# Iron Deficiency in Female Adolescent Athletes -
## Prevalence, Mechanisms and Diagnostics

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UNIVERSITY OF GOTHENBURG

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*"En man med en klocka vet vad klockan är,*
*en man med två klockor är aldrig säker"*

A. Einstein 1875-1955

*To Linda*

## ABSTRACT

# Iron Deficiency in Female Adolescent Athletes – Prevalence, Mechanisms and Diagnostics

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*Background*: Iron deficiency (ID) is a very common condition and the most common nutritional deficiency in the world. ID mostly affects females, both athletes and non-athletes. Several underlying mechanisms are identified, such as insufficient dietary intake and losses by menses. In the athlete group different additional mechanisms are discussed including the existence of "sports anaemia" and in recent years the effect of inflammation due to physical activity and its effect on iron status has been highlighted. The inflammatory response complicates the diagnostic process and alternative laboratory methods have been proposed to improve diagnostics.

*Methods*: To study the prevalence of ID and iron deficiency anaemia (IDA) we used two different populations, first the female national soccer team (individuals aged 19-28 years), secondly a population of adolescent female athletes as well as a control group of adolescent non-athletes in a senior high school was studied. All participants filled in a questionnaire and blood samples comprising blood status, iron status including soluble transferrin receptor and hepcidin, and inflammatory markers as well as *Helicobacter pylori* antibodies were collected. Different methods for detection of ID were compared.

*Results*: The initial study showed a prevalence of ID of 57% and IDA of 29%. In the following study we found ID in 52% of the athletes and 48% of the non-athletes. IDA was seen in 8.6% of the athletes and 3.3% in the control group. The athletes had a significantly better diet and less loss by menses. Serum hepcidin was significantly higher in the athlete group and serum ferritin was the test that identified most individuals with ID.

*Conclusion*: Our studies revealed a high prevalence of ID in both the older elite soccer players as well as in the adolescent young female athletes. The prevalence of IDA was higher in the elite soccer player. In the adolescent athlete group we found a higher iron intake, as well as significantly less menstrual bleeding, but no difference in occurrence of ID. Serum hepcidin was significantly higher in the athlete group compared to the non-athletes. Hepcidin down regulates ferroportin, which results in decreased dietary iron absorption. Thus this could be a mechanism behind sports related iron deficiency. For diagnosis, serum ferritin remains the most sensitive tool, but *Helicobacter pylori* antibodies and serum hepcidin may be used in cases of non-responders to iron treatment.

*Keywords*: iron deficiency, iron deficiency anaemia, female adolescents, physical activity, inflammation, *Helicobacter pylori*

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## LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I Landahl G, Adolfsson P, Börjesson M, Mannheimer C, Rödjer S. Iron Deficiency and Anemia: A Common Problem in Female Elite Soccer Players.
*Int J Sport Nutr Exerc Metab. 2005; 15:689-694*

II Sandström G, Börjesson M, Rödjer S. Iron Deficiency in Adolescent Female Athletes – Is Iron Status Affected by Regular Sporting Activity?
*Clin J Sport Med. 2012; 22:495-500*

III Sandström G, Kaijser B, Rödjer S, Börjesson M. *Helicobacter pylori* antibodies and Iron Deficiency in Female Adolescents.
*Under revision in PLoS one*

IV Sandström G, Rödjer S, Jacobsson S, Börjesson M. Evaluation of Iron Status in Female Adolescent Athletes.
*Submitted to Br J Sport Med*

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## ABBREVIATIONS

| | |
|---|---|
| ACD | Anaemia of chronic disease |
| APR | Acute phase response |
| CLO-test | Campylobacter-like organism test |
| CRP | C-reactive protein |
| ESR | Erythrocyte sedimentation rate |
| ID | Iron deficiency |
| IDA | Iron deficiency anaemia |
| IDE | Iron deficient erythropoiesis |
| FIFA | The Fédération Internationale de Football Association |
| g | gram |
| GIT | Gastrointestinal tract |
| Hp | *Helicobacter pylori* |
| IL-1 | Interleukin 1 |
| IL-6 | Interleukin 6 |
| kg | kilogram |
| MCH | Mean corpuscular haemoglobin |
| MCHC | Mean corpuscular haemoglobin concentration |
| MCV | Mean corpuscular volume |
| mg | milligram |
| sTfR | Soluble transferrin receptor |
| TIBC | Total iron binding capacity |
| TNF-α | Tumor necrosis factor α |
| TS | Transferrin saturation |

## INTRODUCTION

Throughout history, physicians have used iron, the metal of Mars, to treat a variety of symptoms and diseases. Hippocrates, for example, is believed to have been the first physician to use iron salt as a styptic, and this practice still exists in the form of Monsel's solution (Figure 1). Lemery and Geoffroy demonstrated the presence of iron in the blood in 1713. Despite this early discovery, it took almost two hundred years before the metabolism of iron began to be understood. From 1925 and over the next two decades, the existence of non-haemoglobin iron in serum was documented, the transport protein transferrin was identified, and the dissociation of iron from transferrin at low pH levels was discovered. In 1937, ferritin was crystallised from horse spleen tissue (1) and knowledge of how the body maintains its iron homeostasis was gradually accumulated. Over the following years, the site of absorption in the gastrointestinal tract was defined as the upper intestine and with the introduction of hepcidin during the last decade, increasing knowledge has been gained about the turnover of iron in the human body.

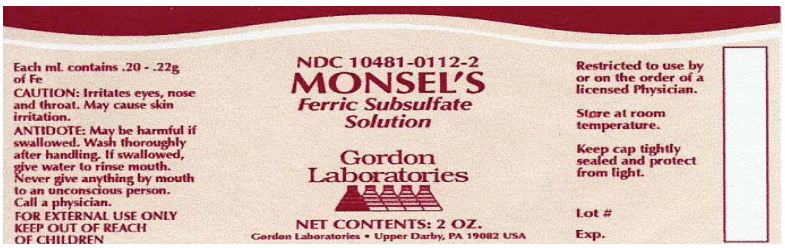


**Figure 1.** Monsel´s solution.

The total amount of iron in the human body ranges from 2.5 g in females to 3.5 g in males. In an adult man, the iron is distributed as follows: haemoglobin (in the erythrocytes), 2.1 g; myoglobin (in muscles), 200 mg; enzymes 150 mg; transport iron (transferrin), 3 mg; depot iron in the form of ferritin, 700 mg; and hemosiderin, 300 mg. Iron performs several important tasks in the body, the most well-known being its role as the oxygen-binding atom in haemoglobin and myoglobin. Iron also plays an important role in the energy-producing parts of the cell, the mitochondria, as a component of enzymes and cytochromes. Heme-containing proteins promote oxidative phosphorylation within the mitochondria (2). Furthermore, iron is an important component of many enzymes and performs vital functions in the synapses in the brain.

Because of the importance of iron, it would seem logical that supplementation to the human body with excessive amounts of iron would be beneficial. However, this is not the case; in fact, iron overload may be dangerous and has a negative impact on several organs and functions in the body. The regulation of the iron content in the body is indeed very exact and iron is preserved within narrow margins. The body has no system for excreting iron and because of the risk of overload; the gut absorbs only 10% of ingested iron. The loss amounts to 1 mg/day for males and 2 mg/day for females. The

most pronounced clinical effect of iron overload is hemochromatosis, with symptoms such as fatigue, cirrhosis of the liver, diabetes, cardiomyopathy, arthritis, testicular failure, bronzing of the skin and joint pain.

## Iron deficiency

Iron deficiency (ID) is the most common nutritional deficiency in the world (3, 4). It affects almost 50% of the population worldwide (5). There is a clear gender difference, with ID being most prevalent among women, as well as in developing countries. ID has an impact on the immune function in infants and children. Among women, ID can lead to decreased work productivity, increased child mortality, increased maternal mortality, and impaired cognitive function. In addition, it has been shown that iron supplementation in iron-deficient females can normalise cognitive function (6), as has also been proposed by Beard and Connor 2003 (7). The end state of ID is iron deficiency anaemia. As shown by several researchers, iron is very important for the developing brain in infants and children (8-10).

ID typically develops over time due to an imbalance between the iron intake and iron loss. Primary losses by menses (11) and inadequate dietary intake of iron (12, 13) are the primary causes of ID. Gastrointestinal bleeding is also a common cause for ID; typically bleeding due to gastritis, ulcus ventriculi and ulcus duodeni and bleeding from different types of cancer of the GI tract, predominantly colon cancer. Other causes are atrophic gastritis (14), mainly in elderly people, blood loss from gastrointestinal parasites such as *Trichuris trichuria* (whipworm) and *Necator americanus* (Hookworm) (15-17), mucosal atrophy in coeliac disease (18, 19) and *Helicobacter pylori* infection (20, 21).

The symptoms of ID are vague and are rarely seen in clinical practice today. The most important and also non-significant symptom remains chronic fatigue. Other traditional symptoms include koilonychias, glossitis and dysphagia.

