Iron absorption in man

—

Diet modification and fortification

by

Michael R Hoppe

UNIVERSITY OF GOTHENBURG

2008

Department of Clinical Nutrition
Institute of Medicine
The Sahlgrenska Academy at University of Gothenburg
Sweden

Title: Iron absorption in man – diet modification and fortification

In Swedish: Järnabsorption hos människa – kostmodifiering och berikning

Illustrator: Jeff Daniels

Printed in Sweden by:
Chalmers Reproservice, Göteborg

ISBN 978-91-628-7498-8

"One thing only I know, and that is that I know nothing"
Socrates

Gewidmet meiner Oma Anne-mi, Hannis, Ludvig, Felix & Wictor

ABSTRACT

Background: Iron (Fe) deficiency is globally the most common form of nutrient deficiency. The approach to combat this problem can be divided into two major strategies *a):* increasing the dietary Fe bioavailability by diet modification, or *b):* increasing the Fe intake through fortification. However, human bioavailability data on the most commonly used form of Fe fortificants, elemental iron powders (Fe_{elem}), are limited. The main reason is the lack of methods for measuring absorption from Fe_{elem}. Furthermore, when it comes to the scientific evidence for the comparative effect from each of these two strategies in improving iron uptake in man, there are still questions.

Aims: To *I):* evolve a method which could be used to characterize the relative bioavailability (RBV) of Fe_{elem} *II):* characterize the RBV of Fe_{elem} fortificants, and finally *III):* study the effect from dietary modifications and/or Fe fortification on Fe absorption and rates of changes in Fe stores.

Methods: To address the given aims the following methods were used in human subjects; Extrinsic labeling with radioactive Fe isotopes and whole-body counting; The area under the serum Fe concentration curve during six hours following administration of 100 mg Fe (S-Fe $AUC_{0\text{-}6h}$). Algorithms based on human data were used as well.

Results: Radioiron absorption and S-Fe $AUC_{0\text{-}6h}$ correlated well (r^2=0.94). The studied Fe_{elem} were all significantly less well absorbed relative ferrous sulfate (RBV=36-65%). Adding 20 g meat, or 20 g meat and 20 mg ascorbic acid to a meal with low Fe bioavailability increased the total Fe absorption with 155% and 227%, respectively. Improvements in Fe status were greater after dietary modifications than after Fe fortification for a diet with low Fe bioavailability.

Conclusions: S-Fe $AUC_{0\text{-}6h}$ following oral administration of 100 mg iron is a valid measure of iron absorption. Dietary modifications of meals with low Fe bioavailability can markedly improve Fe absorption, especially when adding meat which also contributes with highly absorbable heme Fe. Depending on choice of Fe_{elem} in fortification programs effectiveness can differ. Further, if the diet has a low Fe bioavailability, it is difficult to achieve good effects on Fe status by using Fe fortification as the only measure. Thus, the overall conclusion of this thesis is that the best course of action for interventions designed to improve Fe status, firstly must be to ensure an adequate dietary iron bioavailability, and secondly to use a Fe fortificant with high bioavailability.

SAMMANFATTNING

Bakgrund: Järnbrist är globalt sett den vanligaste förekommande näringsbristen. Tillvägagångssätten för att bekämpa detta tillstånd kan huvudsakligen delas in i två strategier; *a):* kostmodifiering för att öka järnets biotillgänglighet, eller *b):* ökning av mängden järn i kosten. Emellertid är kunskapen sparsam gällande biotillgängligheten hos människa för den vanligaste nyttjade gruppen berikningsjärn, elementärt järn (Fe_{elem}). Den huvudsakliga orsaken är brist på metoder för att mäta absorption från Fe_{elem}. Dessutom föreligger frågetecken gällande den jämförande effekten från var och en av dessa två strategier när det gäller att förbättra järnupptaget hos människa.

Syfte: Att *I):* utveckla en metod för att hos människa fastställa relativ biotillgänglighet (RBV) för Fe_{elem}, *II):* fastställa RBV för en grupp Fe_{elem}, samt slutligen *III):* studera effekten av kostmodifiering och järnberikning, samt kombinationen av dessa, på järnabsorption och den tidsmässiga förändringen i förrådsjärn.

Metoder: För att uppnå syftena med denna studie användes följande metoder: inmärkning med radioaktiva järnisotoper och helkroppsräkning, samt arean under kurvan för serumjärnkoncentrationen under sex timmar (S-Fe $AUC_{0\text{-}6h}$) efter oral administrering av 100 mg järn. Även algoritmer baserade på humanstudier användes.

Resultat: Järnabsorption mätt med radioisotoper och med S-Fe $AUC_{0\text{-}6h}$ visade bra överensstämmelse (r^2=0.94). Alla de studerade Fe_{elem} hade sämre biotillgänglighet än ferrosulfat (RBV=36-65%). Tillsats av 20 g kött, eller 20 g kött tillsammans med 20 mg askorbinsyra, till en måltid med låg Fe-biotillgänglighet ökade den totala järnabsorptionen med 155 %, respektive 227 %. Kostmodifiering gav större förbättringar i järnstatus jämfört med järnberikning när det gäller en kost med låg biotillgänglighet.

Konklusioner: S-Fe $AUC_{0\text{-}6h}$ efter oral administrering av 100 mg järn är ett valid mått på järnabsorption. Kostmodifiering av måltider med låg Fe-biotillgänglighet kan markant förbättra järnabsorptionen, framförallt vid tillsats av kött, vilket bidrar med högabsorberbart hem-järn. Beroende på val av Fe_{elem} i interventioner kan utfallet skilja sig. Om den ursprungliga kosten har en låg Fe-biotillgänglighet är det svårt att uppnå positiva effekter på järnstatus genom att enbart använda järnberikning som den enda åtgärden. Således, den övergripande konklusionen i denna avhandling är att den bästa strategin vid interventioner avsedda att förbättra järnstatus, först och främst är att se till att den avsedda kostens Fe-biotillgänglighet är tillfredsställande, och för det andra se till att ett berikningsjärn med hög utnyttjandegrad används.

LIST OF PUBLICATIONS

The thesis is based in the following papers, which are referred to by their Roman numerals.

I. Hoppe M, Hulthén L, Hallberg L. **Serum iron concentration as a tool to measure relative iron absorption from elemental iron powders.** *Scand J Clin Lab Invest* 2003;63:489-496

II. Hoppe M, Hulthén L, Hallberg L. **The validation of using serum iron increase to measure iron absorption in man.** *Br J Nutr* 2004 92:485-488

III. Hoppe M, Hulthén L, Hallberg L. **The relative bioavailability in humans of elemental iron powders for use in food fortification.** *Eur J of Nutr* 2006; 45,(1),37-44.

IV. Hallberg L, Hoppe M, Andersson M, Hulthén L. **The role of meat to improve the critical iron balance during weaning.** *Pediatrics* 2003 Apr;111(4 Pt 1):864-70

V. Hoppe M, Hallberg L, Hulthén L. **The importance of bioavailability of dietary iron in relation to the expected effect on iron fortification.** *Eur J of Clin Nutr* 2007 May 30 [Epub ahead of print]

TABLE OF CONTENTS

ABBREVIATIONS

AA	Ascorbic acid
AGP	Alpha-1-acid glycoprotein
ANOVA	One-way analysis of variance
Ca	Calcium
CI	Confidence interval
CRP	C-reactive protein
DCYTB	Duodenal cytochrome b
DMT1	Divalent metal-ion transporter 1
EDTA	Ethylenediaminetetraacetic acid
ESR	Erythrocyte sedimentation rate
Fe	Iron
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
Fe_{elem}	Elemental iron powders
$FeSO_4$	Ferrous sulfate
FP	Ferroportin
Hb	Hemoglobin
HCl	Hydrochloric acid
HCP1	Heme carrier protein 1
Heph	Hephaestin
ID	Iron deficiency
IDA	Iron deficiency anemia
INACG	International Nutritional Anemia Consultative Group
OITT	Oral iron tolerance test
RBV	Relative bioavailability value
RES	Reticulo endothelial system
SEM	Standard error of the mean
S-Fe	Serum iron concentration
S-Fe $AUC_{0\text{-}6\ h}$	The area under the serum iron concentration curve during six hours
Tf	Transferrin
TfR	Transferrin receptor
TIBC	Total iron binding capacity
TSAT	Transferrin saturation
WBC	Whole body counter
WHO	World Health Organization

INTRODUCTION

Gold is for the mistress – silver for the maid
Copper for the craftsman cunning at his trade.
"Good" said the Baron, sitting in his hall,
"But Iron – Cold Iron – is master of them all!"

This famous (and to "iron scientists" most popular) introduction to the piece of poetry created by Kipling in 1910 could not be truer, although not exactly in the way that the remainder of the poem intends. Iron is, due to its nuanced chemical properties, an absolute necessity for life.

Iron – the element

Iron can alter between two different oxidation states, Fe^{2+} (ferrous iron) and Fe^{3+} (ferric iron). This ability provides iron with a precious quality in biochemistry, namely the ability to accept or donate an electron. This ability is the reason for the role of iron in, not only oxygen transport by hemoglobin which is the main function of iron in the body and the most widely known, but also DNA synthesis and energy production. However, the chemical properties of iron are also an obstacle when it comes to the availability. Due to the drive (read: the law of nature) to decrease free energy, iron easily oxidizes to ferric (trivalent, Fe^{3+}) iron, which in turn precipitates as the insoluble iron hydroxide at pH 7. Thus, in spite of the essential biological importance the major part of the iron in our environment is insoluble, making it unavailable for biological purposes. As a consequence, the human body has since the beginning of time, in interplay with our environment, adapted to this by developing a very limited capacity of secrete/lose iron. The only significant physiological loss of iron, apart from menstruation losses, is the one taking place when worn out enterocytes are being sloughed.

Iron – the nutrient

When going from being "just" an element to being a nutrient, iron passes over to be characterized as either heme iron or non-heme iron. The iron in heme iron is incorporated into a protoporphyrin skeleton forming the most beautiful heme molecule, which in turn is a functional part of, i.e. cytochromes, myoglobin and hemoglobin. The "father" of biochemistry, Felix Hoppe-Seyler (1825-1895), was the first to characterize the crystallized structure of hemoglobin, and its ability to bind oxygen.

The basis for the diet based characterization as heme iron or non-heme iron lies in the stability of the structure the iron is incorporated in. Due to its ability to donate

electrons iron is also able to catalyze Fenton and Haber-Weiss reactions, which create reactive oxygen species. To protect various structures in the organism from oxidative stress, the nonfunctional part of the iron pool is bound to different molecules, such as transport and storage proteins (e.g. transferrin and ferritin). The iron of the functional iron pool is part of numerous enzymes (e.g. catalases and ribonucleotide reductase) and heme proteins. When these iron containing structures, functional and non-functional, are present in the diet they are degraded by our digestive system. However, the heme structure strongly resists degradation. Thus, when presented to the mucosa cell layer in the intestines the iron in the heme is taken up as a complex molecule whereas the non-heme iron is taken up in its elemental form. As a consequence of lacking a protective "shell" like protoporphyrin, the absorption of non-heme iron is substantially affected by the composition of the diet and the iron status, whereas the heme iron is much less affected by these factors (Hallberg*, et al.* 2000b, Hunt 2005). The inorganic non-heme iron is the most dominant form of iron in our diet. It is predominantly found in vegetable foods, mainly cereals, but also present in animal products. Although meat is known as a provider of heme-iron, the major part of iron is in the form of non-heme. In general, the proportions of heme iron in red meat range from ~25-50%. However, when incorporating chicken or fish into a meal or diet the contribution of heme iron will be negligible (Hallberg*, et al.* 2000b).

Factors influencing iron absorption

Dietary factors

Although iron absorption is substantially affected by meal composition it is important to bear in mind that the effect of the influencing dietary factors only is valid when present in the same meal. Some of the most thoroughly studied and recognized dietary factors affecting non-heme iron absorption are calcium, polyphenols, phytate (inositol phosphates), meat (including poultry, fish and seafood), and ascorbic acid. The dose-response relation between these factors and their effects on nonheme-iron absorption differs in appearance. For example, calcium (Ca) has an iron inhibitory "window" of ~50-300 mg. In practice this means that a further Ca intake above, e.g. a large glass of milk (2.5 dl) has, in the same meal, no further inhibitory effect on iron absorption (Hallberg*, et al.* 1991a).

Interestingly, Ca does inhibit both non-heme iron *and* heme iron absorption. Besides meat it is the only dietary factor known to influence heme-iron absorption (Hallberg*, et al.* 1991a). The mechanism seems to be found in the site of action. Unlike other dietary factors which acts in the intestinal lumen, Ca has been proposed to act inside the mucosal cell (Hallberg*, et al.* 1992). As a consequence, the Ca mediated inhibition of iron can not be counteracted by, the otherwise very potent iron absorption enhancers, ascorbic acid and meat (Hallberg*, et al.* 1992). However, it is possible by dietary restructuring to meet the needs for both iron and Ca. This could be achieved by

restricting calcium intake with main meals, which usually contain most of the dietary iron and place the Ca intake in connection to meals low in iron content (Gleerup*, et al.* 1995).

Polyphenolic compounds is a large heterogeneous group of molecules which exist in colored varieties of cereals, legumes, fruits and berries, and as well in wine, tea, coffee and cocoa. Some of the more than 5000 known polyphenolic structures are potent inhibitors of iron absorption. However, their potency regarding their inhibitory effect differs. Thus, the content of iron binding polyphenols in a meal is sometimes expressed as tannic acid equivalents. The major inhibitory effect from polyphenols seems to exists in the first 100 mg tannic acid equivalents (Brune*, et al.* 1989, Gillooly*, et al.* 1983, Tuntawiroon*, et al.* 1991). As an example, a cup of black tea carry approximately 30 mg tannic acid equivalents.

The most frequent existing iron absorption inhibitor is probably phytic acid (or phytate when in the form of salt) (Hallberg*, et al.* 1989a, Siegenberg*, et al.* 1991). Since phytate, the six phosphates ester of inositol, is the most abundant form of phosphorus in cereals it is often expressed as phytate phosphorus (phytate-P). There are also data indicating that the number of phosphate groups is the best expression of the inhibitory effect from phytic acid (Brune*, et al.* 1992). When it comes to intake levels in different populations and diets there are not much data available. The daily median intake of phytate-P in, for example, rural central Mexico has been shown to be as high as ~1150 mg/day (Backstrand*, et al.* 2002). The principal sources in this setting were tortilla and legumes, where the maize based tortillas were responsible for almost 90% of the phytate-P. A more modest intake level has been reported from the UK where the daily phytate-P intake was around 100-200 mg (Wise*, et al.* 1987). The main part was coming from breakfast cereals and whole meal bread. Studies have shown that it is the first 10-20 mg phytate-P in a meal that exerts the largest inhibitory effect. Additionally increase of the amount of phytate-P in the same meal has minor relative effect (Hallberg*, et al.* 1989a, Hallberg*, et al.* 2000b). Consequently, complete elimination of phytate content is recommended. If this is not feasible, the phytic acid to iron molar ratio should be decreased to below 1:1 or 0.4:1 (Hurrell 2004).

An effective way of improving dietary iron bioavailability is by adding animal tissue (Gibson*, et al.* 2003). Recently it was shown that meat intake has an impact on iron status (Tetens*, et al.* 2007). The enhancing effect on iron absorption caused by meat has resulted in the denomination “meat factor” or “factor X”. And although this meat factor has been known for several decades (Layrisse*, et al.* 1969), the full mechanism is still unrevealed. It has been suggested that the effect is due to different amino acids. Some effects have been seen from cysteine (Martinez-Torres*, et al.* 1981) (Taylor*, et al.* 1986) and glutathione (Layrisse*, et al.* 1984). However, there are also results that contradicts the enhancing cysteine effect (Baech*, et al.* 2003b). The animal products, egg albumin and casein, has been shown not to enhance non-heme iron (Bjorn-

Rasmussen, *et al.* 1979, Cook, *et al.* 1976). Recent data also suggests that phospholipids isolated from beef may have enhancing effects on non-heme iron absorption (Armah, *et al.* 2008). Interestingly it was shown that beef stimulates gastric acid secretion, which also enhanced iron absorption in patients with achlorhydria. It was hypothesized that the meat effect is physiological, acting through stimulation of intestinal secretion (Bjorn-Rasmussen, *et al.* 1979). According to these results the meat factor seems not to be attributable to a single factor, but rather to be multifactoral.

Another effective way of improving iron bioavailability is through addition of ascorbic acid, which has been shown to counteract the dose-dependent inhibitory effect of phytates and polyphenols (Hallberg, *et al.* 1989a, Siegenberg, *et al.* 1991). It has been observed that it is the first 25-75 mg of AA added to a meal that is the most important in enhancing iron absorption (Hallberg, *et al.* 1986a). When aiming at enhancing iron absorption a weight ratio of ascorbic acid to iron of 6:1 to 13:1 has been proposed, depending on the amount inhibiting factors (Hurrell 2002, Lynch, *et al.* 2003). Also meat has this counteracting effect on the inhibitory effect of phytates, even though not as well defined compared to ascorbic acid and only seen at higher phytate concentrations (Bjorn-Rasmussen, *et al.* 1979, Hallberg, *et al.* 1989a).

Subject-related factors

As mentioned, the human body has, apart from menstruation losses, no physiological mechanism for iron excretion. This makes iron absorption and its regulation the key mechanism in maintaining a constant iron balance. A failure of this regulation can result in extensive negative health problems due to iron deficiency anemia or hemochromatosis. Consequently, the iron homeostasis is carefully controlled in healthy individuals (Bezwoda, *et al.* 1979, Cook, *et al.* 1974, Taylor, *et al.* 1988). In a healthy individual, the iron status is the predominant physiological factor controlling the iron absorption (Baynes, *et al.* 1987, Magnusson, *et al.* 1981, Taylor, *et al.* 1988). Both heme and non-heme iron absorption has a negative correlation with iron status, although the effect is more pronounced for non-heme iron (Hallberg, *et al.* 1979).

The definition of iron balance is a condition when the amount of absorbed iron from the diet equals the amount of iron needed to cover the physiological iron requirements. This is a condition which the body constantly strives at by effectively controlling the iron absorption (Bothwell 1995, Hallberg, *et al.* 1997, Hallberg, *et al.* 1995, Hallberg, *et al.* 1991b, Hulthén, *et al.* 1995, Hunt, *et al.* 2000, Sayers, *et al.* 1994). Therefore, when iron stores increases, for example owing to a dietary change to a better iron bioavailability, the body starts to negatively regulate iron absorption aimed at reaching new steady-state equilibrium. When the conditions are reversed, i.e. decreased iron stores, the body again strives at a steady-state condition by up-regulating the iron absorption whereupon the decrease in iron stores levels out. However, since the absorption from a diet of low bioavailability or low iron content only can be adaptively

up-regulated to a certain degree, the counteracting effect of iron absorption has its limitations. This limitation is most explicitly manifested as iron deficiency.

Another factor having an extraordinary effect on iron absorption, and the whole iron homeostasis, is the immunological acute phase triggered in the event of infection or inflammation. This will cause an almost instantaneous down-regulation of iron release by enterocytes and macrophages (Fillet*, et al.* 1989), a reduced iron absorption from the intestinal lumen, and at the same time an increased iron uptake by reticulo-endothelial cells (Lynch 2007). The effect of the inflammation-induced hypoferremia is characterized by a decreased serum iron (see figure 1), total iron binding capacity, transferrin saturation, (Beresford*, et al.* 1971, Hoppe*, et al.* 2007, Jurado 1997, Weinberg 1978). Following inflammation and infection there is also a rise in the serum ferritin concentration (S-ferritin) not reflecting iron status (Birgegard*, et al.* 1978, Elin*, et al.* 1977, Eskeland*, et al.* 2002, Hulthen*, et al.* 1998). If this inflammation-induced hypoferremia proceed the erythropoiesis will run short of available iron, which will hamper the production of red blood cells. Thus, a prolonged effect of these alterations will give rise to the so-called anemia of inflammation. The hypoferremia and anemia associated with infection and inflammation was described more than 60 years ago (Cartwright*, et al.* 1946). The mediator responsible for maintaining iron balance, as well as the acute-phase altered iron kinetics, appears to be hepcidin, a recently discovered peptide synthesized in the liver (Ganz*, et al.* 2006, Lynch 2007), and which by some has been referred to as the "holy grail" of iron metabolism.

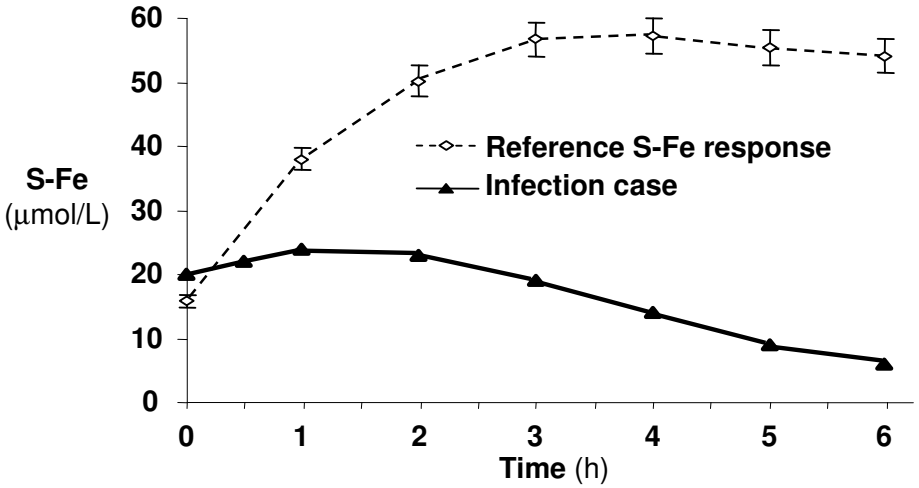

Figure 1. ***The impact of infection on serum iron concentration***.
Outbreak of a common cold during an OITT (100mg Fe) in a 28-year-old male, and the effects on the induced increase in S-Fe. The S-Fe response observed in 41 healthy males during the same test procedure is given as a reference (mean ± SEM) (Hoppe, et al. 2007).

