage: predictive value of metabolic acidosis and the Apgar score. Br Med J 1988;297:24-27.

Sakanaka M, Wen T-C, Matsuda S, Masuda S, Morishita E, Nagao M, et al. *In vivo* evidence that erythropoietin protects neurons from ischemic damage. Proc Natl Acad Sci U S A 1998;95:4635-4640.

Savage R A. The red cell indices - Yesterday, today and tomorrow. Clin Lab Med 1993;13:773-785.

Schaefer R and Schaefer L. Hypochromic red blood cells and reticulocytes. Kidney Int 1999;55:S44-S48.

Scholl T. Iron status during pregnancy: setting the stage for mother and infant. Am J Clin Nutr 2005;81(suppl):1219S-1222S.

Scholl T, Hediger M, Fischer R and Shearer J. Anemia vs iron deficiency: increased risk of preterm delivery in a prospective study. Am J Clin Nutr 1992;55:985-988.

Schümann K, Ettle T, Szegner B, Elsenhans B and Solomons N W. On risks and benefits of iron supplementation recommendations for iron intake revisited. J Trace Elem Med Biol 2007;21:147-168.

Serke S and Huhn D. Identification of CD71 (transferrin receptor) expressing erythrocytes by multiparameter-flow-cytometry (MP-FCM): correlation to the quantitation of reticulocytes as determined by conventional microscopy and by MP-FCM using a RNA-staining dye. Br J Haematol 1992;81:432-439.

Serke S and Huhn D. Improved specificity of determination of immature erythrocytes (reticulocytes) by multiparameter flow-cytometry and thiazole orange using combined staining with monoclonal antibody (anti-glycophorin-A). Clin Lab Haematol 1993;15:33-44.

Serke S, van Lessen A and Huhn D. Quantitative fluorescence flow cytometry: a comparison of the three techniques for direct and indirect inmmunofluorescence. Cytometry 1998;33:179-187.

Shahidi N T. Androgens and erythropoiesis. N Engl J Med 1973;289:72-80.

Shih Y J, Baynes R D, Hudson B G, Flowers C H, Skikne B S and Cook J D. Serum transferrin receptor is a truncated form of tissue receptor. J Biol Chem 1990;265:19077-19081.

Siah C W, Trinder D and Olynyk J K. Iron overload. Clin Chim Acta 2005;358:24-36.

Siddappa A M, Rao R, Long J D, Widness J A and Georgieff M K. The assessment of newborn iron stores at birth: a review of the literature and standards for ferritin concentrations. Neonatology 2007;92:73-82.

Sirén A-L, Fratelli M, Brines M, Goemans C, Casagrande S, Lewczuk P, et al. Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. Proc Natl Acad Sci U S A 2001;98:4044-4049.

Sowade O, Messinger D, Franke W, Sowade B, Scigalla P and Warnke H. The estimation of efficacy of oral iron supplementation during treatment with epoetin beta (recombinant human erythropoietin) in patients undergoing cardiac surgery. Eur J Haematol 1998;60:252-259.

Sposi N M, Cianetti L, Tritarelli E, Pelosi E, Militi S, Barberi T, et al. Mechanisms of differential transferrin receptor expression in normal hematopoiesis. Eur J Biochem 2000;267:2762-2774.

Srai S K S, Bomford A and McArdle H J. Iron transport across cell membranes: molecular understanding of duodenal and placental iron uptake. Best Pract Res Clin Haematol 2002;15:243-259.

Steer P J, Alam M A, Wadsworth J and Welch A. Relation between maternal haemoglobin concentration and birth weight in different ethnic groups. Br Med J 1995;310:489-491.

Stoffman N, Brugnara C and Woods E R. An algorithm using reticulocyte hemoglobin content (CHr) measurement in screening adolescent for iron deficiency. J Adolesc Health 2005;36:529.e1-529.e6.

Stray-Gundersen J, Videman T, Penttilä I and Lereim I. Abnormal hematologic profiles in elite cross-country skiers: blood doping or? Clin J Sport Med 2003;13:132-137.