The basis of the diagnosis of ID is the laboratory tests. The golden standard is still bone marrow aspiration and iron staining (22). This is a well-established method with high specificity, although it is not suitable for screening due to the invasive nature of the procedure. The most common and widely accepted test is serum ferritin, which reflects the iron stores in the body (23, 24). Ferritin is a protein of 450 kDa consisting of 24 subunits and present in every cell type in the human body. The most common cell type where ferritin can be found is in the hepatocytes and in the macrophages. Each ferritin complex can store about 4500 iron ($Fe^{3+}$) ions. Importantly, the cut-off level for ID for serum ferritin is still debated, and especially for athletes still has to be determined (25).

Other frequently used laboratory tests are transferrin saturation; that is, serum iron divided by transferrin or total iron-binding capacity (TIBC), which normally is elevated in ID. The transferrin saturation should typically be less than 10-16%, depending on which method is used for the analysis of serum iron and TIBC. However, serum iron is subject to diurnal variations, with higher concentrations late in the day, and it may also increase after the ingestion of meat. Furthermore, oral contraceptives increase serum

transferrin and result in low transferrin saturation (26). Serum iron is also affected by inflammation, and especially interleukin 6, (IL-6), a cytokine involved in the specific regulation of inflammation, may have an impact on serum iron (27). The IL-6 level is triggered by inflammation (28). The elevated IL-6 concentration has a negative impact on serum iron, which is decreased by IL-6 (29).

A soluble form of the transferrin receptor (sTfR), was first identified in serum in 1986 (30). The sTfR is directly correlated with the mass of erythroid precursors. In aplastic anaemia there is a total absence of sTfR, whereas in thalassemia major there is a marked increase. The potential advantage of using this receptor in the diagnosis of ID is that sTfR is independent of inflammation (31) and therefore not affected by infection or chronic disease. In addition sTfR is unaffected by physical activity (32). Several commercial assays are available, but the use of sTfR in clinical practice has been limited by the lack of an international standard.

"Functional iron deficiency" is defined as a condition where there is an inadequate iron supply to the bone marrow, despite the presence of storage of iron in the cells of the monocyte-macrophage system. The most common condition where functional ID occurs is in patients with chronic renal failure who require parenteral iron supplementation to respond to erythropoietin therapy. Several chronic inflammatory diseases can lead to functional ID, such as rheumatoid arthritis and inflammatory bowel disease. This condition is also named the anaemia of chronic disease (ACD).

### Iron deficiency anaemia

IDA is the end state of ID. IDA is defined as a haemoglobin value below 120 g/l for females and less than 130 g/L for males, according the World Health Organization (WHO) (33). A special group of individuals are those who have a reduction within the reference interval but who do not fall below the WHO cut-off value for anaemia. This is called relative anaemia. As ID is the IDA precursor, 50% of the female population are at risk of developing IDA with negative consequences for physical capacity, as well as other effects of IDA. Common symptoms are fatigue, muscle weakness, hypotension, headache, palpitations, syncope, shortness of breath, and chest pain. IDA is associated with a lower physical capacity. Given the role of ID as the precursor of IDA, the underlying causes are the same.

The principles of diagnosis and management in IDA are well established and have been defined in several detailed guidelines and recommendations (34, 35).

In most cases, IDA is easy to treat. Normally, oral treatment with iron is sufficient and the recommendation is substitution until the haemoglobin value is stable, which could take 1-3 months. After stable haemoglobin level is reached, another three months of treatment are required to replenish the iron stores (34, 36). The situation where the patient does not respond to iron treatment is defined as failure to respond to oral treatment with 100 mg elemental iron daily for 4 to 6 weeks with an increase in haemoglobin by at least 10 g/L (37). There are several reasons for this, such as poor compliance, inaccurate history, false diagnosis and, occasionally, factitious anaemia. ACD, autoimmune gastritis, cancer, status post-gastric bypass surgery and GI bleeding must be

ruled out, as well as *Helicobacter pylori* infection (18, 37-39). In recent years, dietary interventions have been tried to improve the iron status of young women (12).

The anaemia seen in patients with chronic infections, chronic inflammatory diseases and cancer is referred to as inflammation-induced anaemia. The main cause of this anaemia is suppression of the erythropoiesis provided by pro-inflammatory cytokines, such as TNF-α, IL-1 and interferon-γ. The best treatment in ACD is treatment of the underlying cause.

## Physical performance and haemoglobin

It is of great importance for competitive athletes, especially in endurance sports, to maintain adequate body iron stores to be able to preserve their optimum haemoglobin level and thereby the oxygen transport capacity. Of course, many factors influencing success in sports are beyond the individual athlete's control (skill of the opponent, environmental conditions, referee judgements, etc.), but maintaining appropriate levels of body iron is a variable that the athlete can usually control.

Iron is an essential component of the heme molecule, binding to globulins in the bone marrow to form haemoglobin. For maximum aerobic performance, the presence of an adequate quantity of circulating haemoglobin is critical for the transport of oxygen. In the exercising muscle, oxygen is utilised in the metabolic process as an energy source for the oxidation of substrates (carbohydrates and fat). If there is a controversy regarding the effect of ID on physical performance, there is no doubt that an existing IDA has an impact on the maximum aerobic performance (40, 41). Thus, even small reductions in the haemoglobin level may have a negative effect on the exercise capacity (42, 43). It has been shown that the haemoglobin value is linearly correlated with the oxygen uptake capacity (up to haemoglobin values at 200g/L) (40), which has been demonstrated in a classic study by Ekblom et al. from 1972. They demonstrated a high correlation between Hb and performance capacity after venesection and reinfusion (42) (Figure 2). An increase in the haemoglobin concentration overnight by 13%, after reinfusion of three units of stored autologous blood resulted in an increase in maximum oxygen uptake and physical performance capacity by 9% and 23%, respectively. This is in fact, the rationale for blood-doping, where the athletes try to achieve an unnatural high haemoglobin concentration. The use of this method is a serious problem in professional sports. The introduction of the athlete biological passport where the athlete´s individual Hb values are followed, is an important step in the anti-doping efforts against blood-doping. A survey made by the International Ski Federation and the International Olympic Committee in 1989 showed that, in the specific competition, 50% of medal winners and 33% of those finishing from 4$^{th}$ to 10$^{th}$ place had highly abnormal haematological profiles. In contrast, only 3% of skiers finishing from 41$^{st}$ to 50$^{th}$ place had highly abnormal values (44, 45).

The intra-cellular function of iron as a component of the hem-containing cytochromes of the oxidative phosphorylation chain should also be considered. Iron-containing enzymes, such as succinate dehydrogenase and NADH dehydrogenase, are reduced in conditions of ID.

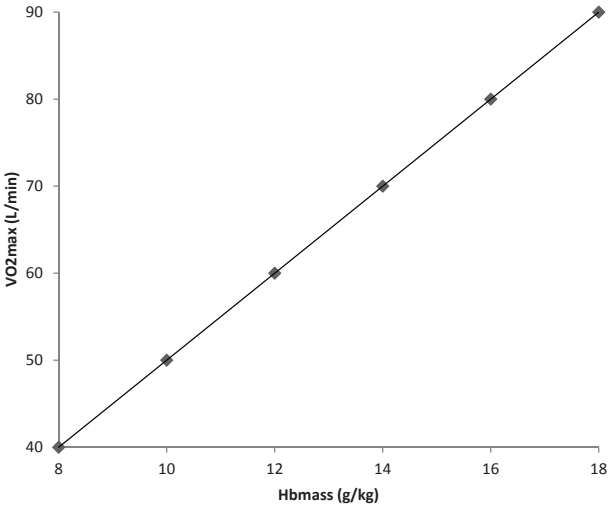


**Figure 2.** The relation between Hbmass (g/kg) and $VO_2$max (L/min).

## Sports anaemia

In 1881 Fleischer described a young soldier who passed dark urine after participating in long field marches (march haemoglobinuria) (46). This may be the first report describing the effect of exercise on the blood. The study of the body's adaptation to physical exercise has since then developed into a major research field. Over the last three decades, the popularity of different training programmes has exploded. A subnormal haemoglobin concentration was reported in athletes, and the phrase *sports anaemia* was first mentioned in 1970 in Yoshimura's review of anaemia in the exercise setting (47). Several hypotheses and mechanisms have been proposed as explanations of this, now established phenomenon.