Iron metabolism

Absorption – the mechanism

Iron in the gastrointestinal lumen is absorbed by the epithelial mucosa cells mainly in the duodenum and upper jejunum. The cellular uptake differs depending on whether the iron is in inorganic (elemental/ionic, i.e. non-heme) or organic (incorporated into the porphyrin skeleton as heme) form. The latter is more efficiently absorbed even though the exact mechanism is enigmatic (Hallberg*, et al.* 1992). However, a brush border heme transporter, heme carrier protein 1 (HCP1), was recently identified. This HCP1 was shown to be saturable as well as regulated by iron status (Shayeghi*, et al.* 2005). Earlier studies have shown that heme iron is absorbed by the mucosa cells as a intact iron-porphyrin-complex (Hallberg*, et al.* 1967, Raffin*, et al.* 1974). In the enterocytes the protoporphyrin is degraded by heme oxygenase, whereupon the iron is released into the same pool as the non-heme iron (Hallberg*, et al.* 1979, Raffin*, et al.* 1974). The two forms of iron partly share the same absorption pathway across the mucosal border. There are findings suggesting that the heme-iron absorption pathway in human is saturable somewhere at 15 mg heme-iron (Pizarro*, et al.* 2003), which could be a result of limited available heme oxygenase and/or HCP1. The influence of iron status on heme-iron absorption is small at low doses, but high at larger doses (Hallberg*, et al.* 1979), suggesting that heme oxygenase, as HCP1, also is regulated by iron status.

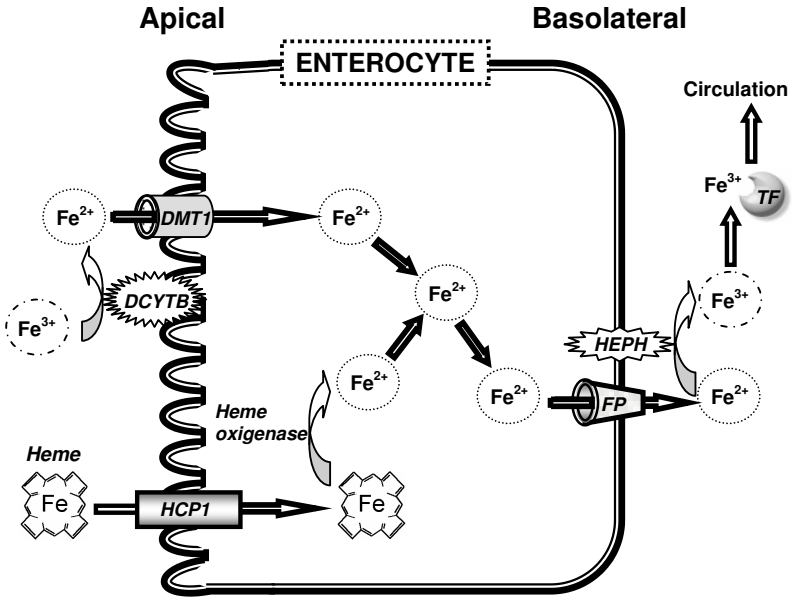

Figure 2. ***Intestinal iron absorption.***
Fe^{2+}: ferrous iron; Fe^{3+}: ferric iron; DMT1: divalent metal-ion transporter 1; DCYTB: duodenal cytochrome b; HCP1: heme carrier protein 1; HEPH: hephaestin; FP: ferroportin; TF: transferrin.

The inorganic non-heme iron is primarily absorbed as Fe^{2+} (ferrous). Thus, in order to pass into the enterocyte, dietary iron in the ferric (Fe^{3+}) form is first reduced to ferrous by the enzyme duodenal cytochrome b (DCYTB). The transport from the apical cellular membrane into the enterocyte is then taken care of by the divalent metal-ion transporter 1 (DMT1). Once inside the cell all iron, independent of its original dietary form (heme or non-heme), enter the same common pool. The last step before entering the circulation is the basolateral membrane transport. This goes via the ferroportin (FP) protein, to the plasma carrier transferrin (Tf), which transports the iron throughout the body and eventually docking with transferrin receptors (TfR) on the surface of iron needing cells (Mackenzie*, et al.* 2008). However, before the iron can be transferred over to Tf, FP must couple with hephaestin (Heph), whose ferroxidase activity oxidizes Fe^{2+} into Fe^{3+}. Although iron carried by Tf (i.e. serum iron) is distributed to all cells in the body the main part is used by the formation of hemoglobin.

Serum iron kinetics

Serum iron or plasma iron is the fraction of iron that is transported in the blood bound to the protein transferrin. Iron that binds to transferrin is either absorbed from the intestinal lumen or released from the iron stores or macrophages as result of the destruction of worn out erythrocytes, which is the main part. As a result of this the actual serum iron concentration (S-Fe) is the sum of the inflow of absorbed iron and the endogenous transport in and out of the circulation (see Figure 3). At the same time as iron is taken up from the intestinal lumen and transported into the circulation there is a simultaneous outflow of iron to the reticulo endothelial system (RES) and other stores. Thus, there is a constant flow of iron through the serum iron pool/compartment. Since the amount of iron in the serum at any given time is almost 10 times lower compared to the daily turn-over, S-Fe can rapidly be affected by various conditions. For example, infection or inflammation is characterized by a significant decrease in S-Fe (Beresford*, et al.* 1971, Hoppe*, et al.* 2007, Jurado 1997, Weinberg 1978), while hemochromatosis can give rise to extremely raised serum ferritin concentrations which affects S-Fe (Pietrangelo 2004).

As a clinical tool S-Fe provides little useful clinical information because of the rather large within-day and day-to-day variations, which makes it unachievable to assess iron status from S-Fe (Borel*, et al.* 1991, Dale*, et al.* 2002, Ekenved*, et al.* 1976b, Hamilton*, et al.* 1950, Hoppe*, et al.* 2003, Høyer 1944, Laurell 1952, Long*, et al.* 1978, Pilon*, et al.* 1981, Romslo*, et al.* 1988, Schwartz*, et al.* 1968, Sinniah*, et al.* 1969, Statland*, et al.* 1976, Waldenström 1946, Wiltink*, et al.* 1973, Winkel*, et al.* 1974). However, there is discrepancy about when the S-Fe peak appears.

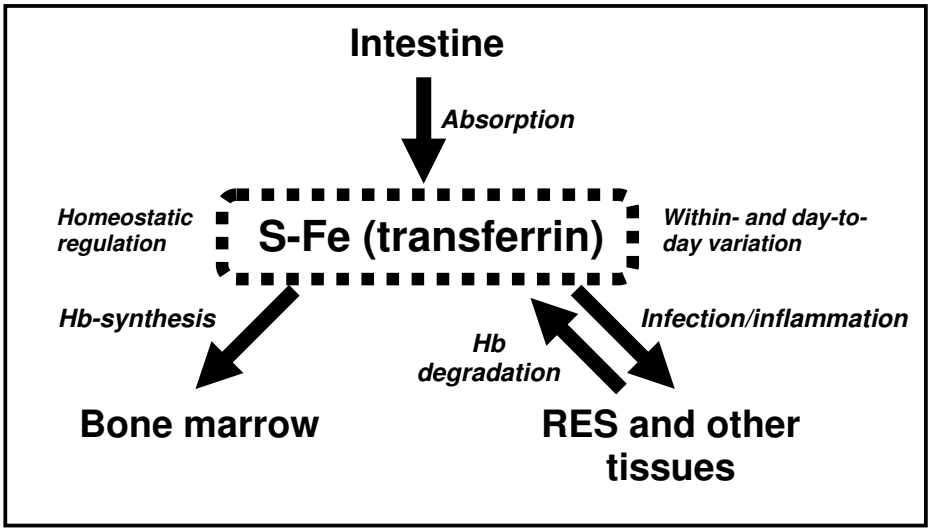

Figure 3. ***Factors controlling the turn-over in serum iron concentration.*** *The serum iron concentration at any given time is primarily determined by the inflow of absorbed iron from the intestine, and the transportation in- and out of the serum due to hemoglobin (Hb) synthesis- and degradation. RES: reticulo endothelial system*

Factors influencing serum iron concentration

Besides the above mentioned major factors there are several additional parameters that theoretically also could influence the serum iron concentration (S-Fe). Studying posture, that is lying, standing or sitting upright during 30 minutes prior blood sampling, did not significantly affect S-Fe (Statland*, et al.* 1974).

When it comes to dietary intake there are some contradictory results (Navarro*, et al.* 2003, Sinniah*, et al.* 1969, Statland*, et al.* 1973b, Wiltink*, et al.* 1973). However, despite these conflicting observations, the relative small amount iron present in a normal meal is not capable to raise the S-Fe to any appreciable extent. In order to use an oral iron tolerance test (OITT) to detect significant changes in iron uptake from different meals or iron preparations, there is need for doses in the pharmacological range. On the other hand it is vital that the iron dose is not too large so it exceeds the TIBC. If this happens a part of the absorbed iron will be deposited in the liver during the first passage (Fawwaz*, et al.* 1967, Wheby*, et al.* 1964). These limitation narrows down the size of the usable-dose-window for the OITT.

As mentioned earlier (Figure 1), inflammation or infection alters iron metabolism (Chiari*, et al.* 1995, Elin*, et al.* 1977, Eskeland*, et al.* 2002, Hoppe*, et al.* 2007, Kemna*, et al.* 2005, Srinivas*, et al.* 1988). The main thread in these studies is the considerably drop in S-Fe below the normal range due to the acute-phase response. The responsible mediator is hepcidin, which acts by down-regulating iron release by enterocytes and macrophages (Ganz 2003), and by internalizing and degrading

ferroportin, leading to decreased export into the circulation of absorbed and released iron (Nemeth, *et al.* 2004).

In the context of physical activity there is lacking consensus regarding the effect on S-Fe (Aruoma, *et al.* 1988, Cordova Martinez, *et al.* 1992, Gimenez, *et al.* 1988, Ricci, *et al.* 1988, Schmid, *et al.* 1996, Taylor, *et al.* 1987, Wirth, *et al.* 1978). Taken together these results indicate that strenuous physical activity can have an effect on S-Fe, whereas mild physical activity most likely does not. Although interleukin-1 has been shown to regulate hepcidin transcription (Lee, *et al.* 2004) and respond to strenuous physical activity, it does not seem like it affects S-Fe (Weight, *et al.* 1991). This suggests that physical activity and acute phase response have different metabolic pathways.

Methods for assessing iron absorption & bioavailability

Bioavailability – definition

The definition of "iron bioavailability" can be expressed as "the part of an orally administered iron dose that reaches the blood circulation and by that can be used for physiological functions". According to this definition, an optimal bioavailability is dependent on an optimal digestion and solubility, an optimal transport over the mucosal layer into the circulation, and finally an optimal incorporation into target organs. Although the ultimate method to assess iron bioavailability should relate to all these steps, any of these steps can be used as an isolated measure to estimate bio-availability.

The second step, i.e. the transport over the mucosal layer into the circulation, is in strict sense "absorption". However, the terms "bioavailability" and "absorption" are commonly used synonymous, which when considering the above described definition is somewhat confusing. Pragmatically, the term "absorption" is primarily a quantitative measure, which can be used to assess bioavailability, whereas the term "bioavailability" is more of a qualitative measure used to express the total utilization of a specific iron compound.

A commonly used concept is the Relative Bioavailability Value (RBV). RBV is obtained by comparing the estimated bioavailability (which in turn can be obtained at any of the three steps mentioned above) for a specific iron compounds to the same from a reference iron powder. A reference iron powder is one that is considered as having the most efficient bioavailability, e.g. ferrous sulfate.

There are a plentiful number of techniques developed to assess iron bioavailability (and absorption). Several of them can be combined creating a vast number of methods with varying accuracy when it comes to accomplish results relevant in humans.

However, all techniques and methods have limitations and advantages, and the balance between these differs depending on the context they are used in.

Solubility and dialyzability

Solubility is the first step in covering the bioavailability definition mentioned above, and thus a necessity for iron absorbability. Consequently, methods designed to study solubility of iron have been developed. However, owing to the complex environmental conditions in the gastrointestinal tract, together with the influence from dietary factors capable of forming iron-ligand complexes, human in vivo conditions can be fairly difficult to mimic.

In order to simulate the gastrointestinal environment a commonly used procedure is to measure the solubility in dilute hydrochloric acid (HCl) (Forbes*, et al.* 1989). To further mimic the iron absorption taking place in vivo, methods also introducing the aspect of dialyzability have been used (Miller*, et al.* 1981). The principle of the dialyzability method is that following an in vitro enzymatic digestion of a test meal the dialyzable iron passes over a dialysis membrane, which then can be quantified by spectrophotometry (Hurrell*, et al.* 1988). Recently it was suggested that the dissolution rate in 0.1 mol/L HCl could be a base for developing a reliable in vitro screening test to predict the RBV of elemental iron compounds (Swain*, et al.* 2003). The evaluated dialyzability method, however, was inadequate in predicting RBV (Swain*, et al.* 2003). The difficulties with using dialyzability to assess RBV has also been reported by Forbes *at al* (Forbes*, et al.* 1989).

Caco-2 cells

A commonly used *in vitro* model is the uptake of iron by Caco-2 cells. Although these cells are derived from human colon adenocarcinoma, they exhibit many features of small intestinal cells, including the influence from dietary factors on iron absorption (Han*, et al.* 1994, Han*, et al.* 1995). One indicator of the iron uptake in Caco-2 cells is the ferritin formation in the cells (Glahn*, et al.* 1998). Another approach to study Caco-2 uptake is to use radioactive iron isotopes (Han*, et al.* 1994). The Caco-2 cell model has in some studies proved to be a rapid screening procedure of the potential maximum bioavailability for iron compounds (Fairweather-Tait 2001).

Balance technique

This method is one of the first used to study iron absorption. The basis for the balance technique is the difference between input and output. After oral administration of a known amount of iron, a fecal collection period is performed. The iron absorption is then measured indirectly by calculating the difference between oral input and fecal output. The primary disadvantage of this method is that it is very laborious and expensive. And although several fecal markers can be used to ensure complete fecal collections, and stable isotopes can be used, the balance technique also has the disadvantage of being imprecise (Rossander*, et al.* 1992).

Hemoglobin repletion test in rats

Studies have shown that dietary inhibitors and enhancers of non-heme iron absorption had different effects in humans and in rats (Reddy*, et al.* 1991). Consequently, rodents can not be considered a suitable model for studying the effect of different dietary factors on human iron absorption. However, if using one and the same standardized meal/diet, and preferably excluding dietary factors known to influence iron absorption, the hemoglobin repletion test in rats has been suggested as a useful technique to assess the RBV of different iron compounds. Different groups of male anemic rats are fed diets fortified with either the iron compound in question, or a control iron which the result from the iron compound is expressed relative to. Each iron compound (including the control iron) is fed to three groups of rats in three different concentrations, and the increase in Hb is measured after 14 days. The RBV of the iron compound in question is obtained by comparing the plotted slope between the iron concentration and the Hb after two weeks, against the same slope obtained from the control iron (Swain*, et al.* 2003).

Radioiron isotopes

Since the first radioiron study almost 70 years ago (Hahn*, et al.* 1939), the two radioactive isotopes of iron (^{55}Fe and ^{59}Fe) have been extensively used in the study of iron absorption. The methodology has been subject for discussion in numerous papers and theses. One of the advantages of using the double radioisotope method is that each subject acts as her/his own control. The basis for this methodology lies in the "pool concept" which was introduced during the development of the extrinsic tag technique. When a single food stuff, which had been biosynthetically labeled with one radioiron isotope, was mixed together with iron salt labeled with the other radioiron isotope, the absorption from the two isotopes were practically identical (Cook*, et al.* 1972, Hallberg*, et al.* 1972). This identical absorption from both the extrinsic (iron salt labeled) isotope and the intrinsic (biosynthetically labeled) isotope remained even when meals having different iron bioavailability were served. The explanation for this is that there is an isotopic exchange through diffusion between the extrinsically added iron and the native iron in the food stuff. This isotopic exchange takes place in a common available pool of iron. However, there are some exceptions from this diffusion controlled isotopic exchange, and that is whole unpolished rice, which hard outer layer inhibits diffusion. Also ferritin, and some iron fortificants with low solubility have been found to be exceptions from this isotopic exchange (Hallberg 1981).

The iron absorption is assessed by calculating the difference between the administered radioactivity and the radioactivity measured either in blood or in the total body. Thus, radioiron isotopes absorption can be assessed either by whole body counting, or by using the iron incorporation in erythrocytes. When using the iron incorporation in erythrocytes to assess iron absorption two important estimations must be done. These are the percentage of absorbed iron that is incorporation in erythrocytes, and the actual

blood volume of the subject. In healthy subjects having normal iron status, approximately 80 % of absorbed iron will be incorporated into erythrocytes. However, this figure can differ depending on e.g. iron status, or presence of inflammation or infection. Estimates for blood volume are usually calculated from sex, weight, and height.

When using the whole body counting to assess iron absorption no such estimation is needed. Two weeks post-meal administration, the iron absorption from the ^{59}Fe (a γ-emitting isotope) labeled meal is calculated as the percentage of detected whole-body radioactivity, corrected for physical decay and background radioactivity. However, absorption from ^{55}Fe, which is a β-emitting isotope, can not be detected by whole-body counting. Thus, after the WBC, a blood sample is drawn in which the relative absorption of each of the isotopes is determined using a liquid scintillator. This relative absorption is then used to calculate the total body ^{55}Fe absorption. Combined with whole body counting, the double isotope method can be considered the present golden standard in iron absorption methodology.

Since iron absorption is affected not only by the composition of the meal but also the individual iron status (Baynes*, et al.* 1987, Magnusson*, et al.* 1981, Taylor*, et al.* 1988), there is a problem when making comparisons between different meals administered to subjects with different iron status. Hence, there has been an informal agreement of normalizing iron absorption results to the 40 % absorption from a reference dose of iron. The normalized meal absorption is the iron absorption for an individual having a reference dose absorption of 40 % (Magnusson*, et al.* 1981), corresponding to borderline iron deficit individuals not having developed anemia. The absorbed amount of iron at this standardized iron status is obtained by multiplying the meal and reference dose ratio with 40 (see page 26). For an example of an implementation of the methodology using radioiron and whole body counting see the Material and method section.

An alternative approach of normalizing the meal absorption values is the usage of serum ferritin as a proxy for iron status. Since a reference dose absorption of 40%, corresponds to a serum ferritin of 23 µg/L (Hallberg*, et al.* 2000b), the same effect is obtained by normalizing iron absorption to a ferritin concentration of 23 µg/L, as when normalizing to 40 % reference dose absorption. However, the influence of a triggered acute phase reaction can impair this normalization (Hulthen*, et al.* 1998).

Iron absorption from single meals, as well as whole diets (Gleerup*, et al.* 1995), can be studied using radioiron labeling. To study heme iron absorption biosynthetically labeled hemoglobin must be used (Hallberg*, et al.* 2003).

Due to the inadequate solubility of elemental iron powders there would be an inadequate isotopic exchange between an extrinsic marker and an elemental iron

powder. Thus, the extrinsic tag method using radioactive iron isotopes can not be used to study absorption from elemental iron. However, a way of using radioisotopes when studying the RBV of elemental iron powders is to label the elemental iron by neutron radiation (Hallberg*, et al.* 1986b). There are only a few such previous studies in human subjects using elemental iron powders labeled with radioiron (Bjorn-Rasmussen*, et al.* 1977, Cook*, et al.* 1973, Forbes*, et al.* 1989, Hallberg*, et al.* 1986b, Hoglund*, et al.* 1969, Rios*, et al.* 1975). In these studies there were some divergent results on bioavailability that most likely derives from the fact that different elemental iron powders differ in physicochemical properties. Even powders manufactured by the same method can vary considerably when it comes to solubility, particle size and reactive surface area (Hurrell*, et al.* 2002).

Stable iron isotopes

There are certain circumstances when the usage of radioactivity could be questioned. Examples of such situations are studies in children and pregnant women. During such conditions the stable iron isotopes ^{54}Fe, ^{57}Fe and ^{58}Fe can be used in various designs (Abrams 1999). A innovative stable isotope method using ^{57}Fe and ^{58}Fe to study iron absorption in infants has been published (Kastenmayer*, et al.* 1994). The isotopic enrichment of iron in erythrocytes was measured by mass spectrometry at baseline and 14 days post administration, and iron absorption from two different meals was calculated based on isotope ratio shifts, total circulating iron and intake of each isotope.