Sunder-Plassmann G, Spitzauer S and Hörl W H. The dilemma of evaluating iron status in dialysis patients - limitations of available diagnostic procedures. Nephrol Dial Transplant 1997;12:1575-1580.

Suominen P, Punnonen K, Rajamaki A and Irjala K. Evaluation of new immunoenzymometric assay for measuring soluble transferrin receptor to detect iron deficiency in anemic patients. Clin Chem 1997;43:1641-1646.

Suominen P, Punnonen K, Rajamäki A and Irjala K. Serum transferrin receptor and transferrin receptor-ferritin index identify healthy subjects with subclinical iron deficits. Blood 1998;92:2934-2939.

Svanberg B, Arvidsson B, Norrby A, Rybo G and Sölvell L. Absorption of supplemental iron during pregnancy - a longitudinal study with repeated bone-marrow studies and absorption measurements. Acta Obstet Gynecol Scand Suppl 1975;48:87-108.

Sweet D G, Savage G A, Tubman R, Lappin T R J and Halliday H L. Cord blood transferrin receptors to assess fetal iron status. Arch Dis Child Fetal Neonatal Ed 2001;85:F46-F48.

Tacchini L, Bianchi L, Bernelli-Zazzera A and Cairo G. Transferrin receptor induction by hypoxia. HIF-1-mediated transcriptional activation and cell-specific post-transcriptional regulation. J Biol Chem 1999;274:24142-24146.

Tamura T, Goldenberg R, Hou J, Johnston K, Cliver S, Ramey S, et al. Cord serum ferritin concentrations and mental and psychomotor development of children at five years of age. J Pediatr 2002;140:165-170.

Taylor D J, Mallen C, McDougall N and Lind T. Effect of iron supplementation on serum ferritin levels during and after pregnancy. Br J Obstet Gynaecol 1982;89:1011-1017.

Teramo K. [Sikiön erytropoietiinintuotanto lisääntyy hapenpuutteessa]. Duodecim 2006;122:2619-2627.

Teramo K, Kari M A, Eronen M, Markkanen H and Hiilesmaa V. High amniotic fluid erythropoietin levels are associated with an increased frequence of fetal and neonatal morbidity in Type 1 diabetic pregnancies. Diabetologia 2004a;47:1695-1703.

Teramo K, Hiilesmaa V, Schwartz R, Clemons G and Widness J. Amniotic fluid and cord plasma erythropoietin levels in pregnancies complicated by preeclampsia, pregnancy-induced hypertension and chronic hypertension. J Perinat Med 2004b;32:240-247.

Thomas C and Thomas L. Biochemical markers and hematologic indices in the diagnosis of functional iron deficiency. Clin Chem 2002;48:1066-1076.

Thomas L, Franck S, Messinger M, Linssen J, Thomé M and Thomas C. Reticulocyte hemoglobin measurement - comparison of two methods in the diagnosis of iron-restricted erythropoiesis. Clin Chem Lab Med 2005;43:1193-1202.

Tsung S H, Rosenthal W A and Milewski K A. Immunological measurement of transferrin compared with chemical measurement of total iron-binding capacity. Clin Chem 1975;21:1063-1066.

Tuomainen T-P, Loft S, Nyyssönen K, Punnonen K, Salonen J T and Poulsen H E. Body iron is a contributor to oxidative damage of DNA. Free Radic Res 2007;41:324-328.

Tuomainen T-P, Punnonen K, Nyyssönen K and Salonen J T. Association between body iron stores and the risk of acute myocardial infarction in men. Circulation 1998;97:1461-1466.

Ullrich C, Wu A, Armsby C, Rieber S, Wingerter S, Brugnara C, et al. Screening healthy infants for iron deficiency using reticulocyte hemoglobin content. JAMA 2005;294:924-930.

van den Broek N. Anaemia in pregnancy in developing countries. BJOG 1998a;105:385-390.

van den Broek N, Letsky E, White S and Shenkin A. Iron status in pregnant women: which measurements are valid? Br J Haematol 1998b;103:817-824.

van den Broek N and Letsky E. Etiology of anemia in pregnancy in south Malawi. Am J Clin Nutr 2000;72:247S-256S.