A potential mechanism behind sports anaemia is *plasma expansion*. This is the effect of several mechanisms, such as vasomotor-mediated extravascular to intravascular fluid movement, individual attempts at hydration and renal fluid conservation. These are all normal responses to the stress of exhaustive exercise. It has been proposed that the plasma expansion at the expense of the number of red cells may decrease viscosity favourably and maximise stroke volume, cardiac output and subsequent oxygen delivery. In 3-5 days, any influence of the plasma expansion on the haemoglobin value should have been corrected (48-51). Another route of blood loss related to physical activity is *losses by the gastrointestinal (GI) tract*. GI complaints are very common in the athlete population with rates from 30% to 70% (52). The most common complaints are heartburn, nausea and vomiting, and epigastric pain. Both the type of sporting activity and the intensity have an impact on the development of GI symptoms. Three major mechanisms contribute to the GI problems: mechanical forces, altered GI blood flow and neuroendocrine changes. The most important problem with regard to GI symptoms that has a direct impact on the iron status is the increased risk of gastritis, ulcers and upper GI bleeding (53). It is very common among athletes to use different types of painkillers and the widespread use of NSAIDs may increase the risk of damage to the GI mucosa. For athletes with an already impaired iron situation, losses by the GI tract could further compromise the athlete's status. Furthermore, *haematu-*

*ria*, the presence of blood in the urine, is observed as a result of sporting activity. The haemolysis and mechanical trauma of the RBCs are indicated in the glomerulus (54), as the excess of haemoglobin is lost in the urine. It is also assumed that the movement of the bladder during running could cause bleeding due to microscopic lesions of the wall (55). The total loss of blood due to GI-bleeding however is considered small in most cases.

Yet another mechanism of iron loss in the athlete is loss due to *haemolysis*. Several potential mechanisms of haemolysis in the context of sporting activity, have been described, such as oxidative stress and mechanical influence on the old RBCs through muscle contraction, accelerating the haemolysis. The contribution to the total haemolysis from these two latter mechanisms is small and the major cause of haemolysis is foot strike (56-58). Telford et al. demonstrated a greater increase in plasma-free haemoglobin levels, as well as decreases in serum haptoglobin levels, in runners compared with cyclists. The significance of the haemolysis for iron loss is not clear and one investigator reports that total body RBC volumes do not differ between trained runners and cyclists (56). A possible explanation, despite the higher rate of RBC destruction in runners, could be that the replacement keeps pace and therefore prevents iron deficiency and anaemia.

Finally, iron loss *through sweating* has been studied (59, 60). Waller et al proposed that iron losses by sweating may have an impact on the iron status in female athletes with a low iron intake (<1.36mg/day) iron intake, but for most athletes it seems unlikely that losses by sweat would result in a significant iron deficiency. Despite all these different explanations of so-called "sports anaemia" many investigators doubt the existence of this phenomenon and its possible effect on physical performance.

## Physical activity and inflammation

Several conditions, such as bacterial infection, surgery, burns, neoplasia, tissue infarction, and inflammatory diseases (61) trigger the acute phase response. Indeed, inflammation is a response to stressful stimuli (62). It is well known that physical exercise initiates the same type of inflammatory response in the human body (63-66). What is the beneficial effect for the organism of the acute inflammatory response? Is it a part of the fight and flight response? Is it a part of the preparation for battle, leading to less bleeding in the battlefield?

Exercise, through the acute phase response, induces changes to several acute phase reactants. In 2001, Fallon et al. demonstrated changes in several of these factors; serum iron, ferritin, transferrin saturation, CRP, ESR and haptoglobin due to physical activity (67). Weight et al. (1991) showed that both WBC and CRP increased after a marathon race (68). They also found an initial decrease in haptoglobin, possibly related to haemolysis, and 24 hours later, an increase in haptoglobin in addition to the initial increase in albumin. Twenty-four hours later, fibrinogen was still increased. The authors concluded that the response to prolonged exercise was similar but not totally analogous to the acute phase response.

In a study on participants in a 1,600 km ultra-marathon race, changes were seen in several mediators of the acute phase response; including increased serum ferritin, reduction in transferrin saturation, increase in haptoglobin, as well as increased WBC and platelet counts (69). Studies of the underlying mechanisms of the acute phase response (APR) support the theory that physical exercise in itself initiates the APR. The major cytokines involved in this reaction are IL-1, IL-6 and tumour necrosis factor (TNF). TNF is elevated 1, 3 and 24 hours following a 2.5-hour run (70) and in another study a 2-3 fold increase in TNF-α was seen (64). IL-1 and IL-6 were increased after a marathon (71) and IL-6 was increased following a 20km race (72). It is assumed that IL-6 is the largest contributor to the systemic cytokine increase (66) with plasma levels rising as high as up to 100 times higher than the levels recorded before initiation of physical activity (63). These acute phase response changes could potentially affect the iron homeostasis in the body.

Hepcidin is a key hormone in the control of the iron homeostasis in the body, regulated by iron stores, inflammation, hypoxia and erythropoiesis. Serum hepcidin has been proposed as a potential diagnostic tool also for iron deficiency (73, 74) (Figure 3).

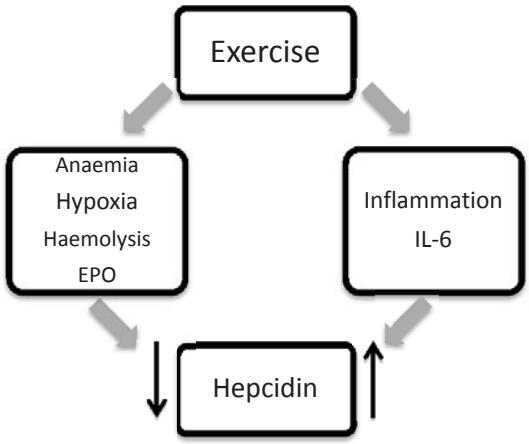


**Figure 3.** The relation between exercise and hepcidin-levels.

ID down-regulates the hepcidin level in serum and the uptake from the gut increases 3-5 times (from 1-2 mg/day to 4 mg/day). Hypoxia also down-regulates serum hepcidin through an increase in erythropoietin activity and increased the iron demand in the bone marrow. The hepcidin effect on ferroportin is a protective reflex and the purpose is to prevent iron overload. Interestingly, mutations of the HFE gene related to hemochromatosis down-regulate the synthesis of hepcidin and more iron is absorbed in the gut in the absence of inhibition of ferroportin.

### *Helicobacter pylori*, iron deficiency and iron deficiency anaemia

*Helicobacter pylori* (Hp) is a very common infection with a high prevalence worldwide (75). Many factors have been shown to be risk factors influencing the incidence

and prevalence, such as age, gender, genetic predisposition, ethnicity, education level and sanitation (76). In 1982, Marshall and Warren suggested that Hp infection was the causative agent of gastritis and peptic ulcer, and today, Hp infection is regarded as the most common cause of gastritis (77). Gastritis may be a benign condition but Hp infection has also been associated with more malignant diseases, such as gastric cancer (78). Recently Hp infection has been identified as a possible cause of unexplained ID (21, 79). Hp has been implicated in several studies as a cause of IDA refractory to oral iron treatment and with a favourable response to Hp eradication (20, 80). Regarding transmission of the infection, it is still not clear exactly how the infection is spread from one individual to another. Childhood has been identified as the time of acquisition, but the route of transmission remains unclear. It has been proposed that intra-familial transmission is far more important than child-to-child transmission outside the family. Investigators state that low socioeconomic status and large family size, as well as the origin of the parents, are of importance. A child's probability of being infected is considerably stronger when the mother is infected than if the father's status is considered. This observation is consistent with a predominant mother-to-child transmission route (81, 82). As shown by Seham et al. (2013), Hp infection up-regulates serum hepcidin levels and is associated with a diminished response to oral iron therapy in children with IDA.

Hp infection in athletes is not well studied and very few studies have been performed (20). It is possible that the infection may have an impact on the iron homeostasis of the athlete.

In summary, ID is common and may have an impact on athletic performance. Several interesting aspects still need to be elucidated regarding ID and athletes. Does sports anaemia exist? Is it, in fact, a sports iron deficiency? The role of the diet and other causes of ID, as well as the impact of Hp infection, need to be clarified. Furthermore, how to establish the diagnosis in athletes is not totally clear, nor is the importance of the inflammatory response for the diagnostic process and the possible influence of hepcidin need to be studied.

## AIM

Thus, the aims of this thesis were:

- To determine the prevalence of iron deficiency and iron deficiency anaemia in a group of adult female athletes and a group of female adolescent athletes, and to compare the latter group with a group of female adolescent non-athletes;

- To study mechanisms that may be involved in the development of iron deficiency in female athletes, including diet-related causes and other lifestyle-related factors, loss by menses, factors related to physical activity; i.a., inflammation, and finally the impact of a *Helicobacter pylori* infection on the iron status;

- To compare different methods of diagnosing iron deficiency in female athlete populations of different age.

## SUBJECTS AND METHODS

### Ethics

The study was approved by the Ethics Committee at Sahlgrenska Academy, Gothenburg University (approval no: Ö-005-01). The subjects who were 18 years of age and older gave their written informed consent to take part in the investigation. For those younger than 18 years, their parents were asked for their informed written consent. The methods used in this investigation were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

We applied for and received complementary approval for Paper III and IV, regarding the widened laboratory testing (approval no: T960-13).

### Study population

#### *Paper I*

The study population in Paper I consists of twenty-eight female soccer players (age 19-28) at national team level. The study was performed as a clinical quality control during the preparations for the FIFA women´s World Cup 1999. The team doctor and the Swedish Football Association initiated the testing according to the FIFA regulation.