Another approach of using stable isotopes has been used to study iron absorption in pregnancy (Whittaker*, et al.* 1991). In this design the subjects are injected intravenously with 187 μg ^{57}Fe, whereupon a solution containing 5 mg ^{54}Fe as $FeSO_4$ together with ascorbic acid is administered orally. Blood samples are then collected every 30 min during 6 h. The fractional absorption of ^{54}Fe is assessed by calculating the area under the serum iron curve for $^{54}Fe/^{56}Fe$ and $^{57}Fe/^{56}Fe$.

However, if aiming at using stable isotopes in the study of elemental iron bioavailability the same aggravating circumstances exists as for radioiron isotopes. In order to label an elemental iron compound the isotope and the elemental iron compound must have the same exact physicochemical properties. Otherwise there will be no isotopic exchange. And, as in the case with radioiron, the only possibility is to synthesize an isotopically enriched form of the iron compound under study.

Oral iron tolerance test

Post-absorption serum iron increase has mostly been used to study iron absorption from iron preparations for pharmaceutical purposes and for diagnosis of iron deficiency (Ekenved 1976a, Ekenved*, et al.* 1976a, Gonzalez*, et al.* 2001, Kelsey*, et al.* 1991, Nielsen*, et al.* 1976). When the purpose has been to evaluate the usefulness of this method in discriminating between normal and iron deficient individuals the

small-dose iron tolerance test, using iron doses of 5-20 mg, is the most commonly used (Costa*, et al.* 1991, Crosby*, et al.* 1984, Jensen*, et al.* 1998, Jensen*, et al.* 1999, Joosten*, et al.* 1997). The small-dose iron tolerance test is based on the fact that low doses of iron do not have the potential to induce any changes in S-Fe in subjects with normal iron status. But for a subject with iron deficiency the iron dose will give a detectable increase in S-Fe. However, this method is not applicable when to study the effect from dietary factors on iron absorption from meals, or the RBV of iron preparations. When using the OITT to study bioavailability of iron compounds there is a need for pharmacological doses which has given raise to questions concerning the usefulness of this method when predicting the outcome in a diet setting when much lower doses of iron are present. However, results suggests that the induced S-Fe increase following 100 mg iron added to a food could predict the iron absorption of a small dose of iron added to the same meal (*paper II*).

Human efficacy

A method similar, although considerably complicated, to the hemoglobin repletion test in rats is used in human efficacy trials. This method is a measure of the efficacy of a specific iron compound in increasing the iron status of a group in comparison of a control group. An efficacy trial is performed under strictly controlled conditions, in contrast to effectiveness trials which can be seen as a real life situation. Thus an effectiveness trial is subject to a variety of factors, e.g. distribution systems, compliance, and other biological factors, which makes it considerable more difficult to evaluate (INACG 2004). A well-designed human efficacy trial is characterized by randomization, double-blinded placebo-control, and a strict compliance control. Iron status parameters, such as hemoglobin, serum ferritin and soluble transferrin receptor concentrations, are measured at baseline and post-trial. Although human efficacy, and especially effectiveness, trials gives the best answer concerning the outcome in a real life situation, the cumbersome and expensive methodology make them unsuitable as screening tools.

Mathematical models

Over the years there have been many attempts to develop mathematical models for estimating iron absorption and bioavailability (Anand*, et al.* 1995, Bhargava*, et al.* 2001, Du*, et al.* 2000, Hallberg*, et al.* 2000b, Monsen*, et al.* 1982, Monsen*, et al.* 1978, Reddy*, et al.* 2000, Tseng*, et al.* 1997). The complexity of the many dietary factors and their interactions has made this challenging. The first model described for estimating iron absorption from different meals required the five factors, total iron, non-heme iron, heme iron, ascorbic acid, and meat (including fish and poultry). From this information any meal could then be classified as having high, medium, or low bioavailability (Monsen*, et al.* 1978). A more extensive algorithm for calculating absorption and bioavailability of dietary iron has been validated and published (Hallberg*, et al.* 2000b). The strength of this algorithm lies in all the known dietary factors, both enhancing and inhibiting, and their interactions that are taken into

account, as well as the possibility of converting the algorithm to any iron status (Hallberg, *et al.* 1997). However, as a result of this complexity, the accuracy of the algorithm is dependent on a total information of all dietary factors influencing iron absorption (Lynch 2005).

Iron deficiency

Definition of iron deficiency

Iron deficiency can be divided into three phases, where the first phase is a decrease in the quantity of storage iron without any influence on the functional iron in the body. Thus, since maintaining an optimal Hb is of high priority for the body, this will take place without giving any clinical symptoms. Phase two is entered when further iron deficiency is taken place, introducing an inability to supply the iron demand from the erythropoiesis. This phase is sometimes referred to as "functional iron deficiency". The last and most critical phase is entered when no iron has been available for the erythrocyte production, and a definite anemia is diagnosed (Figure 4).

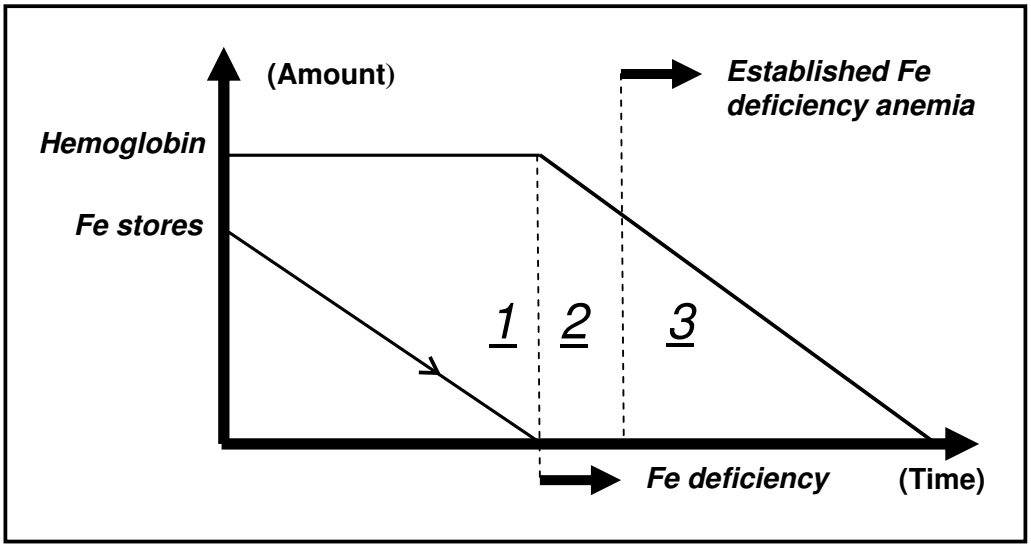

Figure 4. ***The three phases of Fe deficiency***. *1): decrease in storage iron 2): functional iron deficiency 3): established Fe deficiency anemia.*

Historical background

Chlorosis, in descriptive term meaning "pale disease", is an old name of what now generally is believed to be hypochromic and microcytic anemia, which primarily is caused by iron deficiency (together with copper deficiency) (Poskitt 2003). The first time it was described was probably by Hippocrates from Kos about 400 BC. Chlorosis is characterized by a pale, greenish complexion, which also endowed this disease the name "virgin's disease" or green sickness". The first time iron was suggested as a

treatment for chlorosis was in 1577 when the Spanish physician Nicolas Monardes published his "*joyful news out of the new-found world*" (Fox 2003). However, the empirical evidence for this hypothesis was laid forward almost 200 years later (1747) by the Italian physician Vincenzo Menghini. After observing that dried blood could be picked up by a magnet he draw the conclusion that blood must contain iron (Windsor*, et al.* 2007). A conclusion that later on lead physicians to use iron supplements in the treatment of chlorosis (Fox 2003).

Consequences of iron deficiency

Iron deficiency is globally the most common form of nutrient deficiency, affecting an estimated two billion people, and causing approximately 0.8 million deaths a year worldwide. In 2002 WHO ranked iron deficiency among the 10 leading risk factors for disease, disability and death (WHO 2002). And although iron deficiency (ID) is not the only cause of anemia, WHO estimated that iron deficiency is responsible for approximately 50% of all anemia cases. This would mean approximately 1 billion cases of iron-deficiency anemia (IDA) worldwide (WHO 2006). Thus, this nutrient deficiency has a major impact on health worldwide. In a public health perspective iron deficiency anemia can have far-reaching consequences owing to its effect on work capacity (Hunt 2002), resistance to infections, pregnancy and, not least, intellectual performances in the future resource of this world, the children (Cook*, et al.* 1994, WHO 2001).

Region	**Anemia** (total population)	
	No. (millions)	**% of total population**
Africa	244	46
America	141	19
South-East Asia	779	57
Europe	84	10
Eastern Mediterranean	184	45
Western pacific	598	38
TOTAL	**2030**	**37**

Table 1. ***Prevalence of anemia according to WHO.*** *Anemia is based on the proportion of the population with hemoglobin concentrations below established cut-off values (WHO 2006).*

Proposed consequences of iron deficiency, and primarily iron deficiency anemia, are diversified and range from the most commonly known, fatigue (Patterson*, et al.* 2001, Verdon*, et al.* 2003) and decreased aerobic performance (Brownlie*, et al.* 2002, Brutsaert*, et al.* 2003, Friedmann*, et al.* 2001, Hinton*, et al.* 2000), to altered cognitive

functions (Bruner, *et al.* 1996), immunes system alterations (Ahluwalia, *et al.* 2004), increased risk of maternal and child mortality (Brabin, *et al.* 2001a, Brabin, *et al.* 2001b, Stoltzfus 2001), and thermoregulation disorders (Beard, *et al.* 1990, Rosenzweig, *et al.* 1999), to mention some. However, regarding cognitive and behavioral alterations, it has been proposed that it can be confounded by the fatigue and low physical energy level (McCann, *et al.* 2007). Additionally, due to the fact that the predominant iron transporter over the mucosa layer, the divalent metal transporter 1 (DMT-1), has the ability to handle other divalent metal ions than Fe, iron deficiency leads to increased heavy metal absorption. It has been shown that iron deficiency during pregnancy leads to increased cadmium absorption (Akesson, *et al.* 2002), which consequently gives another dimension to ID as a health problem.

Counteracting/combating iron deficiency

"*Food is medicine - hence let your medicine be your food*"
(Hippocrates, approx. 400 BC)

However, as mentioned, during some specific circumstances there is need for more radical measures, i.e. fortification or supplementation. The most common nutritional way of combating iron deficiency (ID) and iron deficiency anemia (IDA) is by increasing the amount of iron in the diet. This is primarily achieved by fortification or supplementation. Since ID mainly is a consequence of poverty (even in some vulnerable groups in developing countries) this could also been accomplished by dietary improvements increasing the intake of iron rich foods (WHO 2001). However, owing to the substantial effect by the composition of the diet on non-heme iron absorption, an additional approach is to modify the composition of the diet, either by reducing the dietary factors having a negative effect on iron absorption, or increasing the factors having an enhancing effect (see page 4).

Efficacy and effectiveness of iron fortification interventions

Iron fortification as a measure of combating iron deficiency has been used for more than half a century and is still mandatory in many industrialized countries. Despite this long-term experience of iron fortification, no large-scale iron fortification interventions have been evaluated for effectiveness. Consequently, there are few data available to show the efficacy of iron fortification interventions in improving iron status (INACG 2004) (Hurrell, *et al.* 2002), especially interventions using iron fortification exclusively (i.e. plain iron-adding without modifying iron bioavailability). Studies of the effects on iron status of smaller iron fortification interventions, where fortification has been the only measure taken, have been controversial and not completely successful (Layrisse, *et al.* 1996, Sivakumar, *et al.* 2001). Studies reporting successful iron fortification interventions have with few exceptions also included encapsulation (Zimmermann, *et al.* 2004) or dietary bioavailability improvements, in the form of EDTA (Ballot, *et al.* 1989, Thuy, *et al.* 2003, Van Thuy, *et al.* 2005, Viteri, *et al.* 1995), fish powder (Lartey, *et al.* 1999) or/and ascorbic acid (Hertrampf,

et al. 1990, Walter*, et al.* 1993), as well. This makes the interpretation of the effects of the iron fortification alone difficult. And since there are observations indicating that low iron bioavailability diets can be a cause of iron deficiency anemia (Zimmermann*, et al.* 2005), this suggests that the bioavailability aspect is of importance.
Interestingly, a recent observation also suggest that the current global trend of increasing overweight and obesity may impair efforts to control iron deficiency (Zimmermann*, et al.* 2008).

Iron fortificants

There are many obstacles to ascend for a successful iron fortification program, which besides logistics also includes safety, technological and economical considerations (Bothwell 1994, Haas*, et al.* 2006). The diet related aspects to consider are primarily: an appropriate vehicle to which the iron can be added, and the actual absorbability of the fortification iron.
There are large differences between different forms of iron fortificants when it comes to absorbability (Hurrell 2002, Hurrell*, et al.* 2002). A factor influencing the absorbability is the solubility in the gastro-intestinal tract. Consequently, iron compounds used as food fortificants can be divided into three categories:

- water soluble
- poorly water soluble but soluble in dilute acid
- water insoluble and poorly soluble in dilute acid,

where elemental iron powders (i.e. iron in its metallic state, Fe^0) represents the third category, which per definition, has the poorest solubility in the gastrointestinal tract (Hurrell 2002). Elemental iron powders can, according to their method of production, be further divided into electrolytic, carbonyl, and reduced iron. Observations indicate that bioavailability of elemental iron can vary considerable. And since physiochemical characteristics can differ between elemental iron powders manufactured even by the same method (Hurrell*, et al.* 2002, Motzok*, et al.* 1978), it is important to evaluate every elemental iron fortificant before using them in fortification purposes.

When choosing iron fortificants, not only the bioavailability is of significance, but also the applicability in the food stuff. For example are ferrous sulfate ($FeSO_4$), and other soluble iron powders having high bioavailability, chemical reactive and acting as pro-oxidants. Thus, they can give rise to unpleasant color-, smell- or taste alterations in the food stuff which they are added to (Hurrell*, et al.* 1989). In many cases this makes ferrous sulfate and other soluble iron powders unsuitable for fortification. Elemental iron powders, on the other hand, is much more stable and do not cause adverse effects on the organoleptic qualities of the food vehicle (Hallberg*, et al.* 1989b).

Consequently, there is a relationship between solubility (and bioavailability) and adverse effects on the organoleptic qualities of the food vehicle. Roughly this means that, the better absorbability, the more unacceptable effects on taste and color. Unfortunately, this makes the choice of iron fortificant a compromise between

chemical reactive iron compounds with high bioavailability and more stabile iron compounds with lower bioavailability. This complicating effect on the organoleptic qualities is also why elemental iron powders, in spite of being water insoluble and poorly soluble in dilute acid, commonly is chosen as fortificants. But since bioavailability data in humans of elemental iron powders available on the market is limited, this calls for studies on their nutritional value in humans.

Background summary

There are three closely-related iron absorption aspects to consider; the iron bioavailability of the diet, bioavailability of the used iron fortificants, and the amount of iron in the diet (iron fortification). When it comes to the scientific evidence for the comparative effect from each of these measures in improving iron uptake in man, there are still questions. Consequently, the comprehensive question arising from this is;

What is the best course of action when it comes to improving iron uptake in man, increasing the amount of iron or enhancing the bioavailability, i.e. iron fortification or diet modification?

AIMS

The overall aim of this thesis was to increase the knowledge regarding the effect of diet modification and fortification on iron absorption in humans.

The specific aims were:

- To standardize and validate an iron tolerance test (*paper I & II*).
- To characterize the relative bioavailability in humans of a collection of samples of elemental iron powders reflecting the elemental iron fortificants available on the market in 2001 (*paper III*).
- To study both the separate effect of a fine particle meat powder, and the combined effect of meat powder and ascorbic acid (AA), on iron absorption when added to a meal with low iron bioavailability (*paper IV*).
- To study both the separate, as well as the combined, effect induced by dietary modifications and iron fortification on the quantity dietary iron absorbed (*paper V*).

MATERIALS AND METHODS

Summarized thesis description

Paper I and paper II describes the development of the method and design, which later on was used in paper III.
Iron absorption from elemental iron fortificants was studied in paper III, whereas paper IV studied the effect of dietary modifications on iron absorption. Finally were both these approaches, alone and in combination, studied in paper V.

Paper I was aiming at describing a standardized design for measuring the induced increase in S-Fe over time in a homogenous group of subjects having a high absorption capacity. Also a modified assay for analyzing serum iron concentration was described. To study the usefulness of this standardized oral iron tolerance test (OITT) the RBV of two different elemental iron powders were studied in two groups of human subjects.

Although Ekenved *et al* (Ekenved 1976a, Ekenved*, et al.* 1976b) had validated the oral iron tolerance test, we decided to control how well our OITT design correlated with radioiron absorption measured with whole-body counting. Thus, in **paper II**, the iron absorption accuracy of the OITT used in paper I was validated.

In **paper III**, the OITT and the design standardized and validated in paper I-II was used to assess the RBV of seven different elemental iron powders used as fortificants in 2001.

The next question, which was put forward in **paper IV**, was how dietary modification using meat, alone or together with ascorbic acid affects the non-heme iron absorption in human. In the case with the meat, not only the non-heme iron but also the heme iron absorption was investigated.

Finally, after observing the very potent enhancing effect from meat and AA on a meal with low dietary iron bioavailability, and the variety in RBV of different elemental iron powders, the following question was raised in **paper V**: How does different degree of dietary modifications and/or iron fortification levels affect Fe absorption and rates of changes in iron stores?

Design & Methods

Paper I

To evaluate weather an OITT could be used to distinguish between the RBV from different compounds, two groups of subjects performed three OITT´s approximately eight weeks apart. The measurement was described as the area under the serum iron concentration curve during six hours (S-Fe $AUC_{0\text{-}6h}$) following administration of 100 mg iron. At the three different occasions each subject was served a meal consisting of one wheat roll fortified with either 100 mg of one of two different elemental iron powders, or as $FeSO_4$. In order to study the diurnal variation in S-Fe a negative control, containing no iron, was also served at one of the occasions.
Eight weeks prior to each occasion the subjects had donated blood (~450ml) at the blood donor centre. After each day of investigation the subjects donated blood after a minimum of two days and a maximum of seven days. Eight weeks later, counting from the day of blood donation, the next day of investigation was scheduled (Figure 5).
The principle for the serum iron assay was that the iron is reduced by thioglycolic acid and is dissolved by hydrochloric acid (HCl). The proteins are precipitated by adding trichloroacetic acid, where after the iron is forming a colored complex together with sodium acetate-bathophenanthroline, which is measured photometrically (λ = 540 nm).

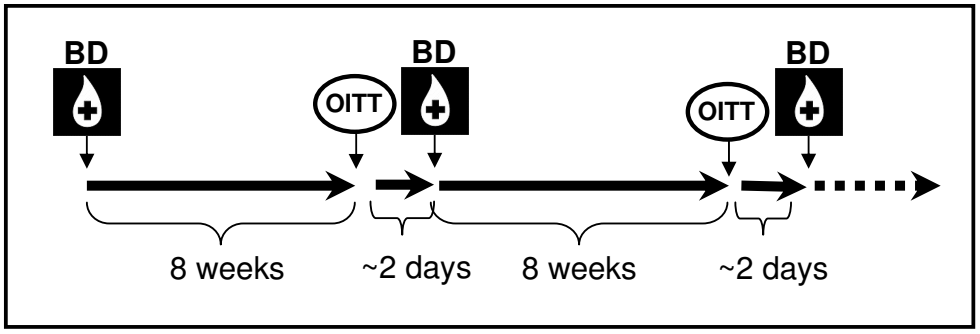

Figure 5. ***Study design in paper I & III.*** *BD: blood donation, OITT: oral iron tolerance test*

Paper II

In order to study the validity of the OITT described in *paper I* the subjects were, on three consecutive mornings, served a wheat roll fortified with $FeSO_4$. On the first two days the roll was fortified with 3 mg iron labeled with ^{59}Fe, whereas on day three the roll was fortified with 100 mg iron labeled with ^{55}Fe. The iron absorption from both these doses, as well as the S-Fe response from the 100 mg dose during six hours (S-Fe $AUC_{0\text{-}6h}$), was studied. Whole-body counting was performed approximately two weeks post test meal where after a blood sample was drawn in order to calculate the whole-body retention of both isotopes (Figure 6). For procedure see *paper IV* below.