Verner A M, Manderson J, Lappin T R J, McCance D R, Halliday H L and Sweet D G. Influence of maternal diabetes mellitus on fetal iron status. Arch Dis Child Fetal Neonatal Ed 2007;92:F399-F401.

Voutilainen P E J, Widness J A, Clemons G K, Schwartz R and Teramo K A. Amniotic fluid erythropoietin predicts fetal distress in Rh-immunized pregnancies. Am J Obstet Gynecol 1989;160:429-434.

Wada H G, Hass P E and Sussman H H. Transferrin receptor in human placental brush border membranes. J Biol Chem 1979;254:12629-12635.

Walters G O, Miller F M and Worwood M. Serum ferritin concentration and iron stores in normal subjects. J Clin Pathol 1973;26:770-772.

Ward J H. The structure, function, and regulation of transferrin receptors. Invest Radiol 1987;22:74-83.

Weiss G, Houston T, Kastner S, Jöhrer K, Grünewald K and Brock J H. Regulation of cellular iron metabolism by erythropoietin: activation of iron-regulatory protein and upregulation of transferrin receptor expression in erythroid cells. Blood 1997;89:680-687.

Whittager P G, Lind T and Williams J G. Iron absorption during normal human pregnancy: a study using stable isotopes. Br J Nutr 1991;65:457-463.

WHO. Iron deficiency anaemia. Assessment, prevention, and control. A guide for programme managers. Geneva: World Health Organisation 2001.

WHO. Standards for maternal and neonatal care: Iron and folate supplementation. Department of making pregnancy safer. Integrated management of pregnancy and childbirth (IMPAC). Geneva: World Health Organisation 2006

Wick M, Pinggera W, and Lehmann P. Iron metabolism, anemias. Diagnosis and Therapy. Novel consepts for renal anemias and rheumatoid arthritis. 4th ed, 2000. Springer-Verlag.

Widness J A, Clemons G K, Garcia J F, Oh W and Schwartz R. Increased immunoreactive erythropoietin in cord serum after labor. Am J Obstet Gynecol 1984;148:194-197.

Widness J A, Schmidt R L and Sawyer S T. Erythropoietin transplacental passage. Review of animal studies. J Perinat Med 1995;23:61-70.

Widness J A, Teramo K A, Clemons G K, Garcia J F, Cavalieri R L, Piasecki G J, et al. Temporal response of immunoreactive erythropoietin to acute hypoxemia in fetal sheep. Pediatr Res 1986;20:15-19.

Wintrobe M M. The size and hemoglobin content of the erythrocyte. J Lab Clin Med 1932;17:899-912.

Withold W, Neumayer C, Beyrau R, Heins M, Schauseil S and Rick W. Efficacy of transferrin determination in human sera in the diagnosis of iron deficiency. Eur J Clin Chem Clin Biochem 1994;32:19-25.

Xiong X, Buekens P, Alexander S, Demianczuk N and Wollast E. Anemia during pregnancy and birth outcome: a meta-analysis. Am J Perinatol 2000;17:137-146.

Yu X, Shacka J J, Eells J B, Suarez-Quian C, Przygodzki R M, Beleslin-Cokic B, et al. Erythropoietin receptor signalling is required for normal brain development. Development 2002;129:505-516.

Zhou S J, Gibson R A, Crowther C A, Baghurst P and Makrides M. Effect of iron supplementation during pregnancy on the intelligence quotient and behavior of children at 4 y of age: long-term follow-up of a randomized controlled trial. Am J Clin Nutr 2006;83:1112-1117.

Ziaei S, Norrozi M, Faghihzadeh S and Jafarbegloo E. A randomised placebo-controlled trial to determine the effect of iron supplementation on pregnancy outcome in pregnant women with haemoglobin $\geq$ 13.2 g/dl. Br J Obstet Gynaecol 2007;114:684-688.

Zwart A, van Assendelft O W, Bull B S, England J M and Lewis S M. Recommendations for reference method for haemoglobinometry in human blood (ICSH standard 1995) and specifications for international haemiglobincyanide standard (4th edition). J Clin Pathol 1996;49:271-274.