#### *Paper II-IV*

The study population in Paper II-IV consists of females from a senior high school in Gothenburg. Besides being a general school for adolescents from the local area, this school is also a senior high school for top athletes recruited both from the local area and from all over Sweden. The students are active in different sports, both individual and team sports. All female athletes at the school (n=71) were invited to take part in the investigation. Fifty-seven females accepted and entered the study. With the assistance of a statistician, a control group consisting of a random sample of age-matched non-athletes was invited to participate in the study. One hundred thirty non-athletes were initially invited. Of these, 92 agreed to take part in the study. Thus, in the study presented in Paper II, 57 student athletes and 92 non-athletes students participated.

For Paper III and IV, the same group of study subjects was used. Because of technical problems related to the blood sampling procedure, not enough serum was obtained for all participants. For this reason, the number of study participants was reduced to 56 athletes and 71 non-athletes in these two papers.

### Study design and settings

#### *Paper I*

This study was performed as a part of a routine clinical evaluation regulated by FIFA. The responsible team doctor initiated it and all the laboratory tests were performed during a team gathering. All team members gave their verbal consent to the testing and the follow-up. The evaluation consisted of a questionnaire, blood samples and a medical consultation. The team members with iron deficiency anaemia and probable

iron deficiency anaemia were their own controls after iron supplementation and the team doctor performed the follow-up.

### *Paper II-IV*

Paper II-IV were performed as clinical trials. Each trial consisted of anthropometric measurements, a questionnaire, and blood samples. The females with iron deficiency anaemia and iron deficiency were offered a medical consultation and treatment for their ID or IDA. For follow-up, the females were referred to the regular school doctor. One third of the females who were treated with iron were not followed up as they had left the school. Because the school accepts students from all over Sweden we were not able to arrange follow-up at the place of residence of each student. Comparisons were made between the female adolescent athletes and non-athletes regarding anthropometric measurements, the results of the questionnaire, and blood tests.

## Methods

### *Questionnaire – Paper I and II*

In connection with entry into the study, all the participants were asked to fill in a questionnaire during the same session as when the blood samples were drawn. The questionnaire was developed by the team doctor as a basis for discussing the soccer player's situation and to identify any possible explanations of ID, if relevant. The questionnaire was not validated, but designed by the team doctor on the basis of his long clinical experience of working with this type of questions. The questionnaire consisted of questions on family history (hereditary disease), smoking habits and dietary habits, including the number of meals per day and whether they were eating breakfast or not. The questionnaire also included items on specific food intake, such as meat, coffee, tea, dietary supplements and medications, including hormonal contraceptives. No dietary registration was done. In addition, there were questions about eating patterns, active weight loss, and if they were trying to gain weight. The subjects were asked to specify their menstrual bleeding as: 1=sparse, 2=normal, 3=abundant (The full questionnaire is included in Appendix).

### *Anthropometric measurements - Paper I-IV*

Anthropometric measurements were performed on the same day as the blood sampling was done. Body weight (kilograms) and height were measured to the nearest 0.5 kilograms and 0.5 centimetres, respectively. Body mass index (BMI) was calculated as the weight (kilograms) divided by the square of the height (metres).

### *Biochemical assays - Paper I-IV*

Venous blood samples for evaluation of iron deficiency, anaemia, *Helicobacter pylori* antibody status and inflammatory status were drawn at the school clinic at certain given times. The subjects had not performed any training on the day of the blood sampling. To minimise dropouts, we offered the study participants three possible testing occasions.

All subjects were fasting from midnight and venous blood samples were drawn between 10 and 12 a.m. with the subjects in the semi-supine position. Blood was drawn

from the antecubital vein. The blood was collected in serum gel (SST) vacutainer tubes and EDTA tubes. All biochemical analyses were performed at the accredited laboratory of the Clinical Chemistry and Bacteriology Department at Sahlgrenska University Hospital (Swedac 1240), according to the manufacturers' protocols.

The concentration of haemoglobin (Hb), the erythrocyte indices (MCV, MCH, MCHC), and erythrocyte particular concentration (EPC) were determined the same day. The concentration of haemoglobin was determined using the Technicon H2 method (Bayer Diagnostics, USA). MCV, MCH and MCHC values were measured on a CellDyn 400 (Abbott Laboratories, USA) by the cytometrical particle count method. Serum was initially kept frozen at -20°C and analyses of serum iron (Fe), TIBC, and serum ferritin were performed at one time to minimise systematic errors. The assays were performed according to standard laboratory procedures. Serum iron was determined with a photometric method as a ferrozine complex on a Hitachi 917 (Boehringer Mannheim, USA). Total iron-binding capacity was calculated from measurements of serum transferrin with an immunochemical method on a Hitachi 917. Transferrin saturation was the ratio of serum iron to TIBC, expressed as a percentage. Serum ferritin was measured by an immunochemical method using a mouse monoclonal anti-ferritin antibody and determined by alkaline phosphate conjugation according to the AxSYM system (Abbot Laboratories, USA).

After the initial analysis, the remaining serum was kept frozen at -70°C for later use. Before study II-IV, the serum was thawed so that the different analyses for the different studies could be performed.

The level of soluble transferrin receptors was analysed using an automated immunoturbidimetric method on a Modular P (Roche) instrument. Latex-bound anti-sTfR antibodies react with the antigen in the sample and form an antibody-antigen complex. The reagents (Roche Diagnostics) have CE marking according to the IVD directive. Hepcidin-25 was analysed with liquid chromatography mass spectrometry LC-MS/MS, LOQ 1 nmol/L, coefficient of variance 15% at a level of 5 nmol/L (83, 84).

Inflammatory activity was assessed by quantification of C-reactive protein with an immuno-turbidimetric method on the Roche Cobas, CRP HS, (Roche Diagnostics Scandinavia AB). Leukocyte counts, WBC, were measured on an ADVIA2120i (Siemens Healthcare Diagnostics AB, Sweden) with an optical cytochemistry, flow cytometry-based analysis.

*Helicobacter pylori* IgG antibodies were analysed using the enzyme-linked immunesorbent assay, ELISA, manufactured by EUROIMMUNE, Lübeck, Germany (www.euroimmune.de). The result was reported as relative units (RU). More than >22 RU/mL was considered a positive result (85).

### *Definitions - used in Paper I-IV*

*Iron deficiency* (ID) was defined as a serum ferritin <16 µg/L (22).

*Probable iron deficiency* was defined as serum ferritin 16 to 20 µg/L and transferrin saturation <20% as in Paper I and II.

*Iron deficiency anaemia* (IDA) was defined as a haemoglobin value <120g/L according to the WHO definition in the presence of ID (33).

*Relative iron deficiency anaemia* was defined as a haemoglobin value >120 g/L and iron deficiency (serum ferritin <16 µg/L) and an increase in haemoglobin >10g/L after iron supplementation as specified in Paper II (86).

## Statistics

We used commercially available statistical software (SPSS 22.0; SPSS Inc. Chicago. IL) to perform the statistical analyses. Descriptive statistics are presented as means ± SD or ranges. For comparison of demographic characteristics we used Student's t-test, the chi-square test and Fisher's exact test. All test were two-sided. $p<0.050$ was considered statistically significant. Comparison between variables was performed with Fisher's permutation test.

The relationships between different variables were investigated using Pearson's moment correlation coefficient. Preliminary analyses were performed to ensure no violation of the assumptions of normality, linearity and homoscedasticity.

## RESULTS

### Paper I

The analyses of the blood samples showed that 7 of 28 (25%) of the subjects had a haemoglobin value below 120g/L according to the WHO definition and therefore were classified as being anaemic. Iron deficiency was seen in 16 of 28 subjects, corresponding to a prevalence of 57%. After iron supplementation to those who were iron deficient, one subject, who initially had a haemoglobin value of 128g/L and was defined as iron deficient, increased her haemoglobin concentration with 14g/L. Since the haemoglobin concentration increased more than 10g/L after iron supplementations, she in had relative anaemia and the total number of subjects defined as having iron deficiency anaemia was 8 corresponding to 29% of the subjects.

Changes in laboratory variables before and after iron supplementation are seen in Table 1.

**Table 1.** Mean values of transferrin saturation and serum ferritin in iron deficient female elite soccer players (n=16) before and after iron supplementation (Tabs Ferromyn 37 mg Fe/tablet, 2 tablets 2 times per day)

| | Before supplementation | After supplementation |
|---|---|---|
| Iron saturation (%) | 13 | 30 |
| Serum ferritin (µg/L) | 10 | 22 |

### Paper II

#### *Baseline characteristics*

The mean age of the participants was 17 years in both groups. There was no significant difference in weight between the two study groups but there was a tendency that the athletes were slightly heavier. We found no significant difference in height between the two groups. The calculated BMI was similar, being with no statistically significant difference between the two groups. All figures on baseline characteristics are shown in Table 2.