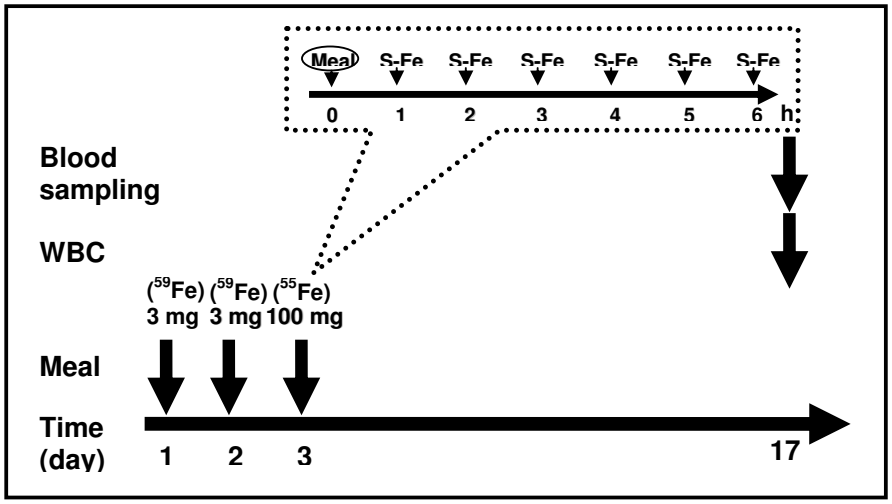

Figure 6. ***Study design in paper II***

Paper III

The, in *paper I-II*, described and validated method and design for measuring induced increase in S-Fe over time (S-Fe $AUC_{0\text{-}6h}$) was used to assess the RBV of seven different Fe_{elem}. One of the Fe_{elem} was also administered together with 50 mg ascorbic acid (AA). The design was the same as described in *paper I*, with the exception that three groups was studied. Further was no negative control served in the third group. The reason was that the result concerning the diurnal variation observed in group 1 and 2 was considered constant enough to be applied in the third group as well.

Paper IV

The study comprised four different experiments where two different dietary modifications of a basal meal with low iron bioavailability were studied. The dietary modifications consisted of adding 20 g red meat (experiment 1 & 2), or adding meat together with 20 mg ascorbic acid (AA) (experiment 3 & 4). Two of the experiments (1 & 3) studied non-heme iron absorption, whereas the other two experiments (2 & 4) studied the total iron absorption (nonheme and heme iron). In experiments 1 & 3 the modified meal (A) and the basal meal (B) were labeled with the 2 radioiron isotopes ^{55}Fe and ^{59}Fe, respectively, and served on four consecutive days (Figure 7). In experiment 2 & 4 the nonheme iron in the modified meal (A) was extrinsically labeled with ^{59}Fe. Heme iron in the same meal was extrinsically labeled by using biosynthetically ^{55}Fe-labeled rabbit hemoglobin. Each meal was served on two consecutive days. The reason for two servings per meal was to reduce the influence from the day-to-day variation in iron absorption (Bjorn-Rasmussen*, et al.* 1976, Brise 1962) .

Trial	Meal		Primary question
	A	B	
1 (4 days)	Basal meal ^{55}Fe	Basal meal ^{59}Fe + 20 g meat	How does 20 g meat affect nonheme-iron absorption?
2 (2 days)	Basal meal + 20 g meat (nonheme-iron labeled with ^{59}Fe and heme-iron labeled with ^{55}Fe)		How does 20 g meat affect total iron absorption?
3 (4 days)	Basal meal ^{55}Fe	Basal meal ^{59}Fe + 20 g meat + 20 mg AA	How does 20 g meat together with 20 mg AA affect nonheme-iron
4 (2 days)	Basal meal + 20 g meat + 20 mg AA (nonheme-iron labeled with ^{59}Fe and heme-iron labeled with ^{55}Fe)		How does 20 g meat together with 20 mg AA affect total iron absorption?

Table 2. ***The four different experiments presented in paper IV.*** *The nonheme-iron absorption and the total iron absorption from two different dietary modifications of a basal meal with low iron bioavailability were studied.*

All meals were served to the subjects after an overnight fast, and the subjects refrained from consuming any food or drink, including water, for three hours after completing the meals. Iron absorption was determined after two weeks by isotope retention, assessed with whole-body counting and erythrocyte radioisotope incorporation. The absorption from the iron labeled with ^{59}Fe (a γ-emitting isotope) was calculated as the percentage of whole-body radioactivity detected after 2 wk, corrected for physical decay and background radioactivity. However, absorption from ^{55}Fe, which is not a γ-emitting isotope, can not be detected by whole-body counting. Thus, after the WBC, a blood sample was drawn in which the relative absorption of each of the isotopes was determined using a liquid scintillator (Tri-Carb, Packard Instruments, Dallas). This relative absorption was used to calculate the total body ^{55}Fe absorption (See figure 7 for study design).

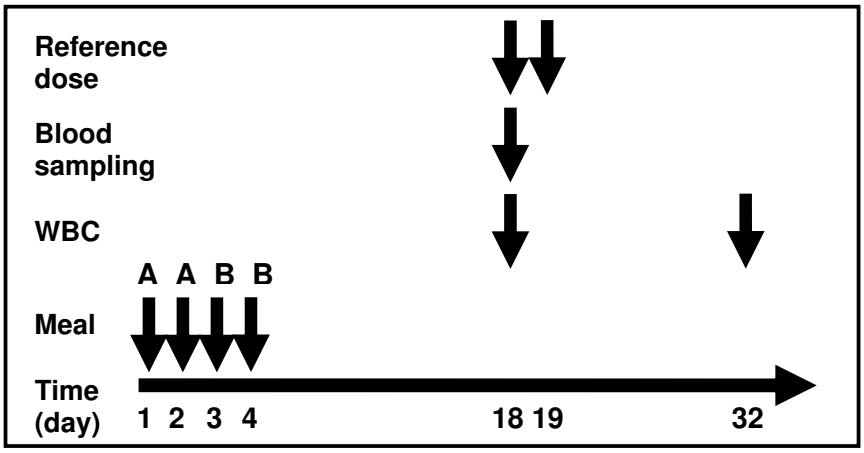

Figure 7. ***Study design for trial 1 and 3 presented in paper IV***

The magnitude of the iron absorption from one and the same meal can differ in large between individuals, primarily due to differences in iron status. Thus, to facilitate comparisons with other studies, the individual iron absorption from the meals was normalized to the same iron absorption capacity. This was done by, after the WBC and taking the blood sample, administering a reference dose (10 ml 0.01 mol HCl containing 3 mg ^{59}Fe-labeled iron(II) + 30 mg ascorbic acid). Iron absorption from this reference dose was assessed with whole-body counting after another two weeks. The iron absorption value from each of the meals was normalized to the iron absorption each individual had had if having a reference dose absorption of 40%. This is the percentage reference dose absorption seen in a subject with low iron stores, i.e. serum ferritin = 23 mg/l. Normalized absorption values for each subject were calculated as follows:

$$\frac{\text{Meal Fe absorption (\%) x 40}}{\text{Reference dose absorption (\%)}} = \text{Normalized meal Fe abs. (\%)}$$

Paper V

When aiming at studying the effect on iron absorption and rates of changes in iron stores following dietary modifications and/or iron fortification, different published mathematical models were used (Hallberg*, et al.* 2000b, Hallberg*, et al.* 1998, Hallberg*, et al.* 2000c). Methodological details concerning iron absorption calculations can be found in the original article (Hallberg*, et al.* 2000b), to which there are three errata (Hallberg*, et al.* 2000a, Hallberg*, et al.* 2001, Hallberg*, et al.* 2006, Hulthén*, et al.* 2006).

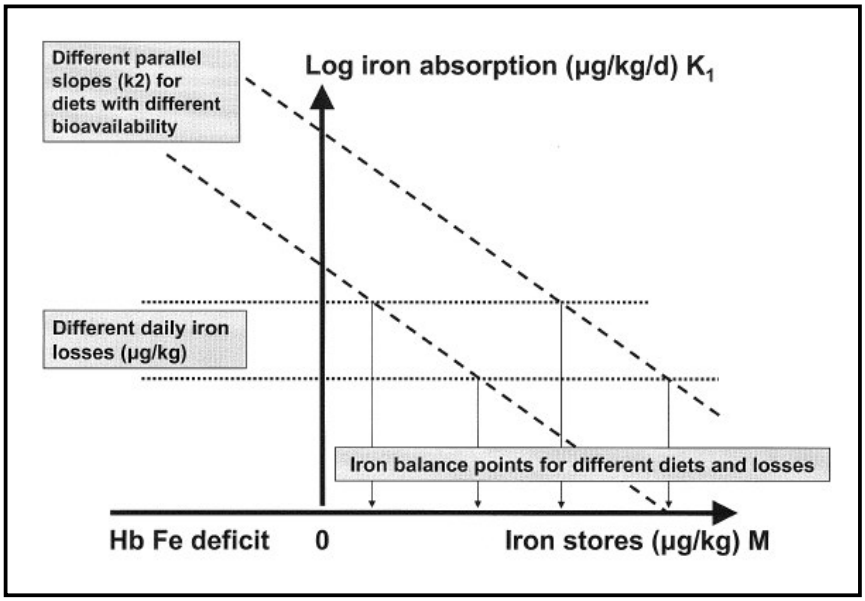

Figure 8. ***Basis for calculations of rate of changes in iron stores***

An equation describing amounts of stored iron at stationary, as well as an equation describing the rate of change in iron stores over time following changes in absorbed amounts of iron, has also been published. Theses equations quantitatively expresses the relationship between the amount of stored iron, iron absorption and iron losses (Hallberg*, et al.* 1998, Hallberg*, et al.* 2000c). The basis for calculations concerning the rate of change in iron stores is the observed linear relationship between log iron absorption and iron stores. The same applies to negative iron stores (i.e. hemoglobin iron deficits due to iron deficiency) (Bezwoda*, et al.* 1979, Cook*, et al.* 1974, Taylor*, et al.* 1988) (Figure 8).

The calculations were based on seven theoretical examples of meals with varying bioavailability, aimed at reflecting meals in developing countries. From these seven meals, five different diets (A to E) were put together (Table 3). The meals and diets are theoretical and have been presented merely according to their content of the, presently known, most important iron absorption enhancers and inhibitors, phytate, polyphenols, ascorbic acid, meat and calcium. The assumption is also made that the meat added to these meals is in the form of fish and chicken that does not contribute to any significant amounts of heme-iron, only meat factor (Hallberg*, et al.* 2000b). Further, the total daily intake of each diet comes from three separate meals, each containing the same amount of iron (1-10 mg/meal).

Dietary component	**Daily diet** (*meal combination*)				
	A	**B**	**C**	**D**	**E**
Ascorbic acid (mg)	35	60	80	95	110
Meat (g)	0	10	20	35	50
Phytate-P (mg)	300	160	75	33	17
Tannin eq. (mg)	145	75	30	15	5
Calcium (mg)	725	610	525	485	460

Table 3. ***Composition of 5 different examples of theoretical developing country diets.***
1 mg phytate-P = 3.53 mg phytic acid = 5.56 µmol phytic acid. Tannin eq

The low bioavailability diets represent the kind of diets seen in some regions of South Africa (Faber*, et al.* 1999), India (Agrahar-Murugkar*, et al.* 2004) and Mexico (Backstrand*, et al.* 2002). These diets contain very small amounts, if any, of meat and are based on, for example, rice, sorghum, maize, cereals, roots and beans, thus also containing high levels of the iron absorption inhibitors tannin and phytate. The calcium content of these five diets ranges between 460 and 725 mg/day, which is common in many regions of the world where dairy products are consumed only rarely (Agrahar-Murugkar*, et al.* 2004, Faber*, et al.* 1999, Nititham*, et al.* 1999, Woo*, et al.* 1998). As an example, a meal consisting of 100 g cooked rice served together with a

portion of stew containing of 100 g cabbage, 100 g mung beans, 3 g fish and 15 g sesame seeds, eaten three times a day together with a cup of tea would roughly represent Diet B in Table 3. A diet having similar bioavailability as Diet E could be accomplished by applying the phytate and tannin reducing strategies discussed under "Future perspectives" on page 42, together with eating slightly more fish and ascorbic acid rich vegetables or fruits.

Iron absorption calculations were made separately for each of the three meals, but the results relate to the total daily intake from these three separate meals. Since one of the main target groups for iron fortification is menstruating women, all the present calculations have been related to an adult menstruating woman weighing 60 kg and having empty iron stores but normal hemoglobin concentration.

Subjects

The studies in *paper I-IV* used human subjects to address the given aims. The results obtained in each of these studies were based on:

- **Paper I**: 32 healthy male blood donors (two groups of 16 subjects)
- **Paper II**: 11 healthy male blood donors
- **Paper III**: 48 healthy male blood donors (three groups of 16 subjects)
- **Paper IV**: 23 healthy women and 19 healthy men.

The number of subjects to include in *paper I -III* were based on the sample size studied by Ekenved *et al* (Ekenved 1976b). In *paper IV* the sample size was based on earlier iron absorption studies at our laboratory, and which rationale has been described (Rossander-Hulthén 1985).

Since **paper V** was using mathematical models (based on human absorption studies), and not clinical studies, only imaginary subjects were used. The calculations were related to adult menstruating women weighing 60 kg and having empty iron stores but normal hemoglobin concentrations.

Statistics

- Descriptive statistics
- Wilcoxon signed ranks test (*paper I*)
- Mann-Whitney U test (*paper I*)
- Pearson correlation test (*paper II*)
- One-way analysis of variance (ANOVA) with Tukey-Kramer as post-hoc test adjusting for multiple comparisons (least squares means) (*paper III*)
- ANOVA with Tukey as post-hoc (*paper III*)
- Student's paired t-test (*paper III & IV*)
- Student's unpaired t-test (*paper III & IV*)
- ANOVA (*paper IV*)

RESULTS AND DISCUSSION

Evaluating bioavailability of elemental iron

Serum iron assay

In *paper I-III* all the S-Fe analyses were performed at our laboratory using an in-house assay. The mean value of the quality control analysis was 20.1 ± 0.6 mmol/L (mean ± SD), mean within day coefficient of variation (CV) was 1.11% and the between day CV was 3.01%. Thus, the used modified assay for analyzing S-Fe has a high precision and accuracy

Diurnal variation in serum iron concentration

When observing the S-Fe during six hours after administering the negative control (the basal meal without any added iron) there was a steady increase from a baseline value of 19.4 ± 1.3 mmol/L (mean ± SEM). After six hours the mean S-Fe level was 25.4 ± 1.3 mmol/L and still climbing. The diurnal variation in the area under the S-Fe curve during six hours (S-Fe AUC_{0-6h}) for the negative control differed significantly from zero ($p<0.001$).

Since the first observation back in 1941 (Vahlquist 1941), this diurnal variation in S-Fe has been observed by numerous investigators (Dale, et al. 2002, Ekenved, et al. 1976b, Hamilton, et al. 1950, Høyer 1944, Laurell 1952, Schwartz, et al. 1968, Sinniah, et al. 1969, Statland, et al. 1973a, Statland, et al. 1976, Waldenström 1946, Wiltink, et al. 1973). However, although the presence of a within-day variation in S-Fe is well established, there is dissonance about when during the 24-hour period the peak appears. Some investigators have observed a peak in the afternoon/evening (Ekenved, et al. 1976b, Statland, et al. 1973a, Statland, et al. 1976, Wiltink, et al. 1973) while others have observed it during the morning hours (Hamilton, et al. 1950, Høyer 1944, Laurell 1952, Sinniah, et al. 1969, Vahlquist 1941, Waldenström 1946). Dale et al observed higher morning S-Fe compared to the afternoon levels for half of the subjects and vice verse for the other half (Dale, et al. 2002). One factor that appears to influence the peak point is the sleeping pattern. Night nurses who slept during the day had the highest S-Fe levels in the evening, in contrast to normal males and females who worked at day and slept by night and having the highest S-Fe levels in the morning (Sinniah, et al. 1969, Waldenström 1946).

The earlier done studies on within-day variation in S-Fe have in many cases monitored the basal cyclic pattern during a 24-hour period. In paper I, where we observed a steady increase in S-Fe, we only monitored a six-hour period (from approximately 8.00 hr to 14.00 hr), but on the other hand more frequently compared to earlier studies. Naturally, the frequency of measurements during a 24-h period will decide when a peak is observed. This could be a reason why there is some divergence regarding the

time for maximum S-Fe between some earlier studies and our. In the study by Laurell et al, they determined the S-Fe at 09.00 and 17.00 and found that the S-Fe was higher in the morning than in the afternoon (Laurell 1952). But since S-Fe only was determined at two occasions it means that there was an invisible 6-hr-period in which the S-Fe theoretically had time to fluctuate a great deal. In order to correspond to our result, the variation in S-Fe between 09.00 and 17.00 should at first rise, where after it falls, i.e. like an upside-down U-shaped curve. This behavior for S-Fe was seen in some of the subjects in the study by Sinniah et al. Although the S-Fe peak appeared in the morning, only looking at the S-Fe levels at 09.00 and 12.00 there was in five of seven males an increase (Sinniah, et al. 1969). Thus, in comparison to our results, these subjects showed the same S-Fe change.

Although a slight decrease in S-Fe from 9.00 am to 09.00 pm has been observed in children 3 to 14 years of age, no variation at all was seen in children under 20 months of age (Schwartz, et al. 1968). Regarding possible distinctions in the within-day variation of S-Fe between the sexes there are also conflicting observations. Sinniah et al saw an decrease between 09.00 and 12.00 in six of the seven females (peak level at 09.00 hr), whereas in five of seven males there was an increase (peak level at 12.00 hr) (Sinniah, et al. 1969). However, In the study by Laurell there was no difference between males and females between the two occasions that the S-Fe was determined (Laurell 1952).

In summary, our results in paper I, together with some earlier published results, show that there is a clear and distinct increase in S-Fe from the morning to the afternoon. This means that behind the visible S-Fe increase when administering an iron dose there are also other events going on, meaning that the visible S-Fe increase is not solely the effect of iron absorption. This basal diurnal S-Fe increase will bring an overestimation of the influence from any iron compound on S-Fe. When the absorption is low this overestimation will be even more marked. Therefore the influence of this diurnal variation in S-Fe was examined and used to calibrate the S-Fe curves obtained from the investigated iron powders.

Validating S-Fe $AUC_{0\text{-}6\ h}$ as a measure of iron absorption

When in *paper II* administering 100 mg iron, the squared correlation coefficient (r^2) for iron absorption and the S-Fe $AUC_{0–6h}$ was 0.94 (p<0.001, n=8). Thus, the S-Fe $AUC_{0\text{-}6\ h}$ was in *paper II* shown to be a valid measure of iron absorption (Figure 9). However, an argument against using a large dose of iron, in this case 100 mg, when conducting the OITT is that it is an pharmacological dose of iron which response perhaps can not be translated into an actual fortification situation when smaller physiological doses are used. However, in *paper II* the r^2 for iron absorption from 3 mg and the S-Fe $AUC_{0–6\ h}$ from 100 mg iron was 0.90 (p<0.001, n=8). And when comparing the absorption from 3 mg iron with the absorption from 100 mg iron, r^2 was 0.88 (p<0.001, n=10). These results implies that the induced serum iron increase

following 100 mg iron added to a food could predict the iron absorption of a small dose of iron added to the same meal. As stated in the introduction section regarding the aims of study II, the 3-mg and the 100-mg iron doses were administered at "roughly" the same time, i.e. on consecutive days. In order to answer the question at issue no other experimental procedure was possible. However, this leaves some things to consider when looking at the results. When administering the 3-mg iron dose on two consecutive days and calculating the mean absorption from those days it reduced the influence of the day-to-day variation in absorption from this iron dose. No such reduction in the day-to-day variation in absorption from 100-mg was possible. Even though the correlation was very good this brings insecurity into the comparison of the study of the 3-mg and the 100-mg iron dose, which could influence the correlation negatively. However, when studying the correlation between S-Fe $AUC_{0\text{-}6\ h}$ and absorption from 100-mg iron the day-to-day variation has no significance since it concerns iron absorption and S-Fe change studied at one and the same point of time.

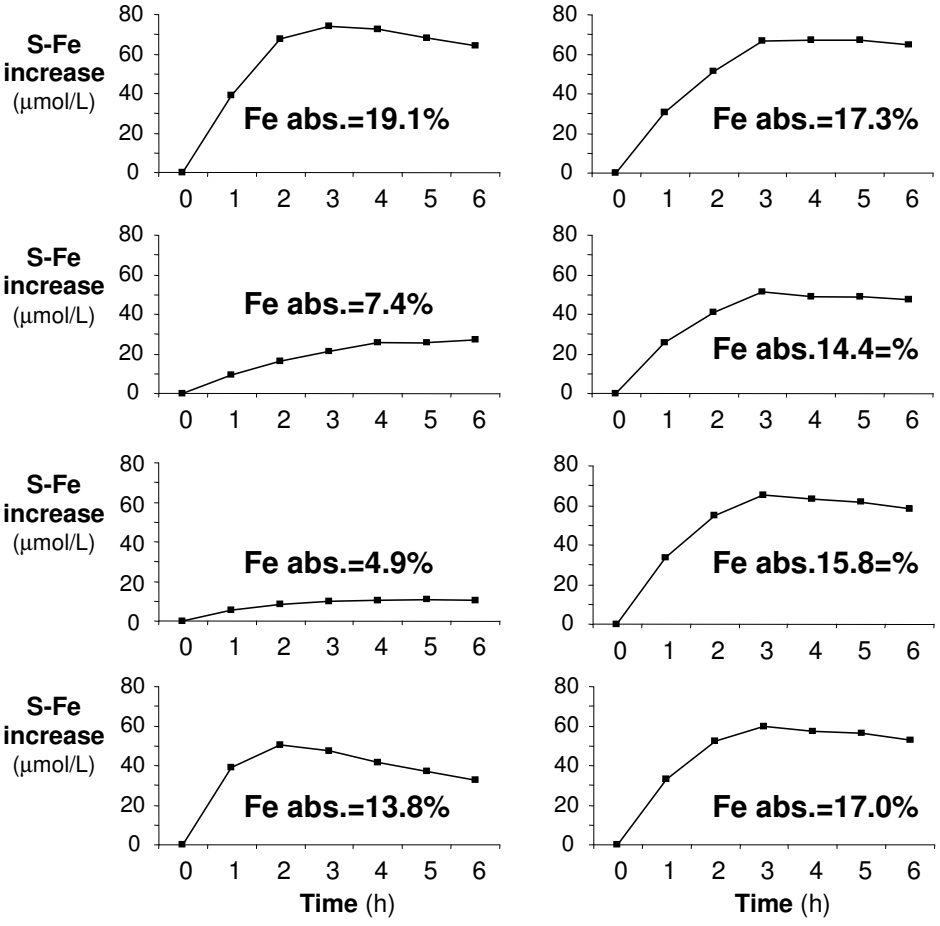

Figure 9. ***Correlation between S-Fe $AUC_{0\text{-}6\ h}$ and Fe absorption observed in paper II (r^2=0.94).*** Illustrated are the individual S-Fe increases for six hours following administration of 100 mg Fe (as $FeSO_4$). The Fe absorption (%) from the same Fe dose measured with radioiron and whole body counting are also given.