APPENDIX: ORIGINAL PUBLICATIONS

I Ervasti M, Kotisaari S, Heinonen S, Punnonen K. Use of advanced red blood cell and reticulocyte indices improves the accuracy in diagnosing iron deficiency in pregnant women at term. Eur J Haematol 2007;79:539-545.

II Ervasti M, Kotisaari S, Sankilampi U, Heinonen S, Punnonen K. The relationship between red blood cell and reticulocyte indices and serum markers of iron status in the cord blood of newborns. Clin Chem Lab Med 2007;45(8):1000-1003.

III Ervasti M, Kotisaari S, Heinonen S, Punnonen K. Elevated serum erythropoietin concentration is associated with coordinated changes in red blood cell and reticulocyte indices of pregnant women at term. Scand J Clin Lab Invest 2008;68(2):160-165.

IV Ervasti M, Sankilampi U, Heinonen S, Punnonen K. Novel red cell indices indicating reduced availability of iron are associated with high erythropoietin concentration and low pH level in the venous cord blood of newborns. Pediatr Res (Article in press).

V Ervasti M, Matinlauri I, Punnonen K. Quantitative flow cytometric analysis of transferrin receptor expression on reticulocytes. Clin Chim Acta 2007;383(1-2):153-7.

Kuopio University Publications D. Medical Sciences

D 420. Stark, Harri. Inflammatory airway responses caused by Aspergillus fumigatus and PVC challenges.
2007. 102 p. Acad. Diss.

D 421. Hintikka, Ulla. Changes in adolescents' cognitive and psychosocial funtioning and self-image during psychiatric inpatient treatment.
2007. 103 p. Acad. Diss.

D 422. Putkonen, Anu. Mental disorders and violent crime: epidemiological study on factors associated with severe violent offending.
2007. 88 p. Acad. Diss.

D 423. Karinen, Hannele. Genetics and family aspects of coeliac disease.
2008. 110 p. Acad. Diss.

D 424. Sutinen, Päivi. Pathophysiological effects of vibration with inner ear as a model organ.
2008. 94 p. Acad. Diss.

D 425. Koskela, Tuomas-Heikki. Terveyspalveluiden pitkäaikaisen suurkäyttäjän ennustekijät.
2008. 253 p. Acad. Diss.

D 426. Sutela, Anna. Add-on stereotactic core needle breast biopsy: diagnosis of non-palpable breast lesions detected on mammography or galactography.
2008. 127 p. Acad. Diss.

D 427. Saarelainen, Soili. Immune Response to Lipocalin Allergens: IgE and T-cell Cross-Reactivity.
2008. 127 p. Acad. Diss.

D 428. Mager, Ursula. The role of ghrelin in obesity and insulin resistance.
2008. 123 p. Acad. Diss.

D 429. Loisa, Pekka. Anti-inflammatory response in severe sepsis and septic shock.
2008. 108 p. Acad. Diss.

D 430. Joukainen, Antti. New bioabsorbable implants for the fixation of metaphyseal bone : an experimental and clinical study.
2008. 98 p. Acad. Diss.

D 431. Nykänen, Irma. Sepelvaltimotaudin prevention kehitys Suomessa vuosina 1996-2005.
2008. 158 p. Acad. Diss.

D 432. Savonen, Kai. Heart rate response to exercise in the prediction of mortality and myocardial infatction: a prospective population study in men.
2008. 165 p. Acad. Diss.

D 433. Komulainen, Pirjo. The association of vascular and neuroprotective status indicators with cognitive functioning: population-based studies.
2008. Acad. Diss.

D 434. Hassinen, Maija. Predictors and consequences of the metabolic syndrome: population-based studies in aging men and women.
2008. Acad. Diss.

D 435. Saltevo, Juha. Low-grade inflammation and adiponectin in the metabolic syndrome.
2008. 109 p. Acad. Diss.

D 436. Ervasti, Mari. Evaluation of Iron Status Using Methods Based on the Features of Red Blood Cells and Reticulocytes.
2008. 104 p. Acad. Diss.