**Table 2.** Baseline characteristics for the female adolescents participating in the study

| Characteristics | Athletes (n=57) | | Non-athletes (n=92) | | |
|---|---|---|---|---|---|
| | Mean±SD | Range | Mean±SD | Range | p |
| Age, years | 16.8±0.9 | 15-18 | 17.1±0.9 | 15-19 | 0.07 |
| Height, m | 1.68±0.1 | 1.5-1.9 | 1.66±0.1 | 1.5-1.8 | 0.06 |
| Weight, kg | 64±6 | 52-78 | 61±10.5 | 45-106 | 0.07 |
| BMI, $kg/m^2$ | 22.5±1.8 | 19-27 | 21.9±3.4 | 17-39 | 0.25 |

### *Iron deficiency*

In the athlete group 30/57 individuals (52%) were iron deficient, compared to 43/92 in the non-athlete group (48%) (p>0.3). There was no difference in ferritin between the two groups (p>0.3). However, there was a significant difference between athletes and non-athletes in serum iron, TIBC, but not in transferrin saturation (Table 3).

**Table 3.** Erythrocyte and Iron Parameters

| | Athletes | | Non-athletes | | |
|---|---|---|---|---|---|
| **Measure** | **Mean±SD** | **Range** | **Mean±SD** | **Range** | **p** |
| Hb, g/L | 138±9.0 | 118-157 | 136±8.5 | 110-169 | >0.3 |
| MCV, fL | 90.0±4.4 | 74-100 | 88.7±6.2 | 50-100 | 0.19 |
| RBC, $10^{12}$/L | 4.6±0.3 | 4.0-5.2 | 4.6±0.4 | 3.8-5.6 | >0.30 |
| S-Fe, mol/L | 14.0±6.0 | 4.0-36.0 | 17.3±7.2 | 7.0-43.0 | 0.004* |
| TIBC, mol/L | 73.3±10.7 | 49-100 | 77.9±11.6 | 48-110 | 0.018* |
| TS, % | 19.5±8.4 | 4.0-47.0 | 22.5±9.9 | 8.0-58.0 | 0.069 |
| Ferritin, µg/L | 21±13.8 | 3.0-63.0 | 21±16.9 | 3.0-86.0 | >0.30 |

*Statistically significant (p<0.05); RBC, red blood cell count; S-Fe, serum iron; TS, transferrin saturation

### *Anaemia*

Comparisons of the two groups showed no significant difference in haemoglobin with results showing a mean value of 136±9 g/L in the athlete group and 138±9 g/L in the non-athlete group (Table 3). There was no difference in erythrocyte particle concentration. There was no difference in mean corpuscular volume (MCV). In total we found that 5/57 (8.6%) of the athletes had iron deficiency anaemia, compared to 3/92 (3.3%) of the non-athletes, the difference being not statistically significant (p=0.24). Among the five athletes with anaemia, two had IDA with haemoglobin <120g/L and three relative anaemia, i.e. their haemoglobin value increased with more than 10 g/L after iron supplementation. In the non-athlete group, all subjects with IDA were defined as having certain IDA.

### *Dietary habits*

The young female athletes, more often than the non-athlete women reported eating breakfast, 81% of the athletes doing so, compared to 52% of the non-athletes (p<0.001). They also reported a significantly higher consumption of milk, with 75% of the athletes reporting drinking milk every day, compared to 52% of the non-athletes (p=0.007). In addition, the athletes ate more often as shown by the number of reported meals per day: 3.4±0.6 for the athletes and 3.0±0.9 for the non-athletes (p=0.003). In the non-athlete group there was a significant correlation between the number of meals and the level of ferritin (p=0.02). This correlation was not significant in the athlete group. There was a trend to more use of dietary supplements in the athlete group, p=0.06, but no difference in the consumption of coffee p=0.21, tea p=0.27, or meat p=0.06 (Table 4).

### *Additional life style factors*

The non-athlete women were smokers at a greater extent than the athletes (27% and 9% respectively, p=0.009). Female athletes did not practice active weight loss as often as non-athletes, (42% compared to 63%, p=0.02). Importantly athlete females reported less menstruation, (1.9±0.5, compared to 2.1±0.5 in the non-athlete group, p=0.02) calculated from the answers in the questionnaire (see methods). The time of menarche was not significantly different, being 12.6±1 (range 9-15) years for the athlete females and 12.4±1 (range 10-15) years for the non-athletes (p<0.30), respectively. Interestingly the female athletes also answered that the menstruation was significantly less painful than in the non-athlete group, p<0.001 (Table 4).

**Table 4.** Lifestyle factors

| Lifestyle Factor | Athletes n=57 | Non-athletes n=92 | p |
|---|---|---|---|
| Breakfast, No. | 46 | 48 | <0.001* |
| Meals per day, (mean±SD) | 3.4±0.6 | 3.0±0.8 | 0.003* |
| Milk, No. | 43 | 48 | 0.007* |
| Coffee, No. | 7 | 20 | 0.21 |
| Tea, No. | 13 | 30 | 0.27 |
| Dietary supplements, No. | 17 | 14 | 0.56 |
| Active weight loss, No. | 24 | 58 | 0.020* |
| Hormonal contraception, No. | 17 | 44 | 0.044* |
| Menstruation, estimated amount (1-2-3) | 1.9±0.5 | 2.1±0.5 | 0.020* |
| Smokers, No. | 5 | 25 | 0.009* |

*Statistically significant (p<0.05).

## Paper III

A total of 18 of 127 adolescent females were positive for Hp IgG (14%). One female in the Hp positive group (6%) and three females in the Hp negative group (3%) had anaemia according to the WHO definition (33), with a haemoglobin <120g/L. The mean haemoglobin value in the Hp positive group was 133.6 g/L and in the Hp negative group 137.0 g/L with no statistically significant difference between the two groups, (p=0.14).

In the whole group, 64 females (50%) had iron deficiency. Of those 64, 12 were Hp positive and 52 Hp negative. In the group of non-iron deficient females (n=63), we found 6 with positive Hp serology. There was a non-statistical significant difference in Hp positive subjects comparing iron deficient subjects with normal subjects, (p=0.22). When specifically looking at athletes and non-athletes, we found none with iron deficiency anaemia and one with iron deficiency among the four Hp positive athletes.

In the group of Hp positive non-athletes (n=14) there were one with iron deficiency anaemia and eleven with iron deficiency.

Regarding laboratory parameters there were no differences in serum iron (p>0.30), TIBC (p=0.27), transferrin saturation (p=0.27), ferritin (p>0.30), or white blood cell count (p>0.30) between Hp positive and negative individuals (Table 5).

There was no significant difference between Hp positive and Hp negative subjects regarding BMI and weight.

**Table 5.** Major laboratory findings. Results expressed as mean and SD

| n | ***Helicobacter*** **positive** **18 (14%)** | ***Helicobacter*** **negative** **109 (86%)** | **p** |
|---|---|---|---|
| Athletes (n) | 4 (7%) | 52 | |
| Non-athlete (n) | 14 (20%) | 57 | |
| Weight (kg) | 60±14 | 61±6 | >0.30 |
| BMI (kg/m$^2$) | 22.1±4.7 | 21.9±2.2 | >0.30 |
| Hb (g/L) | 134±7.6 | 137±8.5 | 0.14 |
| MCV (fL) | 88±6 | 90±4 | 0.13 |
| S-Fe (μmol/L) | 17.1±9 | 15.6±7 | >0.30 |
| TIBC (μmol/L) | 72±11 | 76±12 | 0.27 |
| TS (%) | 24±12 | 21±9 | 0.27 |
| Ferritin (μg/L) | 20.4±19.6 | 20.5±14.5 | >0.30 |

TS; transferrin saturation. p<0.05 statistical significant.

## Paper IV

There was no significant baseline difference between the two groups in age (p=0.06) and BMI (p=0.08) but weight (p=0.006) and length (p=0.04) showed statistical difference (Table 6).

## Haematological parameters

The same comparisons as in Paper II were done but in a smaller number of athletes and non-athletes. Therefore the calculations are presented also in this paper. There was no statistical significant difference between the two groups regarding haemoglobin value with 137g/L in the athlete group and 136g/L in the non-athlete group (p>0.30). No difference was found in MCV, 90fL for the athletes and 89fL for the control group (p>0.30). Finally no difference was found in RBC, 4.6 in the athlete group and 4.7 in the non-athlete group (p>0.30) (Table 6).

One athlete and three non-athletes had anaemia with a haemoglobin value <120 g/L with no statistical difference.