Relative bioavailability of elemental iron powders

In *paper III*, the S-Fe $AUC_{0–6h}$ induced by each of the seven evaluated iron compounds differed significantly from both the diurnal variation S-Fe $AUC_{0–6h}$ ($p<0.001$), and from the $FeSO_4 \cdot H_2O$ S-Fe $AUC_{0–6h}$. However, no overall relative bioavailability (RBV) ranking of the seven iron compounds was possible. The only difference seen was for the reduced iron powder, Atomet 95SP, (0.36±0.04, mean ± SEM) which was significantly lower than both the H-reduced iron powder, AC-325, (0.56±0.04) and the carbonyl iron powder, Ferronyl, (0.58±0.08).

The effect of ascorbic acid on absorption from electrolytic iron

When administered at a weight ratio of AA:Fe ≈ 1:1.8, ascorbic acid (AA) had a marked effect on iron absorption from an electrolytic iron compound. The electrolytic iron powder A-131 given with AA were as well absorbed as $FeSO_4 \cdot H_2O$, and significantly higher compared to the RBV of A-131 administered alone ($p<0.05$). Additionally, compared to when administering A-131 alone, the S-Fe curve observed when also adding AA had a steeper increase and a somewhat faster decrease after passing the maximum S-Fe. The great majority of studies investigating the enhancing effect from AA on non-heme iron absorption have used ferrous sulfate as the source of iron. Accordingly, the knowledge regarding the effect from AA on the absorption from elemental iron compounds are scarce. The observations by Forbes *et al* indicate that the effect from AA on electrolytic iron powder is not as pronounced as when added to ferrous sulfate (Forbes*, et al.* 1989). The recent observations by Swain *et al* showed a even more modest effect from AA when administered together with an electrolytic iron compound (Swain*, et al.* 2006). The weight ratio of ascorbic acid to iron considered necessary to achieve optimal positive effect on iron absorption is, when using ferrous sulfate, between 6:1 and 13:1 (AA:Fe). The higher ratio is needed in the presence of a large amount of iron absorption inhibitors (Hurrell 2002, Lynch*, et al.* 2003). Nevertheless, despite being administered at a weight ratio of ~1:1.8 we observed in *paper III* a marked effect on iron absorption from an electrolytic iron compound. Furthermore, AA not only enhanced the total iron uptake from a wheat roll fortified with 100 mg electrolytic iron powder, but also accelerated the early uptake. Consequently, our results in *paper III* are in some way in contrast to the few earlier observations on elemental iron and ascorbic acid.

Dietary modification of a low bioavailability meal

The effect of adding finely powdered meat

The results, which are based on the pooled data in *paper IV*, are presented in Table 4. Adding freeze dried meat powder (corresponding to 20 g lean meat) to a whole-wheat and milk based meal resulted in a non-heme iron absorption of 0.122 ± 0.014 mg (mean ± SEM), i.e. 85% increase. An additional 0.046 ± 0.004 mg iron was absorbed in the form of heme iron. Together this corresponds to 155 % increase in total iron

absorption. Thus, in relation to earlier observations (Baech*, et al.* 2003a, Baech*, et al.* 2003b), there was an unexpected high enhancing effect on the non-heme iron absorption from this rather small amount of meat. This could be due to a more pronounced local effect on the epithelial mucosa cells in the duodenum and upper jejunum by administering finely powdered meat compared to when meat in relatively larger pieces is exposed to the mucosa cells.

The effect of adding meat and ascorbic acid

When also adding 20 mg ascorbic acid (AA) together with 20 g meat, there was an increase in non-heme iron absorption from 0.122 ± 0.014 mg to 0.170 ± 0.029 mg (mean ± SEM). Compared to the basal meal, there was an increase in non-heme iron absorption and total iron absorption of 158% and 227 %, respectively (Table 4).

The effect of adding ascorbic acid

The iron absorption from adding ascorbic acid (AA) alone was not measured. However, it can be assessed indirectly by calculating the difference in iron absorption when adding meat alone with the iron absorption when adding meat and AA together. When also adding 20 mg AA to the meal containing 20 g meat, there was an increase in non-heme iron absorption of 0.048 mg (39%). By this it can be estimated that the effect of adding AA alone to the basal meal would have resulted in iron absorption of 0.114 mg non-heme iron (0.066 mg + 0.048 mg). This would mean that the expected increase in total iron absorption from adding AA to the basal meal would be 73%. However, since this effect was calculated from the effect seen when serving both meat and AA, it is possible that the reported effect for AA alone can be overestimated due to synergism. On the other hand, a similar study using 50 mg of AA has reported more than tripled iron absorption from a wheat based meal (Cook*, et al.* 1997).

Meal	**Number of Subjects**	**Absorption** (mg Fe)		
		Non-heme	**Heme**	**Total**
Basal meal	8M (7 BD), 8F	0.066 ± 0.019	—	**0.066** ± 0.019
+ AA	9M (6 BD), 8F (1 BD)	0.114 [1]	—	**0.114** [1]
+ Meat	8M (6 BD), 7F (6 BD)	0.122 ± 0.014	0.046 ± 0.004	**0.168** [2]
+ AA + Meat	9M (6 BD), 8F (1 BD)	0.170 ± 0.029	0.046 ± 0.004	**0.216** [2]

Table 4. ***Iron absorption following dietary modification using meat and ascorbic acid.*** *Abbreviations: M, male; F, females; BD, blood donors; AA, ascorbic acid. Values represent mean ± SEM.*
[1] The effect from AA is assessed indirectly, i.e. SEM can not be given.
[2] SEM can not be given since the value is obtained by combining results from two different experiments.

Also the weight ratio of AA to non-heme iron speak against an overestimation. In the AA & meat added meal the ratio was 9:1. However, adding only 20 mg AA to the basal meal results in a weight ratio of 10:1, thus better conditions for a positive effect on iron absorption.
The summarized results from these dietary modifications imply that, although the effect from AA on non-heme iron absorption was almost as good as the effect from the meat if looking at the TOTAL iron absorption, the effect from meat was much more pronounced.

Dietary modification *vs.* iron fortification

The effect on Fe absorption

Results in *paper V* were obtained by using mathematical models to estimate the iron absorption and rates of changes in iron stores from theoretical meals and diets with varying bioavailability. The iron absorption from the five different diets (A-E) varied from 1.6 to 12.7%. This means that when applying the two dimensions, bioavailability and iron content (3-18 mg), the expected absorbed quantities of iron vary from 0.05 to 2.29 mg/day. To meet the average iron requirement (concerning absorbed amount) for women of fertile age weighing 60 kg, the high iron bioavailability Diet E has to have a daily iron content of 11 mg, as compared with 18 and 30 mg for Diets C and B, respectively. Diet A, however, can not meet the requirement even with a daily iron intake of 60 mg (Figure 10).

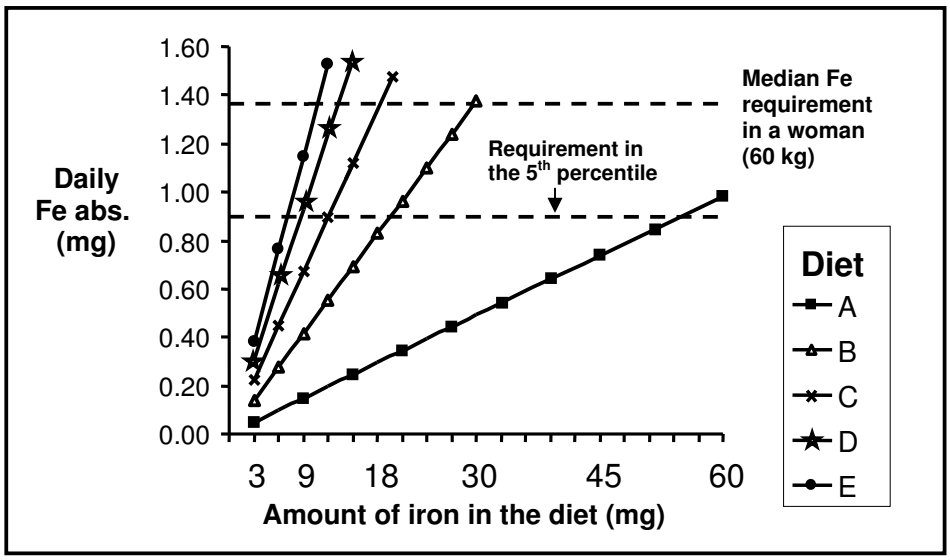

Figure 10. ***Efficacy of diets with different bioavailability and iron contents in satisfying iron requirements.*** *Iron absorption is predicted using an mathematical model (Hallberg, et al. 2000b). Dietary iron bioavailability increases when going from diet A to diet E.*

Hence the conclusion to draw from the results in *paper V* is that dietary modifications of meals with low iron bioavailability can markedly improve iron absorption. Further, when also considering the result in *paper IV*, it can be concluded that this is especially seen when adding red meat which also contributes with highly absorbable heme iron.

The effect on Fe stores

If looking at changes in iron stores over time a prolonged intake of the low bioavailability Diet A containing 15 mg iron (Diet $A_{15\ mg}$) would result in a pronounced iron deficiency. By fortifying this Diet $A_{15\ mg}$ with 30 mg iron per day the amount of stored iron, when reaching the steady-state, would increase by 275 mg. If instead improving bioavailability slightly (switching from Diet A to Diet C, see Table 3), without changing the iron intake, the amount of stored iron should increase by 380 mg. However, neither of these two measures is capable of achieving a positive iron store. When instead combining the action of both modifying and fortifying with 6 mg/day, by switching from Diet $A_{15\ mg}$ to Diet $C_{21\ mg}$, the amount of stored iron increase by 464 mg. This combined action is sufficient to achieve a positive iron store of 37 mg, and consequently, an optimal Hb concentration (Figure 11). Consequently, considering a diet of low iron bioavailability, it is difficult to achieve good effects on iron status by using iron fortification as the only measure.

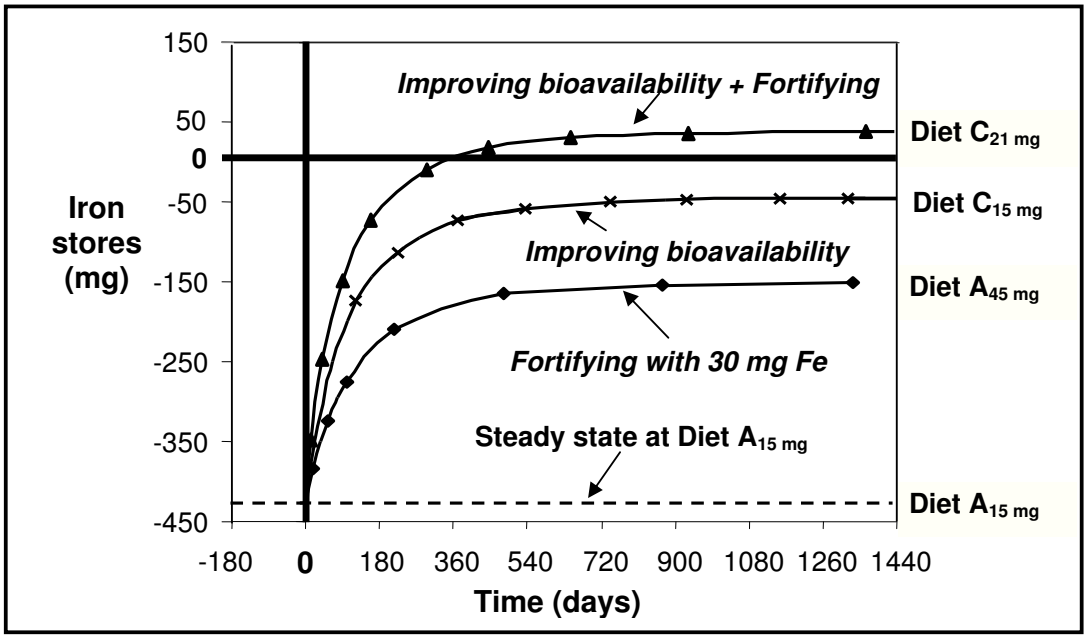

Figure 11. ***Changes in iron stores over time from either fortifying, improving bioavailability, or both fortifying and improving the bioavailability of a diet having low iron bioavailability (Diet $A_{15\ mg}$).*** *Negative iron stores result in a hemoglobin deficit. Results are predicted using mathematical models (Hallberg, et al. 2000b, Hallberg, et al. 1998, Hallberg, et al. 2000c).*

This conclusion could be a likely explanation for the results obtained in a population of South African schoolchildren. When administering brown bread fortified with iron no change in iron status could be observed (van Stuijvenberg*, et al.* 2008). An explanation for this could be that the both the vehicle, as well as the diet as whole, had an iron bioavailability to low to benefit from the iron fortification.

By fortifying and/or modifying Diet E in a similar way as with Diet A, it would have almost the same effects on the relative rates of change in iron status, with optimal effect being achieved after approximately 1 year. However, the final improvements in iron stores would not be as significant after dietary modifications than after iron fortification. Just as has been observed in young adult New Zealand women with mild iron deficiency on a diet having high iron bioavailability (Heath*, et al.* 2001).

Rates of changes in iron stores depending on choice of Fe_{elem}

By applying the method used in *paper V* on the result obtained in *paper III* the importance of Fe_{elem} RBV is elucidated.

Fortification programs are naturally launched in populations having difficulties maintaining a positive iron balance. An example of a diet in such a population could contain a total daily intake of 15 mg native iron, 90 mg ascorbic acid, 75 g fish/poultry, 90 mg phytate-P, 24 mg tannin eqv., and 450 mg calcium, divided into three similar meals. This kind of diet has an iron bioavailability not sufficient to maintain positive iron stores at steady-state in females of reproducing age weighing 60 kg (negative iron stores of 16 mg, i.e. a hemoglobin deficit). By fortifying this fictitious diet with 6 mg iron as carbonyl iron having a RBV=0.58 (*paper III*), instead of a reduced iron with a RBV=0.36 (*paper III*), the time to achieve a positive iron stores would be reduced to almost half, 64 days instead of 117 days. Also the final iron store after reaching steady state would be more than doubled, depending on the choice of fortificant (Figure 12).

Consequently, depending on the choice of Fe_{elem} in fortification programs the efficacy in increasing iron stores can differ distinctly, both in the short- and the long term. Further, the same mentioned choice of iron fortificant would almost halve the amount of iron passing the intestinal lumen in vain, which potential negative effects not can be ignored (Lund*, et al.* 2001, Lund*, et al.* 1999, Younes*, et al.* 1990).

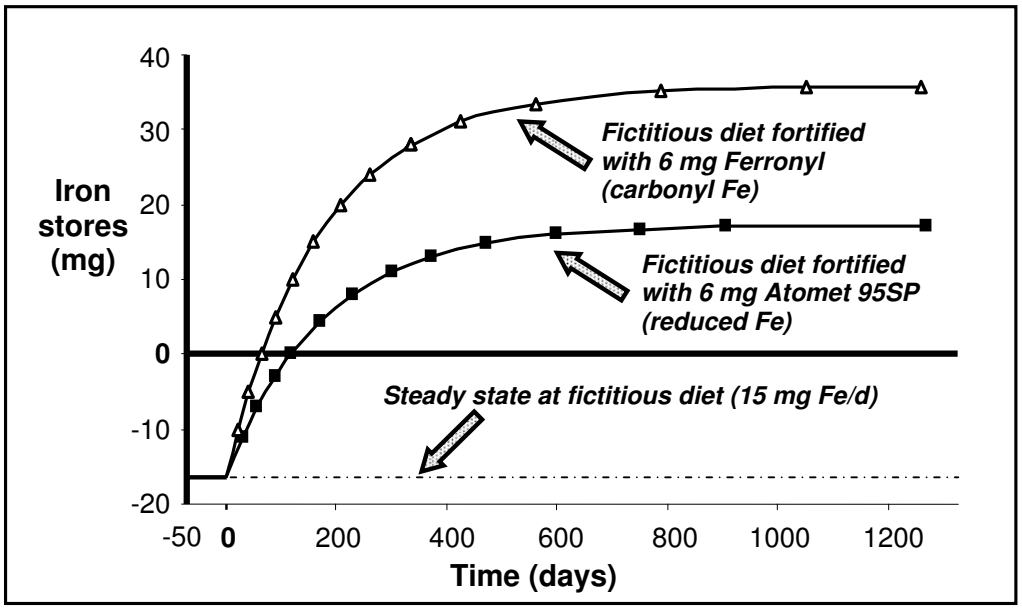

Figure 12. ***Changes in iron stores over time depending on choice of iron fortificants when fortifying a fictitious diet with low bioavailability.*** *By applying the method used in paper V on the result obtained in paper III it is shown that efficacy in increasing iron stores can differ distinctly depending on the RBV of the fortificant. Results are predicted using mathematical models (Hallberg, et al. 2000b, Hallberg, et al. 1998, Hallberg, et al. 2000c).*

Methodological considerations

Extrinsic labeling technique (paper II & IV)

Since this methodology has been so extensively used in the study of iron absorption it has been subject for discussion in numerous papers and theses, i.e. (Rossander-Hulthén 1985).

Deviations in iron absorption from two identical meals can originate from the potential sources of error one needs to consider in these kinds of studies. Random sources of error are present in everything from preparation of isotopic solutions and meals, to the very handling of the blood samples. Also individual day-to-day variations in iron absorption contributes to deviations (Bjorn-Rasmussen*, et al.* 1976, Brise 1962). This can be reduced by assessing the mean value following administering of the study meal on two consecutive days. An example of a random error is the use of an iron compound with low solubility, which accordingly does not undergo isotopic exchange with the native iron in the food stuff. This random error will result in overestimation of the iron absorption. To get round this, the native iron in the meals studied in *paper IV* was replaced with $FeSO_4$, which have a high solubility and bioavailability. The inclusions of subjects can also be a source of error, especially in studies using control subjects. By using two isotopes every subject acts as her/his own control, thus

minimizing this source of error. This also goes for when comparisons where done in the same group. Very low, and thus unreliable, iron absorption values can be observed in iron replete individuals. Consequently, to achieve a variation in iron absorption it is important to include different subjects representing a broad range in iron status. In *paper IV*, blood donors, as well as females and males were selected as to represent such a broad range in iron status. However, there is also a potential risk in using blood donors who have recently donated blood. The risk lies in a possible higher iron absorption capacity at the time point of serving the study meals, compared to the time point for serving the reference dose, which is two weeks later. At the time for the reference dose the blood donors can have advanced towards a steady-state, which may not have been the case when eating the study meals. An additional weak point could be to include women of fertile age since they have a monthly cycle in which a periodical iron loss occurs. Although the median blood loss during menstruation is relatively low, approximately 20-30 mg (Bothwell 1996), it can differ greatly between individuals (Hallberg*, et al.* 1966, Hallberg*, et al.* 1964). Thus, this can possess a risk when including only a small number of women in an iron absorption study. Accordingly, the optimal approach would be to administer the reference dose and the study meals at the same time point in the menstruation cycle. However, the source of error in risk of having the largest impact is the compliance of the subjects, which includes heeding to the instructions regarding fasting when served the study meals and the reference dose.

Iron absorption algorithm (paper V)

Methodological considerations of the presently used algorithm by Hallberg & Hulthén (Hallberg*, et al.* 2000b) has been discussed in *paper V*, resulting in the conclusion that the algorithm is valid enough to assess *mean* iron absorption from single meals. However, a conference abstract mentioned in this recent discussion has since then been restructured and published. In this published paper by Beard *et al* (Beard*, et al.* 2007) six different prediction algorithms for iron bioavailability, including the Hallberg & Hulthén algorithm, were compared with the assessed iron absorption (based on increase in ferritin and iron intake from 3-day food records) in a intervention study in the Philippines (Beard*, et al.* 2007). The conclusion by the authors was that all used algorithms underestimated iron absorption. However, the efficacy results by Beard *et al* are compromised by a major selection bias. By including only the subjects who gained in ferritin (4 out of 10 subjects were excluded), the inclusion procedure favored subjects who either had a iron requirement below median requirement, or had an "absorption capacity" higher than the average. Then by comparing only the result of the subjects who gained in ferritin with the results of the iron absorption algorithms, which apply to an individual with an "average" requirement and an "average" iron absorption capacity, a major over-estimation of the efficacy was done.

This algorithm is valid from meals with the lowest iron bioavailability, to meals with high iron bioavailability. However, there are limits to how far into the high bioavailability region the algorithm is valid. In meals constituting practically no iron

absorption inhibitors and very high amounts of enhancers the algorithm tends to give unrealistically high absorption values. The reason is that the algorithm has been validated in the ~0-45% non-hem iron absorption region (i.e. bringing unreliability beyond this absorption), and for certain amounts of dietary factors. The equations constituting the algorithm are based on meals containing 0-85 mg ascorbic acid, 0-175 g meat, fish, sea food or poultry, 0-600 mg calcium, 0-271 phytate phosphorus, 0-100 mg tannic acid equivalents, 0-3 eggs or 0-20 g soy protein. Thus, these levels of dietary factors show in what range the algorithm is valid.

Oral iron tolerance test (S-Fe $AUC_{0-6\ h}$) (paper I-III)

There are several complicating circumstances when using the S-Fe to assess iron absorption. At the same time as the iron is taken up from the intestinal lumen and transported into the circulation there is a simultaneous outflow of iron to the reticulo endothelial system (RES) and other stores (Figure 3). Further, in the event of an acute phase reaction, which can be present at varying degrees, iron can be detained in the mucosa cells and thus not be further transported into the body and the circulation. And in an anemic condition due to iron deficiency the iron outflow to the bone marrow can be up-regulated in such a degree that large iron absorption does not reflect on the increase in S-Fe. Consequently, it is crucial to reduce the influence from these factors, and also to standardize the procedures as to minimize the influence from confounding factors.

Although S-Fe $AUC_{0-6\ h}$ is a valid measure of iron absorption (*paper II*) when administering 100 mg iron, the large intra-individual variations made it impossible to establish statistical significant differences in RBV between the different investigated iron powders (*paper III*). The day-to-day variation in iron absorption (Bjorn-Rasmussen*, et al.* 1976, Brise 1962), is one of the plausible causes for these intra-individual differences in S-Fe $AUC_{0-6\ h}$ ratios for a $S\text{-}Fe_{elem}$ and the control iron. A possible approach to reduce this day-to-day variation in iron absorption would be to administer a 100 mg iron dose on two consecutive days to get a mean value like when using the extrinsic tag method. However, since the absorption across the mucosa seems to be blocked after an oral iron dose of >30 mg (O'Neil-Cutting*, et al.* 1987), this approach is not possible.

In order to examine possible reasons for the observed inter-individual variations in RBV we have examined a number of factors including lean body mass, initial serum iron concentration ($S\text{-}Fe_0$), initial total iron binding capacity ($TIBC_0$), weight, height, body mass index, number of blood donations before entering the study, age, hemoglobin concentration (Hb) and serum ferritin concentration. Only serum ferritin concentration and TIBC had a negative correlation with AUC_{0-6h} after oral administration of 100 mg iron as $FeSO_4 \cdot H_2O$ (r^2=0.42, p<0.001 and r^2=0.25, p<0.001). This indicates that the observed intra-individual difference in mean S-Fe AUC_{0-6h} for $FeSO_4$ in *paper III*, to some extent (i.e. 42%), is an expression of differences in iron status. Another factor contributing to this inter-individual difference

could be a difference in clearance efficiency of iron from the serum. This would most likely have a more pronounced impact on a fast increasing S-Fe compared to a more modest S-Fe increase. As a result, an S-Fe $AUC_{0\text{-}6\ h}$ ratio obtained in a subject with a highly efficient S-Fe clearance should differ from the S-Fe $AUC_{0\text{-}6\ h}$ ratio obtained in a subject with a more modest S-Fe clearance capacity. According to this proposed explanation, the inter-individual difference in the S-Fe $AUC_{0\text{-}6\ h}$ ratio should be wider for the electrolytic iron powders compared to the reduced ones. This was not the case, but on the other hand, a high or low S-Fe clearance phenotype could by chance be concentrated to only one of the groups.

Improving the S-Fe $AUC_{0\text{-}6\ h}$ methodology

Besides dietary factors and amount of iron present, there are several other factors influencing the iron absorption capacity, where iron status (Bezwoda*, et al.* 1979, Cook*, et al.* 1974, Taylor*, et al.* 1988) and infection/inflammation (Lynch 2007) are the most important. And since these factors can be more or less present in one and the same individual at different time points (e.g. 8 weeks apart), it gets difficult to compare repeated absorption measurement. When using the radioiron extrinsic labeling technique this is dealt with by normalizing to a standardized iron reference dose absorption of 40% (Magnusson*, et al.* 1981).

Consequently, the suggested procedure to enhance the precision of the S-Fe $AUC_{0\text{-}6h}$ method would be to introduce the usage of a reference dose. In order to support this hypothesized improvement in S-Fe $AUC_{0\text{-}6h}$ methodology, the data in *paper IV* of this thesis was reanalyzed. By doing this, it is possible get a preview of how a reference dose normalization would improve the methodology. As seen in *paper II*, the median (95% CI) value for S-Fe $AUC_{0\text{-}6h}$ following administration of $FeSO_4$ (when not normalized) was 263 (149-325) area units. After normalizing the S-Fe $AUC_{0\text{-}6h}$ to 60% absorption from the radioiron labeled 3-mg iron dose administered the preceding days, the median (95% CI) value for S-Fe $AUC_{0\text{-}6h}$ was 261 (227-299) area units (the 60 % absorption was arbitrary chosen as to get a median value of the same size/magnitude as the non-normalized value). As illustrated in figure 13, after normalizing for radioiron absorption, the data gets more symmetrically distributed, at the same time as the range in variation narrows down considerably.

An alterative normalization approach, and quite simpler compared to introducing the usage of a reference dose, could be to normalize to one and the same ferritin concentration. After normalizing the S-Fe $AUC_{0\text{-}6h}$ obtained in *paper II* to ferritin =23 µmol/l, the median (95% CI) value for the normalized S-Fe $AUC_{0\text{-}6h}$ was 389 (134-699) area units. Thus, this normalizing to ferritin actually increased the range of variation, i.e. giving an even poorer precision. A presumptive explanation could be a previous infection/inflammation. Although iron status and iron absorption correlates well in healthy individuals (Baynes*, et al.* 1987, Magnusson*, et al.* 1981, Taylor*, et al.* 1988), the impact of infection/inflammation can aggravate this relationship. The

reason is that ferritin can be falsely increased for weeks after an infection (Hulthen, *et al.* 1998), whereas the iron absorption capacity might supposedly not to be decreased for as long. However, to our knowledge, the effect of infection on time-course changes in iron absorption has not been studied directly.
Although there are several biomarkers available for establishing infection and inflammation (e.g. C-reactive protein, alpha-1-acid glycoprotein and interleukin-6) no one seems to correlate with ferritin over a longer time period. Consequently, although procedures were taken in *paper II* to minimize bias by infection or inflammation, no complete approached is available, making it difficult to completely avoid this source of error.

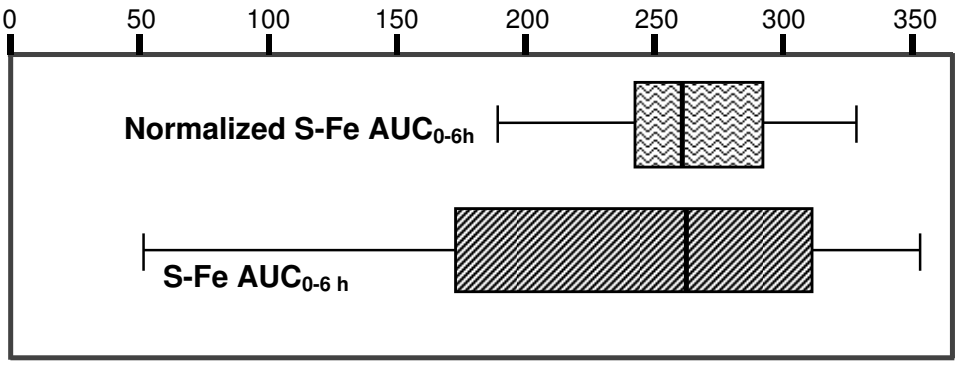

Figure 13. ***Comparison of S-Fe $AUC_{0\text{-}6\ h}$ and S-Fe $AUC_{0\text{-}6\ h}$ normalized to the absorption from a 3 mg oral iron dose.***

In *paper III* male blood donors were used as a model. The purpose of using only males was to avoid the confounding effect from menstruation loss, which can differ greatly between individuals (Hallberg, *et al.* 1966, Hallberg, *et al.* 1964). By including only blood donors who had donated blood eight weeks prior to each oral iron loading, the intention was to control the blood losses, as well as maintaining a high absorption capacity. An alternative approach of using male blood donors would be to use female subjects, and by that perform the repeated oral iron loadings preferably at the same time in the menstruation cycle (roughly 30 days) for each individual. These female subjects should have low iron status, although not anemia, since this would affect the normal S-Fe turn-over.

Future perspectives

Suggested improved S-Fe $AUC_{0\text{-}6\ h}$ study design

Thus, when aiming at assessing RBV of e.g. an elemental iron powder, the suggested improved study design would be a cross-over design composed of three sub-studies. The subjects, females of reproductive age having low iron stores, but not anemia, would at three different time points be served a test meal fortified with either no iron, FeSO4 or elemental iron. Each of the sub-studies should be performed at the same time in the menstruation cycle for each subject. Each sub-study would comprise of three consecutive mornings where on days 1 and 2 the subjects would be served a reference dose of 3 mg iron labeled with ^{59}Fe. In order to reduce the influence from the day-to-day variation in absorption the mean absorption from these 2 d should be calculated. The subjects will then be instructed not to eat or drink anything for the next three hours.

On day 3, the oral iron loading would take place as previously described in *paper I*. Two weeks later a whole body counting will be made to determine the basal iron absorption from the reference doses in each individual. By expressing the S-Fe $AUC_{0\text{-}6h}$ corresponding to a 40% reference dose absorption, variations owing to differences in iron absorption capacity at each studied time point would be reduced.

One menstruation cycle later the next sub-study would be carried out in the same way. As a measure to avoid systematic errors introduced by infection/inflammation, blood samples would be analyzed for C-reactive protein (CRP), alpha-1-acid glycoprotein (AGP) and erythrocyte sedimentation rate (ESR). The subjects will also be asked about any indications of infection during the preceding weeks. If that's the case, the oral iron loading would be postponed one menstruation cycle.

Ways of improving iron bioavailability of the diet

As demonstrated in *paper V*, the effect of improving a diet with poor iron bio-availability has a more marked effect on the absorbed amount of iron compared to fortifying this diet. The two main measures available when aiming at improving the dietary iron bioavailability is either by reducing the factors inhibiting iron absorption or/and increasing the amounts of factors with enhancing effect. In most cases is it possible by promoting local food production (including livestock) (Ayele*, et al.* 2003) and usage of local available ingredients to do the dietary modifications needed to improve the iron bioavailability. However, as previously pointed out (Faber*, et al.* 1999), in order to carry out effective intervention programs, agriculturists, health professionals and social scientists should work together in a multi-disciplinary way. Concrete strategies for enhancing the absorption of fortification iron has been presented by the SUSTAIN Task Force (Hurrell*, et al.* 2004). Recently the WHO has also presented a report on guidelines on food fortification (WHO 2006).

In the search for economical, non-conventional ways of combating iron deficiency in developing countries there are promising results from adding marine algae to the diet (Garcia-Casal*, et al.* 2007). Phytate degradation aimed at improving iron absorption is possible by using the hydrolytic enzyme phytase (Greiner*, et al.* 2003, Hurrell*, et al.* 2003). Furthermore, when it comes to phytate and tannins it has been shown that by combining enzymatic oxidation with traditional food preparation techniques, such as cooking, soaking, germination and lactic acid fermentation it is possible to enhance the iron bioavailability in the diet (Hurrell 2004, Larsson*, et al.* 1996, Manary*, et al.* 2002, Matuschek*, et al.* 2001). Also the incubation with crude extracts of fruits containing polyphenol oxidase (PPO) has been shown to result in significantly lower amounts of phenolic compounds (Matuschek 2005). In rural Malawi a community-based method to remove dietary phytate has been used which resulted in an improved iron status (Manary*, et al.* 2002). Another way of reducing phytate content is by genetical modification of crops, which has been shown effective in reducing the phytate content and enhancing the iron absorption (Mendoza*, et al.* 1998). However, it is important to bear in mind that even if the phytate content of a meal was to be considerably reduced the inhibitory effect on iron absorption would still be present due to its significant effect even at very small amounts (Hallberg*, et al.* 1989a, Siegenberg*, et al.* 1991). Thus, complete degradation is recommended. If this is not possible, the phytic acid to iron molar ratio should be decreased to below 1:1 or 0.4:1 (Hurrell 2004).

Addition of animal tissue is an effective way of improving iron bioavailability (Gibson*, et al.* 2003). Since 50% of the total iron content in red meat can constitute of heme iron, which is highly absorbable, incorporation of red meat can make a marked contribution to total amount of iron absorbed (*paper IV*). However, when incorporating chicken or fish into a meal or diet, as assumed in the meals in *paper V*, the contribution of heme iron will be negligible. Even though effective, the presented approach of improving iron bioavailability with meat can, due to economical reasons, unfortunately be rather hard to accomplice in developing countries.

Future new innovative strategies for iron fortification may be able to overcome the problems constituted by poor dietary bioavailability. Examples of such novel approaches is the use of microencapsulated iron (Tondeur*, et al.* 2004, Zlotkin*, et al.* 2001), or administration of iron stored in the ferritin of recombinant yeast (Chang*, et al.* 2005), which protects the iron fortificant from the factors in the diet that inhibits iron absorption. However, until these approaches are fully evaluated and practically functioning, it is of outermost importance to improve the diet bioavailability before initiating an iron fortification intervention.

Another interesting strategy, which in some studies has shown good potential in enhancing iron fortificant absorption, is the usage of EDTA [ethylenediaminetetra-acetic acid]. The positive characteristic of EDTA, i.e. the ability to prevent the iron from binding to factors inhibiting iron absorption, has earlier given rise to a

recommendation of NaFeEDTA [sodium iron (Fe^{3+}) ethylenediaminetetraacetic acid] as the most suitable iron fortificant for use in developing countries by the International Nutritional Anemia Consultative Group (INACG) (INACG 1993). The two most important determinants for the positive effect seen by EDTA is; *I):* the molar ratio of EDTA to iron, *II):* and that the food must contain iron inhibitors which EDTA can compete with when to chelate bind with iron. It seems like the optimal ratio of EDTA to iron is somewhere between 1:2 and 1:1. Hurrell et al observed that a ratio were iron were in excess (i.e. 1:3) rendered a low effect, whereas a more equal ratio of 1:1 improved iron absorption (Hurrell*, et al.* 2000). INACG reported that a ratio of EDTA to iron of 1:4 to 1:1 increases iron absorption, with a maximum absorption at 1:2. Further increased ratio does not significantly increase or decrease iron absorption (INACG 1993). Despite the promising effect on iron absorption, EDTA has, just like meat, an important disadvantage which in a developing country context is a major limiting factor, the price tag.

GENERAL CONCLUSIONS

From the data obtained in the studies presented in this thesis the following conclusions could be drawn:

- For males with low iron stores there is a steady increasing diurnal variation in S-Fe from the morning to the afternoon.
- The S-Fe $AUC_{0\text{-}6h}$ following oral administration of 100 mg iron is a valid measure of iron absorption. However, in order to compare S-Fe $AUC_{0\text{-}6h}$ obtained at different time points, or in different individuals, normalization to one and the same iron absorption capacity is necessary.
- An effective procedure to enhance the precision of the S-Fe $AUC_{0\text{-}6h}$ would be to normalize the S-Fe $AUC_{0\text{-}6h}$ to the absorption from a reference dose of radioiron labeled iron.
- The S-Fe $AUC_{0\text{-}6h}$ following 100 mg iron added to a meal can predict the iron absorption of a small dose of iron added to the same meal.
- The seven evaluated iron fortificants, representing the market in 2001, were all significantly less well absorbed compared to $FeSO_4 \cdot H_2O$.
- Co-fortifying electrolytic iron with ascorbic acid seems to give an enhanced, as well as an accelerated early iron uptake.
- Since finely pulverized freeze-dried meat seems to have a more pronounced effect on iron absorption, compared to meat in ordinary meals where it is present in larger pieces, it could be a clue in finding the answer behind the “meat factor”.
- Dietary modifications of meals with low iron bioavailability can markedly improve iron absorption. Especially when adding meat which also contributes with highly absorbable heme-Fe.
- Depending on choice of iron fortificant in fortification interventions the effectiveness can differ.
- Dietary modifications of meals with low iron bioavailability can markedly improve iron absorption. This is especially seen when adding meat which also contributes with highly absorbable heme iron.
- If the original diet has a low iron bioavailability, it is difficult to achieve good effects on iron status by using iron fortification as the only measure.

Overall main conclusion

The best course of action for interventions designed to improve iron status must firstly be to ensure an adequate dietary iron bioavailability and secondly to use a type of iron fortificant with high bioavailability

ACKNOWLEDGEMENT

Life is like a game of pinball. Where you finally end up is entirely dependent upon the encounters you do on the way. So, where to start when so few (me, myself and I) have so many to acknowledge?… Firstly, eftersom min älskade farmor skall kunna läsa det här så släpper jag engelskan… För som Albert Einstein sa: ” You do not really understand something unless you can explain it to your grandmother”.

Ett speciellt stort **TACK** vill jag rikta till:

Mina eminenta handledare, Professor **Lena Hulthén** och Professor emeritus **Leif Hallberg**, för att ni gav mig möjligheten att genomföra denna utvecklande resa.
Lena, min huvudhandledare (med den fantastiska förmågan att ha mer järn i elden än det rimligen borde finnas eld för…), för din avgörande roll i den (h)järnberikning som denna intellektuellt utvecklande resa inneburet för mig, samt för att du delat med dig av dina ämnesöverskridande kunskaper, inte minst under våra trevliga resor.
Leif, för ditt stöd, engagemang, och otroliga vetenskapliga expertis, samt för att du bekantade mig med min nuvarande älsklingsdessert ”sweet sticky rice with mango”…
Frode Slinde, min inofficielle mentor…för all hjälp, stöd och förmåga att kritiskt ifrågasätta ALLT… samt, ej att förglömma, alla trevliga dagar vi bl.a. ägnat åt spaning efter snöleoparder…
Hilde Brekke, för alla våra utvecklande diskussioner om allt från vetenskap till vardagliga världsliga ting, samt moraliskt stöd, både före och efter, men framförallt under, vår tid i ”Gränslandet”....
”Föredettingarna”, **Frode**, **Hilde**, **Stine**, **Jenny**, **Agneta**… och numera förnämliga Medicine Doktorer! som bidrog till en lärorik och gemytlig tillvaro som doktorand-wannabe. Era gedigna avhandlingar har satt standarden för vad jag ödmjukast hoppas nå upp till…
Elisabeth Gramatkovski, för att du från min första stund på AKN har varit till så stor hjälp och stöd.
Vibeke Malmros, för att du varit så mån om mig och hjälpt mig med allt från blodprovstagningar till VAB-bryderier.
Mona, **Pascal** & **Christel** för all hjälp och stöd.
Alla arbetskamrater vid Avdelningen för Klinisk Näringslära (AKN), för en trevlig och konstruktiv arbetsmiljö, samt överseende med en stundom asocial natur…
Oma, för att du alltid funnits där för mig och för att du lärde kleine Michaelus att man inte behöver vara rädd i den stora, mörka skogen…
Hannis, min livspartner och skyddande hamn…
Ludvig, **Felix** & **Wictor**, mitt hjärtas fröjd och glädje. Det finns inte ord för hur mycket ni betyder för mig. Ni är allt jag någonsin kunnat önska mig, utan att jag själv visste om det…
Ozzy *et al*……
Sist, men inte minst, **alla Ni** som ej nämnts ovan, men som med er närvaro i olika skeden i livet påverkat den jag är och därmed indirekt bidragit till denna avhandling. Ni vet vilka ni är…

REFERENCES

"Furuki o tazune atarashiki o shiru"

Abrams SA. (1999) Using stable isotopes to assess mineral absorption and utilization by children. *Am J Clin Nutr* 70:955-64.

Agrahar-Murugkar D, Pal PP. (2004) Intake of nutrients and food sources of nutrients among the Khasi tribal women of India. *Nutrition* 20:268-73.

Ahluwalia N, Sun J, Krause D, Mastro A, Handte G. (2004) Immune function is impaired in iron-deficient, homebound, older women. *Am J Clin Nutr* 79:516-21.

Akesson A, Berglund M, Schutz A, Bjellerup P, Bremme K, Vahter M. (2002) Cadmium exposure in pregnancy and lactation in relation to iron status. *Am J Public Health* 92:284-7.

Anand AN, Seshadri S. (1995) A quantitative model for prediction of iron bioavailability from Indian meals: an experimental study. *Int J Food Sci Nutr* 46:335-42.

Armah CN, Sharp P, Mellon FA, Pariagh S, Lund EK, Dainty JR, Teucher B, et al. (2008) L-alpha-glycerophosphocholine contributes to meat's enhancement of nonheme iron absorption. *J Nutr* 138:873-7.