**Table 6.** Anthropometric measurements, haematological and iron-related parameters (mean±SD and range), *=p<0.05, statistically significant

| | **Athletes** | **Range** | **Non-athletes** | **Range** | **p** |
|---|---|---|---|---|---|
| **n** | **56** | | **71** | | |
| Iron deficiency | 28 (50 %) | | 34 (48 %) | | >0.30 |
| Anaemia | 1 | | 4 | | >0.30 |
| Haemoglobin g/L | 137±8.7 | 118-157 | 136±7.8 | 110-169 | 0.30 |
| MCV (fL) | 89.8±4.3 | 74-100 | 89.4±4.7 | 70-100 | >0.30 |
| RBC ($10^{12}$/L) | 4.6±0.3 | 4.0-5.2 | 4.7±0.3 | 3.8-5.4 | >0.30 |
| Serum iron (µmol/L) | 14.0±6 | 4.0-27.0 | 17.6±7 | 7.0-40.0 | 0.003* |
| TIBC (µmol/L) | 73±11 | 49-100 | 77±12 | 61-110 | 0.06 |
| Ferritin (µg/L) | 20.9±14 | 3-63 | 20.9±17 | 3-86 | >0.30 |
| sTfR (mg/L) | 3.74±0.97 | 2.4-6.9 | 3.65±1.25 | 1.9-9.0 | >0.30 |
| Hepcidin (nmol/L) | 4.7±3.0 | 2.0-23.0 | 3.3±1.9 | 0.7-14.1 | 0.001* |
| WBC ($10^9$) | 7.4±1.9 | 4.2-13.0 | 6.7±1.6 | 3.2-11.4 | 0.03* |
| CRP (mg/L) | 1.93±4.2 | 0.15-30 | 2.06±4.1 | 0.15-27 | >0.30 |

## Iron status

In the athlete group there was a statistical significant lower serum iron, 14 µmol/L compared to the non-athlete group, 17.6 µmol/L (p=0.003). There was no difference regarding TIBC, iron saturation, serum ferritin and sTfR. Hepcidin was significant higher in the athlete group, 4.7 nmol/L compared to 3.3 nmol/L (p<0.001).

Iron deficiency defined as serum ferritin < 16µg/L was found in 28 athletes (50%) and in 34 non-athletes (48). The difference was not statistical significant (p>0.30) (Table 6).

*Inflammatory parameters*

There was no difference between the two groups in high sensitive CRP (p>0.30) but a significant difference was found in WBC with higher values in the athlete group (p=0.03) (Table 6).

## DISCUSSION

### The prevalence of ID and IDA in female athletes

Our pilot study on the female soccer players in the national team showed a surprisingly high prevalence of both ID and IDA (87). The prevalence of ID was 55%, much higher than compared to the average female population in Sweden of the same age, having a prevalence of 33% (88) and compared to females in the United States, with a prevalence of 30% (89), as well as figures in Europe (90). The expectation was that the prevalence in the group of soccer players possibly would be lower, due to better medical monitoring and an assumed greater awareness among the soccer players about nutrition. Even more serious though, is the finding of IDA being present in almost a third of the female elite soccer players in Paper I. The prevalence of ID and IDA in a sporting female population has been debated. Studies performed during a period of 15 years (1998-2013) fail to provide a consistent answer. The results diverge from studies reporting a better iron status in female athletes (91, 92) to studies showing a worse situation in female athletes (93, 94). Finally, there are studies reporting no difference at all (95).

In our second study, the aim was to determine the prevalence of ID and IDA among a group of young female athletes (86). We were able to establish collaboration with a senior high school for athletes, to perform a study on female adolescent athletes and a control group for comparison of the prevalence figures. This study also showed that ID also is common in a group of adolescent female athletes, with an ID frequency of 52%. In comparison, Hallberg et al. showed that the prevalence among Swedish teenagers was 40% (88). The prevalence figure of 52% almost equals the figure from our first study, even though the females in the first study were between 19 and 25 years of age and the females in the second study were between 15 and 19 years old. ID is very common in our first and second study, in both adolescent female athletes and non-athletes and is thus a problem not only for female athletes but for all females.

Interestingly, the presence of IDA in the second study group was much less evident. Only 8.6% in the group of young athletes had IDA, compared with almost a third in the first study. A possible explanation could be that it takes some time to develop IDA, as illustrated below (Figure 4). Potentially, the female may need, at least, a couple of years with menstruation and loss of iron before presenting with IDA. The adolescence itself consumes a great amount of iron. One could speak of a so-called “anaemia career”, starting with the menarche and developing over time due to a continuous negative iron balance. It may not be until the female athlete reaches the mid-twenties that the imbalance between her iron intake and the iron loss results in IDA.

While there is doubt about the effect of pure ID on physical performance (27, 96), the importance of a lowered haemoglobin value on the performance of the athlete is unquestioned (40, 97). When one third of a team entering a soccer field suffers from IDA, you are truly giving the opponent an advantage. Good performance in soccer depends of several factors, including technique and tactics, but aerobic capacity is certainly of importance (98, 99). Helgerud et al. showed that a better maximum aerobic

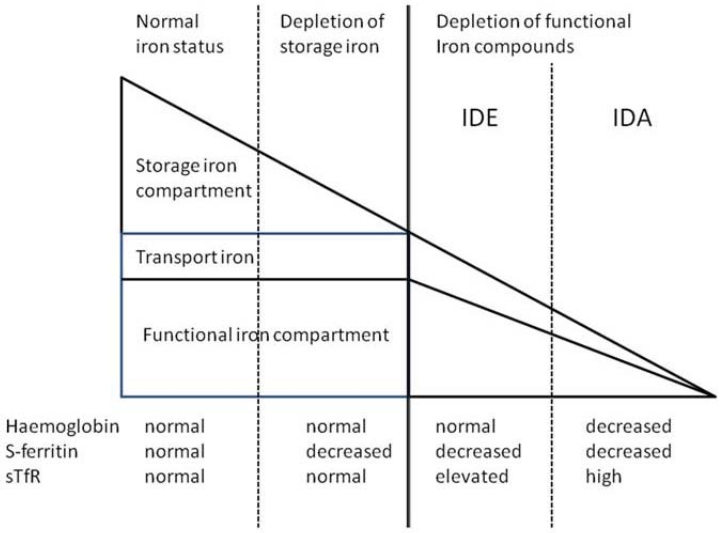


**Figure 4.** The development of IDA over time with uncompensated iron losses. IDE = iron depleted erythropoiesis. IDA = iron deficiency anaemia. sTfR = soluble transferrin receptor.

capacity ($VO_{2max}$) improves soccer performance, measured as the distance covered by a player during a game, as well as increased involvement with the ball (97).

## Underlying mechanisms of ID--does sporting activity play a role?

### *Nutrition and iron losses*

The two major causes of ID is insufficient intake of iron and increased loss, primarily through the menses (90, 100). The human body maintains the iron balance within very narrow margins, due to the potentially toxic effects of iron overload. Importantly, it is not possible for the body to increase the excretion of iron. In the sedentary female, these mechanisms are the most likely reasons for the ID and IDA found in otherwise healthy individuals. Interestingly, in our second study, we demonstrated that the adolescent female athletes had better dietary habits, assessed as a more frequent intake of breakfast, more meals per day and a higher intake of meat. If the mean loss by menses were the same for all females, then the athlete group would present with a better iron status. When adding the finding of significantly sparser menses in athletes, there would, indeed, be a difference. Surprisingly, we could not demonstrate a difference between the adolescent female athletes and the control group, in serum ferritin. Interestingly, the finding of significantly less menstrual bleeding does not correspond to the finding of significantly less use of oral contraceptives in the athlete group, which usually is associated with a reduced amount of menstrual bleeding (101). To be considered in this context is the female athlete triad (FAT), a syndrome that is characterised by low energy availability, functional hypothalamic amenorrhoea and osteoporosis (102). One or more of these conditions pose significant health risks to physically active girls and females. In a study on female adolescent athletes, Barrack

et al. demonstrated a significantly lower bone mineral density in endurance runner athletes compared with non-runner athletes (participating in ball, power or antigravitational sports) (103). In our second study the female athletes tended to be slightly heavier (p=0.06), and the non-athletes were practicing active weight loss significantly more often than the athletes; hence, we do not believe that FAT has any major impact on our study group. In antigravitional sports bone mineral density is higher in spite of low fat deposition (104)**.**

The lack of an expected difference between groups, in iron status led to the question of whether there are other mechanisms that may explain why the athlete female group failed to present a better iron status.

### *"Sports anaemia"*

During the past forty years, many investigators have tried to explain the lowered haemoglobin value among athletes with the so-called "sports anaemia" condition (47). The pseudo-anaemia demonstrated after physical activity, due to haemodilution, gives a spuriously low haemoglobin value, but the haemoglobin mass is unchanged and the iron status is not affected at all; at least, no iron loss takes place. The other traditionally proposed explanations of "sports anaemia" are foot strike haemolysis, blood loss from the GI tract due to ischaemic damage to, primarily, the gastric mucosa, haemolysis due to oxidative stress and mechanical influence on the erythrocytes and loss by sweat. However, pseudo-dilution does not result in iron loss and we do not believe that the other proposed mechanisms of iron loss can explain the observed lack of a better iron status in the athlete group in our second study. So there must be something else.....