Aruoma OI, Reilly T, MacLaren D, Halliwell B. (1988) Iron, copper and zinc concentrations in human sweat and plasma; the effect of exercise. *Clin Chim Acta* 177:81-7.

Ayele Z, Peacock C. (2003) Improving access to and consumption of animal source foods in rural households: the experiences of a women-focused goat development program in the highlands of Ethiopia. *J Nutr* 133:3981S-3986S.

Backstrand JR, Allen LH, Black AK, de Mata M, Pelto GH. (2002) Diet and iron status of nonpregnant women in rural Central Mexico. *Am J Clin Nutr* 76:156-64.

Baech SB, Hansen M, Bukhave K, Jensen M, Sorensen SS, Kristensen L, Purslow PP, et al. (2003a) Nonheme-iron absorption from a phytate-rich meal is increased by the addition of small amounts of pork meat. *Am J Clin Nutr* 77:173-9.

Baech SB, Hansen M, Bukhave K, Kristensen L, Jensen M, Sorensen SS, Purslow PP, et al. (2003b) Increasing the cooking temperature of meat does not affect nonheme iron absorption from a phytate-rich meal in women. *J Nutr* 133:94-7.

Ballot DE, MacPhail AP, Bothwell TH, Gillooly M, Mayet FG. (1989) Fortification of curry powder with NaFe(111)EDTA in an iron-deficient population: report of a controlled iron-fortification trial. *Am J Clin Nutr* 49:162-9.

Baynes RD, Bothwell TH, Bezwoda WR, MacPhail AP, Derman DP. (1987) Relationship between absorption of inorganic and food iron in field studies. *Ann Nutr Metab* 31:109-16.

Beard JL, Borel MJ, Derr J. (1990) Impaired thermoregulation and thyroid function in iron-deficiency anemia. *Am J Clin Nutr* 52:813-9.

Beard JL, Murray-Kolb LE, Haas JD, Lawrence F. (2007) Iron absorption prediction equations lack agreement and underestimate iron absorption. *J Nutr* 137:1741-6.

Beresford CH, Neale RJ, Brooks OG. (1971) Iron absorption and pyrexia. *Lancet* 1:568-72.

Bezwoda WR, Bothwell TH, Torrance JD, MacPhail AP, Charlton RW, Kay G, Levin J. (1979) The relationship between marrow iron stores, plasma ferritin concentrations and iron absorption. *Scand J Haematol* 22:113-20.

Bhargava A, Bouis HE, Scrimshaw NS. (2001) Dietary intakes and socioeconomic factors are associated with the hemoglobin concentration of Bangladeshi women. *J Nutr* 131:758-64.

Birgegard G, Hallgren R, Killander A, Stromberg A, Venge P, Wide L. (1978) Serum ferritin during infection. A longitudinal study. *Scand J Haematol* 21:333-40.

Bjorn-Rasmussen E, Hallberg L. (1979) Effect of animal proteins on the absorption of food iron in man. *Nutr Metab* 23:192-202.

Bjorn-Rasmussen E, Hallberg L, Magnusson B, Rossander L, Svanberg B, Arvidsson B. (1976) Measurement of iron absorption from compositite meals. *Am J Clin Nutr* 29:772-8.

Bjorn-Rasmussen E, Hallberg L, Rossander L. (1977) Absorption of 'fortification' iron. Bioavailability in man of different samples of reduced Fe, and prediction of the effects of Fe fortification. *Br J Nutr* 37:375-88.

Borel MJ, Smith SM, Derr J, Beard JL. (1991) Day-to-day variation in iron-status indices in healthy men and women. *Am J Clin Nutr* 54:729-35.

Bothwell TH. (1994) The pros and cons of iron fortification. *S Afr Med J* Suppl:24-6.

Bothwell TH. (1995) Overview and mechanisms of iron regulation. *Nutr Rev* 53:237-45.

Bothwell TH. Iron balance and the capacity of regulatory systems to prevent the development of iron deficiency and overload. In: Hallberg LA, N-G, ed. Iron nutrition in health and disease. London: John Libbey & Co Ltd, 1996:3-16.

Brabin BJ, Hakimi M, Pelletier D. (2001a) An analysis of anemia and pregnancy-related maternal mortality. *J Nutr* 131:604S-614S; discussion 614S-615S.

Brabin BJ, Premji Z, Verhoeff F. (2001b) An analysis of anemia and child mortality. *J Nutr* 131:636S-645S; discussion 646S-648S.

Brise HH, L. (1962) A method for comparative studies on iron absorption in man using two radioiron isotopes. *Acta Med Scand* 171:7-22.

Brownlie Tt, Utermohlen V, Hinton PS, Giordano C, Haas JD. (2002) Marginal iron deficiency without anemia impairs aerobic adaptation among previously untrained women. *Am J Clin Nutr* 75:734-42.

Brune M, Rossander-Hulten L, Hallberg L, Gleerup A, Sandberg AS. (1992) Iron absorption from bread in humans: inhibiting effects of cereal fiber, phytate and inositol phosphates with different numbers of phosphate groups. *J Nutr* 122:442-9.

Brune M, Rossander L, Hallberg L. (1989) Iron absorption and phenolic compounds: importance of different phenolic structures. *Eur J Clin Nutr* 43:547-57.

Bruner AB, Joffe A, Duggan AK, Casella JF, Brandt J. (1996) Randomised study of cognitive effects of iron supplementation in non-anaemic iron-deficient adolescent girls. *Lancet* 348:992-6.

Brutsaert TD, Hernandez-Cordero S, Rivera J, Viola T, Hughes G, Haas JD. (2003) Iron supplementation improves progressive fatigue resistance during dynamic knee extensor exercise in iron-depleted, nonanemic women. *Am J Clin Nutr* 77:441-8.

Cartwright GE, Lauritsen MA, Jones PJ, Merrill IM, Wintrobe MM. (1946) The Anemia of Infection. I. Hypoferremia, Hypercupremia, and Alterations in Porphyrin Metabolism in Patients. *J Clin Invest* 25:65-80.

Chang YJ, Jo MY, Hwang EH, Park CU, Kim KS. (2005) Recovery from iron deficiency in rats by the intake of recombinant yeast producing human H-ferritin. *Nutrition* 21:520-4.

Chiari MM, Bagnoli R, De Luca PD, Monti M, Rampoldi E, Cunietti E. (1995) Influence of acute inflammation on iron and nutritional status indexes in older inpatients. *J Am Geriatr Soc* 43:767-71.

Cook JD, Layrisse M, Martinez-Torres C, Walker R, Monsen E, Finch CA. (1972) Food iron absorption measured by an extrinsic tag. *J Clin Invest* 51:805-15.

Cook JD, Lipschitz DA, Miles LE, Finch CA. (1974) Serum ferritin as a measure of iron stores in normal subjects. *Am J Clin Nutr* 27:681-7.

Cook JD, Minnich V, Moore CV, Rasmussen A, Bradley WB, Finch CA. (1973) Absorption of fortification iron in bread. *Am J Clin Nutr* 26:861-72.

Cook JD, Monsen ER. (1976) Food iron absorption in human subjects. III. Comparison of the effect of animal proteins on nonheme iron absorption. *Am J Clin Nutr* 29:859-67.

Cook JD, Reddy MB, Burri J, Juillerat MA, Hurrell RF. (1997) The influence of different cereal grains on iron absorption from infant cereal foods. *Am J Clin Nutr* 65:964-9.

Cook JD, Skikne BS, Baynes RD. (1994) Iron deficiency: the global perspective. *Adv Exp Med Biol* 356:219-28.

Cordova Martinez A, Escanero JF. (1992) Iron, transferrin, and haptoglobin levels after a single bout of exercise in men. *Physiol Behav* 51:719-22.

Costa A, Liberato LN, Palestra P, Barosi G. (1991) Small-dose iron tolerance test and body iron content in normal subjects. *Eur J Haematol* 46:152-7.

Crosby WH, O'Neil-Cutting MA. (1984) A small-dose iron tolerance test as an indicator of mild iron deficiency. *Jama* 251:1986-7.

Dale JC, Burritt MF, Zinsmeister AR. (2002) Diurnal variation of serum iron, iron-binding capacity, transferrin saturation, and ferritin levels. *Am J Clin Pathol* 117:802-8.

Du S, Zhai F, Wang Y, Popkin BM. (2000) Current methods for estimating dietary iron bioavailability do not work in China. *J Nutr* 130:193-8.

Ekenved G. (1976a) Absorption from different types of iron tablets - correlation between serum iron increase in total absorption of iron. *Scand J Haematol Suppl* 28:51-63.

Ekenved G. (1976b) Iron absorption studies. Studies on oral iron preparations using serum iron and different radioiron isotope techniques. *Scand J Haematol Suppl* 28:1-97.

Ekenved G, Arvidsson B, Solvell L. (1976a) Influence of food on the absorption from different types of iron tablets. *Scand J Haematol Suppl* 28:79-88.

Ekenved G, Norrby A, Solvell L. (1976b) Serum iron increase as a measure of iron absorption - studies on the correlation with total absorption. *Scand J Haematol Suppl* 28:31-49.

Elin RJ, Wolff SM, Finch CA. (1977) Effect of induced fever on serum iron and ferritin concentrations in man. *Blood* 49:147-53.

Eskeland B, Baerheim A, Ulvik R, Hunskaar S. (2002) Influence of mild infections on iron status parameters in women of reproductive age. *Scand J Prim Health Care* 20:50-6.

Faber M, Smuts CM, Benade AJ. (1999) Dietary intake of primary school children in relation to food production in a rural area in KwaZulu-Natal, South Africa. *Int J Food Sci Nutr* 50:57-64.

Fairweather-Tait SJ. (2001) Iron. *J Nutr* 131:1383S-6S.

Fawwaz RA, Winchell HS, Pollycove M, Sargent T. (1967) Hepatic iron deposition in humans. I. First-pass hepatic deposition of intestinally absorbed iron in patients with low plasma latent iron-binding capacity. *Blood* 30:417-24.

Fillet G, Beguin Y, Baldelli L. (1989) Model of reticuloendothelial iron metabolism in humans: abnormal behavior in idiopathic hemochromatosis and in inflammation. *Blood* 74:844-51.

Forbes AL, Arnaud MJ, Chichester CO, Cook JD, Harrison BN, Hurrell RF, Kahn SG, et al. (1989) Comparison of in vitro, animal, and clinical determinations of iron bioavailability: International Nutritional Anemia Consultative Group Task Force report on iron bioavailability. *Am J Clin Nutr* 49:225-38.

Fox PL. (2003) The copper-iron chronicles: the story of an intimate relationship. *Biometals* 16:9-40.

Friedmann B, Weller E, Mairbaurl H, Bartsch P. (2001) Effects of iron repletion on blood volume and performance capacity in young athletes. *Med Sci Sports Exerc* 33:741-6.

Ganz T. (2003) Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood* 102:783-8.

Ganz T, Nemeth E. (2006) Iron imports. IV. Hepcidin and regulation of body iron metabolism. *Am J Physiol Gastrointest Liver Physiol* 290:G199-203.

Garcia-Casal MN, Pereira AC, Leets I, Ramirez J, Quiroga MF. (2007) High iron content and bioavailability in humans from four species of marine algae. *J Nutr* 137:2691-5.

Gibson S, Ashwell M. (2003) The association between red and processed meat consumption and iron intakes and status among British adults. *Public Health Nutr* 6:341-50.

Gillooly M, Bothwell TH, Torrance JD, MacPhail AP, Derman DP, Bezwoda WR, Mills W, et al. (1983) The effects of organic acids, phytates and polyphenols on the absorption of iron from vegetables. *Br J Nutr* 49:331-42.

Gimenez M, Uffholtz H, Paysant P, Belleville F, Nabet P. (1988) Serum iron and transferrin during an exhaustive session of interval training. *Eur J Appl Physiol Occup Physiol* 57:154-8.

Glahn RP, Lee OA, Yeung A, Goldman MI, Miller DD. (1998) Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an in vitro digestion/Caco-2 cell culture model. *J Nutr* 128:1555-61.

Gleerup A, Rossander-Hulthen L, Gramatkovski E, Hallberg L. (1995) Iron absorption from the whole diet: comparison of the effect of two different distributions of daily calcium intake. *Am J Clin Nutr* 61:97-104.

Gonzalez H, Mendoza C, Viteri FE. (2001) Absorption of unlabeled reduced iron of small particle size from a commercial source. A method

to predict absorption of unlabeled iron compounds in humans. *Arch Latinoam Nutr* 51:217-24.

Greiner R, Egli I. (2003) Determination of the activity of acidic phytate-degrading enzymes in cereal seeds. *J Agric Food Chem* 51:847-50.

Haas JH, Miller DD. (2006) Overview of experimental biology 2005 symposium: food fortification in developing countries. *J Nutr* 136:1053-4.

Hahn PF, Bale WF, Lawrence EO, Whipple GH. (1939) Radioactive iron and its metabolism in anemia. Its absorption, transportation, and utilization. *The Journal of Experimental Medicine* 69:739-753.

Hallberg L. (1981) Bioavailability of dietary iron in man. *Ann Rev Nutr* 1:123-47.

Hallberg L, Bjorn-Rasmussen E. (1972) Determination of iron absorption from whole diet. A new two-pool model using two radioiron isotopes given as haem and non-haem iron. *Scand J Haematol* 9:193-7.

Hallberg L, Bjorn-Rasmussen E, Howard L, Rossander L. (1979) Dietary heme iron absorption. A discussion of possible mechanisms for the absorption-promoting effect of meat and for the regulation of iron absorption. *Scand J Gastroenterol* 14:769-79.

Hallberg L, Brune M, Erlandsson M, Sandberg AS, Rossander-Hulten L. (1991a) Calcium: effect of different amounts on nonheme- and heme-iron absorption in humans. *Am J Clin Nutr* 53:112-9.

Hallberg L, Brune M, Rossander L. (1986a) Effect of ascorbic acid on iron absorption from different types of meals. Studies with ascorbic-acid-rich foods and synthetic ascorbic acid given in different amounts with different meals. *Hum Nutr Appl Nutr* 40:97-113.

Hallberg L, Brune M, Rossander L. (1986b) Low bioavailability of carbonyl iron in man: studies on iron fortification of wheat flour. *Am J Clin Nutr* 43:59-67.

Hallberg L, Brune M, Rossander L. (1989a) Iron absorption in man: ascorbic acid and dose-dependent inhibition by phytate. *Am J Clin Nutr* 49:140-4.

Hallberg L, Hogdahl AM, Nilsson L, Rybo G. (1966) Menstrual blood loss--a population study. Variation at different ages and attempts to define normality. *Acta Obstet Gynecol Scand* 45:320-51.

Hallberg L, Hoppe M, Andersson M, Hulthen L. (2003) The role of meat to improve the critical iron balance during weaning. *Pediatrics* 111:864-70.

Hallberg L, Hulten L, Gramatkovski E. (1997) Iron absorption from the whole diet in men: how effective is the regulation of iron absorption? *Am J Clin Nutr* 66:347-56.

Hallberg L, Hulthen L. (2000a) Erratum concerning the article "Prediction of dietary iron absorption: an algorithm for calculating absorption and bioavailability of dietary iron." *Am J Clin Nutr* 72:1242.

Hallberg L, Hulthen L. (2000b) Prediction of dietary iron absorption: an algorithm for calculating absorption and bioavailability of dietary iron. *Am J Clin Nutr* 71:1147-60.

Hallberg L, Hulthen L. (2001) Erratum concerning the article "Prediction of dietary iron absorption: an algorithm for calculating absorption and bioavailability of dietary iron." *Am J Clin Nutr* 74:274.

Hallberg L, Hulthen L. (2006) Erratum concerning the article "Prediction of dietary iron absorption: an algorithm for calculating absorption and bioavailability of dietary iron." *Am J Clin Nutr* 84:1253.

Hallberg L, Hulthen L, Bengtsson C, Lapidus L, Lindstedt G. (1995) Iron balance in menstruating women. *Eur J Clin Nutr* 49:200-7.

Hallberg L, Hulthen L, Garby L. (1998) Iron stores in man in relation to diet and iron requirements. *Eur J Clin Nutr* 52:623-31.

Hallberg L, Hulthen L, Garby L. (2000c) Iron stores and haemoglobin iron deficits in menstruating women. Calculations based on variations in iron requirements and bioavailability of dietary iron. *Eur J Clin Nutr* 54:650-7.

Hallberg L, Nilsson L. (1964) Constancy of Individual Menstrual Blood Loss. *Acta Obstet Gynecol Scand* 43:352-9.

Hallberg L, Rossander-Hulten L. (1991b) Iron requirements in menstruating women. *Am J Clin Nutr* 54:1047-58.

Hallberg L, Rossander-Hulten L, Brune M, Gleerup A. (1992) Calcium and iron absorption:

mechanism of action and nutritional importance. *Eur J Clin Nutr* 46:317-27.

Hallberg L, Rossander-Hulthen L, Gramatkovski E. (1989b) Iron fortification of flour with a complex ferric orthophosphate. *Am J Clin Nutr* 50:129-35.

Hallberg L, Solvell L. (1967) Absorption of hemoglobin iron in man. *Acta Med Scand* 181:335-54.

Hamilton LD, Gubler CJ, Cartwright GE, Wintrobe MM. (1950) Diurnal Variation in the plasma Iron Level of Man. *Proceedings of the Society for Experimental Biology and Medicine* 75:65-8.

Han O, Failla ML, Hill AD, Morris ER, Smith JC, Jr. (1994) Inositol phosphates inhibit uptake and transport of iron and zinc by a human intestinal cell line. *J Nutr* 124:580-7.

Han O, Failla ML, Hill AD, Morris ER, Smith JC, Jr. (1995) Ascorbate offsets the inhibitory effect of inositol phosphates on iron uptake and transport by Caco-2 cells. *Proc Soc Exp Biol Med* 210:50-6.

Heath AL, Skeaff CM, O'Brien SM, Williams SM, Gibson RS. (2001) Can dietary treatment of non-anemic iron deficiency improve iron status? *J Am Coll Nutr* 20:477-84.

Hertrampf E, Olivares M, Walter T, Pizarro F, Heresi G, Llaguno S, Vega V, et al. (1990) [Iron-deficiency anemia in the nursing infant: its elimination with iron-fortified milk]. *Rev Med Chil* 118:1330-7.

Hinton PS, Giordano C, Brownlie T, Haas JD. (2000) Iron supplementation improves endurance after training in iron-depleted, nonanemic women. *J Appl Physiol* 88:1103-11.

Hoglund S, Reizenstein P. (1969) Studies in iron absorption. V. Effect of gastrointestinal factors on iron absorption. *Blood* 34:496-504.

Hoppe M, Hulthen L. (2007) Capturing the onset of the common cold and its effects on iron absorption. *Eur J Clin Nutr* 61:1032-4.

Hoppe M, Hulthén L, Hallberg L. (2003) Serum iron concentration as a tool to measure relative iron absorption from elemental iron powders. *Scand J Clin Lab Invest* 63:489-96.

Hulthén L, Gramatkovski E, Gleerup A, Hallberg L. (1995) Iron absorption from the whole diet. Relation to meal composition, iron requirements and iron stores. *Eur J Clin Nutr* 49:794-808.

Hulthén L, Hoppe M, Hallberg L. (2006) Letter to the Editor: Reply to H Jang and M Jeon. *Am J Clin Nutr* 84:1242.

Hulthen L, Lindstedt G, Lundberg PA, Hallberg L. (1998) Effect of a mild infection on serum ferritin concentration--clinical and epidemiological implications. *Eur J Clin Nutr* 52:376-9.

Hunt JM. (2002) Reversing productivity losses from iron deficiency: the economic case. *J Nutr* 132:794S-801S.

Hunt JR. (2005) Dietary and physiological factors that affect the absorption and bioavailability of iron. *Int J Vitam Nutr Res* 75:375-84.

Hunt JR, Roughead ZK. (2000) Adaptation of iron absorption in men consuming diets with high or low iron bioavailability. *Am J Clin Nutr* 71:94-102.

Hurrell R. (2002) How to ensure adequate iron absorption from iron-fortified food. *Nutr Rev* 60:S7-15; discussion S43.

Hurrell R, Bothwell T, Cook JD, Dary O, Davidsson L, Fairweather-Tait S, Hallberg L, et al. (2002) The usefulness of elemental iron for cereal flour fortification: a SUSTAIN Task Force report. Sharing United States Technology to Aid in the Improvement of Nutrition. *Nutr Rev* 60:391-406.

Hurrell RF. (2004) Phytic acid degradation as a means of improving iron absorption. *Int J Vitam Nutr Res* 74:445-52.

Hurrell RF, Furniss DE, Burri J, Whittaker P, Lynch SR, Cook JD. (1989) Iron fortification of infant cereals: a proposal for the use of ferrous fumarate or ferrous succinate. *Am J Clin Nutr* 49:1274-82.

Hurrell RF, Lynch S, Bothwell T, Cori H, Glahn R, Hertrampf E, Kratky Z, et al. (2004) Enhancing the absorption of fortification iron. A SUSTAIN Task Force report. *Int J Vitam Nutr Res* 74:387-401.

Hurrell RF, Lynch SR, Trinidad TP, Dassenko SA, Cook JD. (1988) Iron absorption in humans: bovine serum albumin compared with beef muscle and egg white. *Am J Clin Nutr* 47:102-7.

Hurrell RF, Reddy MB, Burri J, Cook JD. (2000) An evaluation of EDTA compounds for iron fortification of cereal-based foods. *Br J Nutr* 84:903-10.

Hurrell RF, Reddy MB, Juillerat MA, Cook JD. (2003) Degradation of phytic acid in cereal porridges improves iron absorption by human subjects. *Am J Clin Nutr* 77:1213-9.

Høyer K. (1944) Physiologic variations in the iron content of human blood serum. *Acta Med Scandinavica* 119:562-76.

INACG. International Nutritional Anemia Consultative Group. Iron EDTA for Food Fortification. Washington, DC: ILSI Human Nutrition Institute, 1993.

INACG. International Nutritional Anemia Consultative Group. Efficacy and Effectiveness of Interventions to Control Iron Deficiency and Iron Deficiency Anemia. Washington, DC: ILSI Human Nutrition Institute, 2004.

Jensen NM, Brandsborg M, Boesen AM, Yde H, Dahlerup JF. (1998) Low-dose oral iron absorption test: establishment of a reference interval. *Scand J Clin Lab Invest* 58:511-9.

Jensen NM, Brandsborg M, Boesen AM, Yde H, Dahlerup JF. (1999) Low-dose oral iron absorption test in anaemic patients with and without iron deficiency determined by bone marrow iron content. *Eur J Haematol* 63:103-11.

Joosten E, Vander Elst B, Billen J. (1997) Small-dose oral iron absorption test in anaemic and non-anaemic elderly hospitalized patients. *Eur J Haematol* 58:99-103.

Jurado RL. (1997) Iron, infections, and anemia of inflammation. *Clin Infect Dis* 25:888-95.

Kastenmayer P, Davidsson L, Galan P, Cherouvrier F, Hercberg S, Hurrell RF. (1994) A double stable isotope technique for measuring iron absorption in infants. *Br J Nutr* 71:411-24.

Kelsey SM, Hider RC, Bloor JR, Blake DR, Gutteridge CN, Newland AC. (1991) Absorption of low and therapeutic doses of ferric maltol, a novel ferric iron compound, in iron deficient subjects using a single dose iron absorption test. *J Clin Pharm Ther* 16:117-22.

Kemna EH, Pickkers P, Nemeth E, van der Hoeven H, Swinkels D. (2005) Time-course analysis of hepcidin, serum iron, and plasma cytokine levels in humans injected with LPS. *Blood* 106:1864-6.

Larsson M, Rossander-Hulthen L, Sandstrom B, Sandberg AS. (1996) Improved zinc and iron absorption from breakfast meals containing malted oats with reduced phytate content. *Br J Nutr* 76:677-88.

Lartey A, Manu A, Brown KH, Peerson JM, Dewey KG. (1999) A randomized, community-based trial of the effects of improved, centrally processed complementary foods on growth and micronutrient status of Ghanaian infants from 6 to 12 mo of age. *Am J Clin Nutr* 70:391-404.

Laurell CB. (1952) The diurnal variation of the serum iron concentration. *J Clin Lab Invest* 5:118-21.

Layrisse M, Chaves JF, Mendez C, Bosch V, Tropper E, Bastardo B, Gonzalez E. (1996) Early response to the effect of iron fortification in the Venezuelan population. *Am J Clin Nutr* 64:903-7.

Layrisse M, Cook JD, Martinez C, Roche M, Kuhn IN, Walker RB, Finch CA. (1969) Food iron absorption: a comparison of vegetable and animal foods. *Blood* 33:430-43.

Layrisse M, Martinez-Torres C, Leets I, Taylor P, Ramirez J. (1984) Effect of histidine, cysteine, glutathione or beef on iron absorption in humans. *J Nutr* 114:217-23.

Lee P, Peng H, Gelbart T, Beutler E. (2004) The IL-6- and lipopolysaccharide-induced transcription of hepcidin in HFE-, transferrin receptor 2-, and beta2-microglobulin-deficient hepatocytes. *Proc Natl Acad Sci U S A*.

Long R, Delaney KK, Siegel L. (1978) Diurnal variation of serum iron in normal individuals. *Clin Chem* 24:842.

Lund EK, Fairweather-Tait SJ, Wharf SG, Johnson IT. (2001) Chronic exposure to high levels of dietary iron fortification increases lipid peroxidation in the mucosa of the rat large intestine. *J Nutr* 131:2928-31.

Lund EK, Wharf SG, Fairweather-Tait SJ, Johnson IT. (1999) Oral ferrous sulfate supplements increase the free radical-generating capacity of feces from healthy volunteers. *Am J Clin Nutr* 69:250-5.

Lynch S. (2005) The precision of in vitro methods and algorithms for predicting the bioavailability of dietary iron. *Int J Vitam Nutr Res* 75:436-45.

Lynch S. (2007) Influence of infection/inflammation, thalassemia and nutritional status on iron absorption. *Int J Vitam Nutr Res* 77:217-23.

Lynch SR, Stoltzfus RJ. (2003) Iron and ascorbic Acid: proposed fortification levels and recommended iron compounds. *J Nutr* 133:2978S-84S.

Mackenzie EL, Iwasaki K, Tsuji Y. (2008) Intracellular iron transport and storage: from molecular mechanisms to health implications. *Antioxid Redox Signal* 10:997-1030.

Magnusson B, Bjorn-Rassmussen E, Hallberg L, Rossander L. (1981) Iron absorption in relation to iron status. Model proposed to express results to food iron absorption measurements. *Scand J Haematol* 27:201-8.

Manary MJ, Krebs NF, Gibson RS, Broadhead RL, Hambidge KM. (2002) Community-based dietary phytate reduction and its effect on iron status in Malawian children. *Ann Trop Paediatr* 22:133-6.

Martinez-Torres C, Romano E, Layrisse M. (1981) Effect of cysteine on iron absorption in man. *Am J Clin Nutr* 34:322-7.

Matuschek E, Towo E, Svanberg U. (2001) Oxidation of polyphenols in phytate-reduced high-tannin cereals: effect on different phenolic groups and on in vitro accessible iron. *J Agric Food Chem* 49:5630-8.

Matuschek ESU. (2005) The effect of fruit extracts with polyphenol oxidase (PPO) activity on the in vitro accessibility of iron in high-tannin sorghum. *Food chemistry* 90:765-71.

McCann JC, Ames BN. (2007) An overview of evidence for a causal relation between iron deficiency during development and deficits in cognitive or behavioral function. *Am J Clin Nutr* 85:931-45.

Mendoza C, Viteri FE, Lonnerdal B, Young KA, Raboy V, Brown KH. (1998) Effect of genetically modified, low-phytic acid maize on absorption of iron from tortillas. *Am J Clin Nutr* 68:1123-7.

Miller DD, Schricker BR, Rasmussen RR, Van Campen D. (1981) An in vitro method for estimation of iron availability from meals. *Am J Clin Nutr* 34:2248-56.

Monsen ER, Balintfy JL. (1982) Calculating dietary iron bioavailability: refinement and computerization. *J Am Diet Assoc* 80:307-11.

Monsen ER, Hallberg L, Layrisse M, Hegsted DM, Cook JD, Mertz W, Finch CA. (1978) Estimation of available dietary iron. *Am J Clin Nutr* 31:134-41.

Motzok I, Verma RS, Chen SS, Rasper J, Hancock RG, Ross HU. (1978) Bioavailability, in vitro solubility, and physical and chemical properties of elemental iron powders. *J Assoc Off Anal Chem* 61:887-93.

Navarro M, Wood RJ. (2003) Plasma changes in micronutrients following a multivitamin and mineral supplement in healthy adults. *J Am Coll Nutr* 22:124-32.

Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T, et al. (2004) Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* 306:2090-3.

Nielsen JB, Ikkala E, Solvell L, Bjorn-Rasmussen E, Ekenved G. (1976) Absorption of iron from slow-release and rapidly-disintegrating tablets - a comparative study in normal subjects, blood donors and subjects with iron deficiency anaemia. *Scand J Haematol Suppl* 28:89-97.

Nititham S, Srianujata S, Rujirawat T. (1999) Dietary intake of phytate, zinc and calcium of self-selected diets of Ubon Ratchathani and Bangkok subjects, Thailand. *J Med Assoc Thai* 82:855-61.

O'Neil-Cutting MA, Crosby WH. (1987) Blocking of iron absorption by a preliminary oral dose of iron. *Arch Intern Med* 147:489-91.

Patterson AJ, Brown WJ, Roberts DC. (2001) Dietary and supplement treatment of iron deficiency results in improvements in general health and fatigue in Australian women of childbearing age. *J Am Coll Nutr* 20:337-42.

Pietrangelo A. (2004) Hereditary hemochromatosis--a new look at an old disease. *N Engl J Med* 350:2383-97.

Pilon VA, Howanitz PJ, Howanitz JH, Domres N. (1981) Day-to-day variation in serum ferritin concentration in healthy subjects. *Clin Chem* 27:78-82.

Pizarro F, Olivares M, Hertrampf E, Mazariegos DI, Arredondo M. (2003) Heme-iron absorption is saturable by heme-iron dose in women. *J Nutr* 133:2214-7.

Poskitt EM. (2003) Early history of iron deficiency. *Br J Haematol* 122:554-62.

Raffin SB, Woo CH, Roost KT, Price DC, Schmid R. (1974) Intestinal absorption of hemoglobin iron-heme cleavage by mucosal heme oxygenase. *J Clin Invest* 54:1344-52.

Reddy MB, Cook JD. (1991) Assessment of dietary determinants of nonheme-iron absorption in humans and rats. *Am J Clin Nutr* 54:723-8.

Reddy MB, Hurrell RF, Cook JD. (2000) Estimation of nonheme-iron bioavailability from meal composition. *Am J Clin Nutr* 71:937-43.

Ricci G, Masotti M, De Paoli Vitali E, Vedovato M, Zanotti G. (1988) Effects of exercise on haematologic parameters, serum iron, serum ferritin, red cell 2,3-diphosphoglycerate and creatine contents, and serum erythropoietin in long-distance runners during basal training. *Acta Haematol* 80:95-8.

Rios E, Hunter RE, Cook JD, Smith NJ, Finch CA. (1975) The absorption of iron as supplements in infant cereal and infant formulas. *Pediatrics* 55:686-93.

Romslo I, Talstad I. (1988) Day-to-day variations in serum iron, serum iron binding capacity, serum ferritin and erythrocyte protoporphyrin concentrations in anaemic subjects. *Eur J Haematol* 40:79-82.

Rosenzweig PH, Volpe SL. (1999) Iron, thermoregulation, and metabolic rate. *Crit Rev Food Sci Nutr* 39:131-48.

Rossander-Hulthén L. Absorption of non-heme iron from meals. Doctoral Thesis. Dept of Medicine II. Göteborg: University of Gothenburg, 1985.

Rossander L, Sandberg A-S, Sandström B. The influence of dietary fibre on mineral absorption and utilisation. In: Schweizer TFE, C.A., ed. Dietary fibre - a component of food. Nutritional function in health and disease. London, 1992:197-216.

Sayers MH, English G, Finch C. (1994) Capacity of the store-regulator in maintaining iron balance. *Am J Hematol* 47:194-7.

Schmid A, Jakob E, Berg A, Russmann T, Konig D, Irmer M, Keul J. (1996) Effect of physical exercise and vitamin C on absorption of ferric sodium citrate. *Med Sci Sports Exerc* 28:1470-3.

Schwartz E, Baehner RL. (1968) Diurnal variation of serum iron in infants and children. *Acta Paediatr Scand* 57:433-5.

Shayeghi M, Latunde-Dada GO, Oakhill JS, Laftah AH, Takeuchi K, Halliday N, Khan Y, et al. (2005) Identification of an intestinal heme transporter. *Cell* 122:789-801.

Siegenberg D, Baynes RD, Bothwell TH, Macfarlane BJ, Lamparelli RD, Car NG, MacPhail P, et al. (1991) Ascorbic acid prevents the dose-dependent inhibitory effects of polyphenols and phytates on nonheme-iron absorption. *Am J Clin Nutr* 53:537-41.

Sinniah R, Doggart JR, Neill DW. (1969) Diurnal variations of the serum iron in normal subjects and in patients with haemochromatosis. *Br J Haematol* 17:351-8.

Sivakumar B, Brahmam GN, Madhavan Nair K, Ranganathan S, Vishnuvardhan Rao M, Vijayaraghavan K, Krishnaswamy K. (2001) Prospects of fortification of salt with iron and iodine. *Br J Nutr* 85 Suppl 2:S167-73.

Srinivas U, Braconier JH, Jeppsson B, Abdulla M, Akesson B, Ockerman PA. (1988) Trace element alterations in infectious diseases. *Scand J Clin Lab Invest* 48:495-500.

Statland BE, Bokelund H, Winkel P. (1974) Factors contributing to intra-individual variation of serum constituents: 4. Effects of posture and tourniquet application on variation of serum constituents in healthy subjects. *Clin Chem* 20:1513-9.

Statland BE, Winkel P, Bokelund H. (1973a) Factors contributing to intra-individual variation of serum constituents. 1. Within-day variation of serum constituents in healthy subjects. *Clin Chem* 19:1374-9.

Statland BE, Winkel P, Bokelund H. (1973b) Factors contributing to intra-individual variation of serum constituents. 2. Effects of exercise and diet on variation of serum constituents in healthy subjects. *Clin Chem* 19:1380-3.

Statland BE, Winkel P, Bokelund H. (1976) Variation of serum iron concentration in young healthy men: Within-day and day-to-day changes. *Clin Biochem* 9:26-9.

Stoltzfus RJ. (2001) Iron-deficiency anemia: reexamining the nature and magnitude of the public health problem. Summary: implications for research and programs. *J Nutr* 131:697S-700S; discussion 700S-701S.

Swain JH, Johnson LK, Hunt JR. (2006) An irradiated electrolytic iron fortificant is poorly absorbed by humans and is less responsive than FeSO4 to the enhancing effect of ascorbic acid. *J Nutr* 136:2167-74.

Swain JH, Newman SM, Hunt JR. (2003) Bioavailability of elemental iron powders to rats is less than bakery-grade ferrous sulfate and predicted by iron solubility and particle surface area. *J Nutr* 133:3546-52.

Taylor C, Rogers G, Goodman C, Baynes RD, Bothwell TH, Bezwoda WR, Kramer F, et al. (1987) Hematologic, iron-related, and acute-phase protein responses to sustained strenuous exercise. *J Appl Physiol* 62:464-9.

Taylor P, Martinez-Torres C, Leets I, Ramirez J, Garcia-Casal MN, Layrisse M. (1988) Relationships among iron absorption, percent saturation of plasma transferrin and serum ferritin concentration in humans. *J Nutr* 118:1110-5.

Taylor PG, Martinez-Torres C, Romano EL, Layrisse M. (1986) The effect of cysteine-containing peptides released during meat digestion on iron absorption in humans. *Am J Clin Nutr* 43:68-71.

Tetens I, Bendtsen KM, Henriksen M, Ersboll AK, Milman N. (2007) The impact of a meat- versus a vegetable-based diet on iron status in women of childbearing age with small iron stores. *Eur J Nutr*.

Thuy PV, Berger J, Davidsson L, Khan NC, Lam NT, Cook JD, Hurrell RF, et al. (2003) Regular consumption of NaFeEDTA-fortified fish sauce improves iron status and reduces the prevalence of anemia in anemic Vietnamese women. *Am J Clin Nutr* 78:284-90.

Tondeur MC, Schauer CS, Christofides AL, Asante KP, Newton S, Serfass RE, Zlotkin SH. (2004) Determination of iron absorption from intrinsically labeled microencapsulated ferrous fumarate (sprinkles) in infants with different iron and hematologic status by using a dual-stable-isotope method. *Am J Clin Nutr* 80:1436-44.

Tseng M, Chakraborty H, Robinson DT, Mendez M, Kohlmeier L. (1997) Adjustment of iron intake for dietary enhancers and inhibitors in population studies: bioavailable iron in rural and urban residing Russian women and children. *J Nutr* 127:1456-68.

Tuntawiroon M, Sritongkul N, Brune M, Rossander-Hulten L, Pleehachinda R, Suwanik R, Hallberg L. (1991) Dose-dependent inhibitory effect of phenolic compounds in foods on nonheme-iron absorption in men. *Am J Clin Nutr* 53:554-7.

Vahlquist B. (1941) Das serumeisen: Eine pädiatrisch-klinische und experimentelle studie. *Acta paediatrica* 28 suppl 5:1-358.

Waldenström J. (1946) The incidence of iron deficiency (sideropenia) in some rural and urban populations. *Acta Med Scand suppl* 170:252-79.

Walter T, Dallman PR, Pizarro F, Velozo L, Pena G, Bartholmey SJ, Hertrampf E, et al. (1993) Effectiveness of iron-fortified infant cereal in prevention of iron deficiency anemia. *Pediatrics* 91:976-82.

van Stuijvenberg ME, Smuts CM, Lombard CJ, Dhansay MA. (2008) Fortifying brown bread with sodium iron EDTA, ferrous fumarate, or electrolytic iron does not affect iron status in South African schoolchildren. *J Nutr* 138:782-6.

Van Thuy P, Berger J, Nakanishi Y, Khan NC, Lynch S, Dixon P. (2005) The use of NaFeEDTA-fortified fish sauce is an effective tool for controlling iron deficiency in women of childbearing age in rural Vietnam. *J Nutr* 135:2596-601.

Weight LM, Alexander D, Jacobs P. (1991) Strenuous exercise: analogous to the acute-phase response? *Clin Sci (Lond)* 81:677-83.

Weinberg ED. (1978) Iron and infection. *Microbiol Rev* 42:45-66.

Verdon F, Burnand B, Stubi CL, Bonard C, Graff M, Michaud A, Bischoff T, et al. (2003) Iron supplementation for unexplained fatigue in non-anaemic women: double blind randomised placebo controlled trial. *Bmj* 326:1124.

Wheby MS, Umpierre G. (1964) Effect of Transferrin Saturation on Iron Absorption in Man. *N Engl J Med* 271:1391-5.

Whittaker PG, Lind T, Williams JG. (1991) Iron absorption during normal human pregnancy: a study using stable isotopes. *Br J Nutr* 65:457-63.

WHO. Iron deficiency anaemia: Assessment, prevention, and control. Geneva: WHO/NHD/01.3, 2001.

WHO. The World Health Report 2002 - Reducing Risks, Promoting Healthy Life. Geneva: World Health Organization, 2002.

WHO. Guidelines on food fortification with micronutrients. Geneva: World Health Organization, 2006.

Wiltink WF, Kruithof J, Mol C, Bos MG, van Eijk HG. (1973) Diurnal and nocturnal variations of the serum iron in normal subjects. *Clin Chim Acta* 49:99-104.

Windsor JS, Rodway GW. (2007) Heights and haematology: the story of haemoglobin at altitude. *Postgrad Med J* 83:148-51.

Winkel P, Statland BE, Bokelund H. (1974) Factors contributing to intra-individual variation of serum constituents: 5. Short-term day-to-day and within-hour variation of serum constituents in healthy subjects. *Clin Chem* 20:1520-7.

Wirth JC, Lohman TG, Avallone JP, Jr., Shire T, Boileau RA. (1978) The effect of physical training on the serum iron levels of college-age women. *Med Sci Sports* 10:223-6.

Wise A, Lockie GM, Liddell J. (1987) Dietary intakes of phytate and its meal distribution pattern amongst staff and students in an institution of higher education. *Br J Nutr* 58:337-46.

Viteri FE, Alvarez E, Batres R, Torun B, Pineda O, Mejia LA, Sylvi J. (1995) Fortification of sugar with iron sodium ethylenediaminotetraacetate (FeNaEDTA) improves iron status in semirural Guatemalan populations. *Am J Clin Nutr* 61:1153-63.

Woo J, Leung SS, Ho SC, Lam TH, Janus ED. (1998) Dietary intake and practices in the Hong Kong Chinese population. *J Epidemiol Community Health* 52:631-7.

Younes M, Trepkau HD, Siegers CP. (1990) Enhancement by dietary iron of lipid peroxidation in mouse colon. *Res Commun Chem Pathol Pharmacol* 70:349-54.

Zimmermann MB, Chaouki N, Hurrell RF. (2005) Iron deficiency due to consumption of a habitual diet low in bioavailable iron: a longitudinal cohort study in Moroccan children. *Am J Clin Nutr* 81:115-21.

Zimmermann MB, Wegmueller R, Zeder C, Chaouki N, Biebinger R, Hurrell RF, Windhab E. (2004) Triple fortification of salt with microcapsules of iodine, iron, and vitamin A. *Am J Clin Nutr* 80:1283-90.

Zimmermann MB, Zeder C, Muthayya S, Winichagoon P, Chaouki N, Aeberli I, Hurrell RF. (2008) Adiposity in women and children from transition countries predicts decreased iron absorption, iron deficiency and a reduced response to iron fortification. *Int J Obes (Lond)*.

Zlotkin S, Arthur P, Antwi KY, Yeung G. (2001) Treatment of anemia with microencapsulated ferrous fumarate plus ascorbic acid supplied as sprinkles to complementary (weaning) foods. *Am J Clin Nutr* 74:791-5.