### *Helicobacter pylori and ID*

Several papers report a correlation between Hp infection and ID (21, 39, 105). One study has been carried out regarding physical activity, ID and Hp in children and adolescents (20). For this reason, we investigated the possibility of Hp infection being a part of the explanation in the possible sports related relative iron "deficiency" of our studied athletes. In the group of females having ID, we found 12 individuals who were positive for Hp antibodies compared to six in the group without ID. This difference was not statistically significant, but the figures might well have been significant with a larger group of subjects. Most Hp-positive individuals acquire the infection during childhood and it is supposed that the infection is transmitted from mother to child (76). The family background plays a role, especially overcrowded living and low socioeconomic status. One study from Korea points out overcrowding during training camps as a reason for young athletes getting infected with Hp (20). Hp infection does not seem to increase the blood loss from the ventricular mucosa, and normally, no gastritis is seen on gastroscopy, even if there is an infection. The infection is generally asymptomatic and in studies on functional dyspepsia, there was only a small decrease in symptoms in the group where Hp was eradicated, compared with the placebo group. In Paper III we found Hp prevalence of 14% with no difference between the athlete´s group and the controls. No gastrointestinal symptoms were reported in any of the two groups in the study. Ethnicity, expressed as origin in a high-risk area seems to be the most important risk factor for being infected with Hp (82). In our study we

noted a high proportion of individuals in the group of non-athletes, origin from high and intermediate risk areas. According to international guidelines, Hp serology is the recommended screening test (105). A false negative test is very unusual. The problem with serology is that the tested person will not necessarily have an active infection at the time of the test. Active infection is best diagnosed with the CLO test or biopsy from the ventricular mucosa.

However, in patients where gastrointestinal evaluation fails to disclose a likely cause of IDA in the clinic situation, or in patients refractory to oral iron supplementation, screening for coeliac disease (anti-tissue transglutaminase antibodies), autoimmune gastritis (gastrin, anti-parietal or anti-intrinsic factor antibodies), and *Helicobacter pylori* (IgG antibodies and urease breath test) are recommended (107, 108).

### *Inflammatory activity and ID*

It is well known that intense physical activity results in an acute inflammatory response. Hypothetically, the physiological effect of the acute phase reaction, APR, is to prepare the individual for possible battle by increasing the blood volume and the cardiac output, as well as mobilising the coagulation system and preventing bleeding. Many research groups have studied the inflammatory response related to physical activity and coherent data from these groups point to a chain of action, from physical activity to the APR through several cytokines, such as IL-1, IL-6 and TNF-α. Secondary to the IL-6 activity, increased levels of hepcidin, the major regulator of iron absorption in the gut, and release from the macrophages are seen (109-111). The major effect of an elevated serum hepcidin concentration is a decrease in iron absorption from the gut.

Studies on athletes have shown an increased level of inflammatory cytokines and of hepcidin after physical activity. In some studies, increased levels were seen as long as 24 hours after the activity (111) and in another, the levels were still increased after 72 hours of rest (112). One possible scenario, due to the frequency and intensity of training sessions, is that the athlete suffers from chronically elevated hepcidin levels, spanning the whole season. This persistent increase could, at least theoretically, lead to a situation of decreased iron uptake during long periods of time. In our second study we found a difference in several life-style factors, which should favour a better iron status in the athlete group (86). However to our surprise, this was not seen, as there was no difference in the prevalence of ID between the two groups. In Paper IV, we demonstrated a significant increase in serum hepcidin concentration in the adolescent female athletes, compared to the control group. It is possible that this elevation of hepcidin is the explanation for the lack of a difference in serum ferritin. Thus, physical activity in itself may contribute to the development of clinical iron deficiency, by way of activating the acute phase reaction.

Several studies have shown an increased CRP level after athletic activity, with a peak after 24 hours (113) and even 48 hours post-activity (68), however, this was not seen in our study. Thus, as mentioned earlier, repeated training sessions over time could potentially result in a chronically elevated serum hepcidin level, with a subsequent negative impact on the iron homeostasis. An interesting observation is that the type

of sporting activity may influence the inflammatory response. It has been shown that in court and field sports, an acute phase response did not occur in female netball and soccer players. In the study by Dufaux et al. (67), they reported CRP values similar to those found in our study. The white blood cell count (WBC) increased significantly in the present study, which has also been shown earlier by other researchers (28, 114).

## How to diagnose iron deficiency in general and in athletes

Usually it is not difficult to establish the diagnosis of ID in otherwise healthy patients. Most clinicians use traditional markers of ID, such as serum ferritin and transferrin saturation for the identification of ID. For defining anaemia, the cut off level of 120g/L set by the World Health Organization is used in combination with erythrocyte indices (MCV, MCH and MCHC) (33). This cut-off level for anaemia is set on group level. However, the individual optimum haemoglobin concentration is more important. One possible scenario, and an example of the clinical importance of the individual haemoglobin levels, is a drop in the Hb level within the reference interval; e.g., from 155 g/L to 125 g/L. This individual would not be considered anaemic according to the WHO definition, but the Hb drop would have a negative impact on the athlete's physical performance.

### *Iron stained bone marrow*

The golden standard for the diagnosis of ID, iron-stained bone marrow smears, is not appropriate to use in the screening setting due to the invasive nature of the test (22). This method is today only used occasionally, primarily when it is difficult to establish the diagnosis with the traditional tests.

### *Serum ferritin*

The most common screening test today, is serum ferritin and in guidelines published in 2012, the authors state that serum ferritin is the most powerful test for iron deficiency (35). However, the cut off for defining ID may vary. In clinical trials, the cut-off value ranges from <10 µg/L to <30 µg/L, which sometimes makes it difficult to compare different studies. A low serum ferritin level is highly specific of iron deficiency, while no disorders present with a low serum ferritin without ID. Serum ferritin is a well-standardised method but this value is affected by both inflammation and liver disease which might confuse the diagnosis. However, if the patient presents with a serum ferritin level below 16 µg/L the diagnosis of ID is certain. In Paper IV, we compared the different methods for diagnosing ID. The method that identified most patients with ID, was serum ferritin followed by transferrin saturation. When specifically looking at the different individuals, in the study, we identified four female athletes with a serum ferritin level >16 µg/L and a simultaneously low transferrin saturation. These women would have been "missed" if only serum ferritin had been used for the screening test. Interestingly, these four individuals also presented with a high concentration of serum hepcidin.

These findings illustrate well the diagnostic difficulties when inflammation is present. The presence of inflammation possibly related to physical activity affects diagnosis, as the traditional markers of iron deficiency are affected by inflammation. Serum fer-

ritin, being an acute phase reactant, is increased by inflammation and ID, could thus be misinterpreted in the inflammatory patient. Serum iron and transferrin decrease in the presence of inflammation, thereby reducing the reliability.

The relationship between ferritin and hepcidin is well described, with coherent data from several studies showing an association between an increase in IL-6, hepcidin and ferritin (115-117). The significant difference in the serum hepcidin concentration between athletes and non-athletes found in our study is most probably an effect of physical exercise. For each given ferritin value, the athlete presents with a higher hepcidin concentration.

### *Soluble transferrin receptor*

Because of the problems related to inflammation and the diagnosis of ID, researchers have tried to find new methods that would ideally be independent of inflammation. One method that have interested the researchers is the soluble transferrin receptor (118). The transferrin receptor is expressed on the surface of the erythrone and the amount increases when the need of iron increases. There is a soluble form of the receptor that is related to the amount in the bone marrow (30). Thus, the soluble transferrin receptor increases when the erythropoiesis is iron-deficient (119). However, this is a late event and does not happen until the iron stores are empty or almost depleted. Therefore, when the sTfR increases, there is a great risk that the haemoglobin value has already started to decrease. The main advantage of using this method, is the independency of inflammation (120, 121) and it is highly likely that the sTfR level is unaffected by physical activity (32). This was demonstrated in one study where ferritin, IL-6 and hepcidin increased after physical activity but the sTfR remained unchanged (115). A problem is the lack of standardisation of different methods and cut-off levels that prevents comparisons between different studies. High values are also seen in other disorders with increased erythropoiesis as haemolytic disorders.

### *Serum hepcidin*

A great deal of interest has been shown in the molecule of hepcidin. This is a small protein produced in the liver, which has been labelled the “great conductor” of the iron regulation in the body. In Paper IV, we found a significant difference in the serum hepcidin concentration between adolescent female athletes and the control group. Interestingly, this was on the basis of no difference in the frequency of ID, assessed by serum ferritin or by transferrin saturation and sTfR. Normally, the hepcidin concentration is low in the presence of ID. Even though many subjects, both athletes and non-athletes, were defined as having ID, not many had greatly reduced levels of serum hepcidin, in fact some presented with a normal but low serum ferritin, reduced transferrin saturation and an elevated serum hepcidin. Our theory, is that the acute inflammatory reaction secondary to physical activity leads to an increase in the serum hepcidin concentration, which, in turn, leads to ID through the negative effects of hepcidin; i.e., sports iron deficiency. Investigators report that elevated levels of serum hepcidin persists in one study three hours and in another study 24 hours after a marathon race in females (111, 122). Zieman et al. showed that the level of hepcidin is elevated also after 72 hours of rest (112). It is possible that the higher serum hepcidin concentrations seen in the athlete group is a marker of an inflammatory state, related

to physical activity and hence not really are related to the athlete´s iron situation. In clinical practice, hepcidin levels can be used to distinguish a true IDA from an ACD. A true IDA presents with low serum hepcidin concentration, while an ACD presents with high values. The role of serum hepcidin in clinical practice is not yet well defined and the method is not available in all health care facilities.

In summary, according to our results, the most sensitive way to diagnose ID and IDA in athletes is to use the Hb value and serum ferritin, using the cut-off levels of 120 g/L for Hb and <16 $\mu$g/L for serum ferritin. For female athletes with a serum ferritin level between 16 and 30 $\mu$g/L, analysis of sTfR and serum hepcidin could give additional information. Regarding the time and interval between regular check-ups, we recommend at least once a year. When treating ID and IDA, it takes 4-6 weeks to reset the Hb value and an additional three months to replenish the iron stores. For patients not responding to iron supplementation and where a lack of compliance has been ruled out, as well as in patients where gastrointestinal evaluation fails to disclose a likely cause of IDA, or in patients who are refractory to oral iron supplementation, screening for coeliac disease, autoimmune gastritis, and *Helicobacter pylori* (IgG antibodies and urease breath test) is recommended.

## CONCLUSION

- In our studies we found a very high prevalence of ID, both in adult female elite soccer players and in adolescent female athletes as well as in non-athlete adolescent females. The prevalence of IDA was higher in the elite soccer player group compared to the adolescent athletes, indicating the possibility of an anaemia career.

- We found a higher iron intake, assessed as the number of meals per day and the frequency of eating breakfast, as well as significantly less menstrual bleeding, assessed as days of bleeding and estimated volumes, in the athletes compared with the non-athletes. Thus, a better iron status in the female athletes would have been expected, on the basis of a better iron intake/iron loss balance. The fact that their iron status was not better suggests that some other mechanism may have an impact on the iron status.

- We demonstrated a significantly higher serum hepcidin concentration in female adolescent athletes compared with non-athletes and this could be one mechanism behind sports iron deficiency.

- Serum ferritin still seems to be the best method to screen for and diagnose ID, having the highest sensitivity.

- For those with an unclear cause for their ID or those not responding to iron supplementation, Hp infection must be out ruled as a cause for ID.

## CLINICAL RECOMMENDATIONS

- All female athletes should check their Hb value and iron status annually. Health interview including questions regarding eating habits and menses should been done simultaneously.

- Use traditional markers for ID and IDA; haemoglobin, MCV, MCH, MCHC, s-iron, TIBC, s-ferritin.

- Hb <120g/L indicates anaemia → investigate and treat

- S-ferritin <16µg/L → treatment for 4-6 weeks + subsequent control

- For s-ferritin 16-30 µg/L → treatment trial, control of Hb after 6 weeks, relative anaemia?

- For those not responding to iron treatment:
  - Renew personal and medical history and physical examination
  - check sTfR, s-hepcidin (if available), Hp antibodies (especially for individuals originating from high risk areas)

- If Hp positive → gastroscopy and biopsy, or CLO-test. Eradication?

- If elevated s-hepcidin → investigate inflammatory status, causes for inflammation? Intensified iron treatment? Break in training?

## FUTURE PERSPECTIVES

For the future, it is of great interest to:

- Study serum hepcidin concentrations in athletes over a complete training and competitive season - to establish if chronically elevated serum hepcidin concentration is present

- Define the role of serum hepcidin in the clinical practice

- More closely study the group of athletes with a serum ferritin level between 16-30 μg/L, their levels of iron status markers and their response to iron treatment and, if possible, a break in training

- Study physical performance in athletes with serum ferritin level between 16-30 μg/L and if they improve on iron supplementation

- Study how patients with signs of ID and elevated serum hepcidin concentrations respond to intravenous iron treatment

## SAMMANFATTNING PÅ SVENSKA

Järnbrist är den vanligaste bristsjukdomen i världen, och vanligast bland kvinnor. Obehandlad kan den leda till blodbrist, vilket medför en sänkt prestationsförmåga. I en undersökning av elitfotbollsspelare fann vi att 55% hade järnbrist och 1/3 hade blodbrist. I vår följande studie fann vi att det är vanligt med järnbrist bland unga elitidrottande kvinnor och vid jämförelse med icke elitidrottande kvinnor fanns det ingen skillnad i förekomst. Däremot visade det sig att de idrottande kvinnorna hade ett bättre kosthåll och mindre menstruation. Båda dessa faktorer skulle gynna en bättre järnsituation hos de idrottande kvinnorna men studien visade ingen skillnad. Hur skall man förklara denna skillnad?

Orsakerna till järnbrist hos kvinnor är i huvudsak bristande intag via kosten och förluster via menstruation. Vad gäller idrottare har man dessutom under lång tid studerat ”sports anaemia”, en blodbrist utlöst av fysisk träning. Många mekanismer har föreslagits, t ex pseudo-dilution, förluster via svett, hemolys (sönderfallande blodkroppar), och blod- och järnförluster via mag-tarm kanalen.

I en studie omfattande elitidrottande unga kvinnor fann vi ingen skillnad i förekomst av järnbrist. Dock fanns det en signifikant skillnad i livsstilsfaktorer där idrottare åt frukost i större omfattning och också fler måltider per dag. Dessutom fanns det en signifikant skillnad i menstruation räknat som antal blödningsdagar och volym. Sammantaget skulle detta gynna ett bättre järnintag hos idrottande kvinnor.

Det tycks finnas en faktor som påverkar järnbalansen i kroppen. Det är visat att hård fysisk träning utlöser en inflammatorisk reaktion i kroppen. Denna leder till en höjning av hepcidin i serum. Hepcidin har en central roll i kroppen vad gäller reglering av järnupptag från tarmen och frisättning av järn från depåer. Vi visar att det finns en signifikant skillnad i serum hepcidin mellan idrottare och icke-idrottare där idrottare har höga nivåer trots att det inte är någon skillnad i förekomst av järnbrist. Denna hepcidinstegring blockerar upptaget av järn i tarmen och kan förklara att idrottarna inte har bättre järnstatus än icke-idrottarna. Slutligen jämfördes olika metoder att diagnosticera järnbrist och slutsatsen är att serum ferritin är det prov som identifierar flest individer med järnbrist vid screening. HP infektion är inte vanligare hos idrottare men förekommer hos 14% och bör uteslutas om ingen annan orsak till anemi hittas.

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## APPENDIX

## Hälsofrågeformulär

1. Finns ärftlig (hos föräldrar eller syskon) sjukdom som du känner till?

2. Har du någon kronisk sjukdom (ex diabetes, reumatisk sjukdom, astma, medfödd hjärtsjukdom) eller annat?

3. Har du någon sjukdom just nu (t.ex. infektion, blodbrist, ansträngningsastma)?

4. Aktuella mediciner (ej kosttillskott)
   - p-piller?
   - annan regelbunden medicinering?
   - annan medicinering vid behov?

5. Hur många veckor under det senaste året har du avstått helt från träning/tävling på grund av sjukdom?

6. Snusar du?

7. Röker du?

8. Har du någon gång försökt att gå upp i vikt?

9. Har du någon gång försökt att gå ned i vikt?

10. Anser du att du har en ätstörning?

11. Anser du, baserat på antal måltider du äter per dag samt måltidernas kvalité och sammansättning att dina kostvanor är bra?

12. Har dina måltider en bra näringsmässig sammansättning?

13. Hur många måltider äter du per dag?

14. Äter du frukost varje?

15. Äter du en lagad lunch/middag med fisk/kött, potatis/ris/pasta varje dag?

16. Äter du kött?

17. Dricker du mjölk i anslutning till måltider? Om ja, antal glas/dag?

18. Dricker du kaffe? Om ja, antal koppar per dag?

19. Dricker du te? Om ja, antal muggar per dag?

20. Äter du kosttillskott? Om jag gå till fråga 21, annars 22.

21. Specificera din användning av kost/näringstillskott med ett x vid hur ofta du tar ett visst preparat (% av antal dagar per år)

| | Regelbundet (>70%) | Ibland (15-70%) | Sällan (<15%) |
|---|---|---|---|
| Järn | | | |
| Multivitaminer | | | |
| Mineraler | | | |
| C-vitamin | | | |
| Ginseng | | | |
| Q-10 | | | |
| Protein/aminosyror | | | |
| Fisk/omega-3 olja | | | |
| Antioxidanter | | | |
| Kreatin | | | |
| Anti-förkylning | | | |
| Övriga | | | |

22. Ålder vid första menstruation?

23. Aktuell mensrytm? Intervall, antal dagar?

24. Mensmängd? Sparsam – måttlig – riklig

25. Har du haft oregelbunden mens eller uppehåll i mens >3månader?

26. Längsta mensuppehåll (ej graviditet)?

27. Hur många menstruationsblödningar har du haft de senaste 12 månaderna?

28. Har du råkat ut för stressfraktur någon gång?

29. Övriga upplysningar om ditt hälsotillstånd som du tror har betydelse för dig i ditt dagliga liv och under idrottsutövning?